Sphingoid long chain bases prevent lung infection by *Pseudomonas aeruginosa*

Yael Pewzner-Jung¹**, Shaghayegh Tavakoli Tabazavareh², Heike Grassmé², Katrin Anne Becker², Lukasz Japtok³, Jörg Steinmann⁴, Tammar Joseph¹, Stephan Lang⁵, Burkhard Tuemmler⁶, Edward H Schuchman⁷, Alex B Lentsch⁸, Burkhard Kleuser³, Michael J Edwards⁸, Anthony H Futerman¹ & Erich Gulbins²,⁸,*

Abstract

Cystic fibrosis patients and patients with chronic obstructive pulmonary disease, trauma, burn wound, or patients requiring ventilation are susceptible to severe pulmonary infection by *Pseudomonas aeruginosa*. Physiological innate defense mechanisms against this pathogen, and their alterations in lung diseases, are for the most part unknown. We now demonstrate a role for the sphingoid long chain base, sphingosine, in determining susceptibility to lung infection by *P. aeruginosa*. Tracheal and bronchial sphingosine levels were significantly reduced in tissues from cystic fibrosis patients and from cystic fibrosis mouse models due to reduced activity of acid ceramidase, which generates sphingosine from ceramide. Inhalation of mice with sphingosine, with a sphingosine analog, FTY720, or with acid ceramidase rescued susceptible mice from infection. Our data suggest that luminal sphingosine in tracheal and bronchial epithelial cells prevents pulmonary *P. aeruginosa* infection in normal individuals, paving the way for novel therapeutic paradigms based on inhalation of acid ceramidase or of sphingoid long chain bases in lung infection.

Keywords cystic fibrosis; long chain base; lung infection; *Pseudomonas aeruginosa*; sphingosine

Subject Categories Microbiology, Virology & Host Pathogen Interaction; Respiratory System

DOI 10.15252/emmm.201404075 | Received 17 March 2014 | Revised 10 July 2014 | Accepted 11 July 2014 | Published online 1 August 2014

EMBO Mol Med (2014) 6: 1205–1214

Introduction

Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene and is found with a prevalence of 1 in 2,500–3,500 Caucasian newborns. CF patients, and patients with chronic obstructive pulmonary disease (COPD), are particularly susceptible to *Pseudomonas aeruginosa* infection, with approximately 80% of CF patients suffering from chronic *P. aeruginosa* pneumonia by 25 years of age, and one-third of patients with COPD hosting the bacteria (Rakhimova et al, 2009; Cystic Fibrosis Foundation, 2011). However, the increasing rate of *P. aeruginosa* resistance to many antibiotics necessitates the development of alternative therapies, which might also be beneficial in preventing *P. aeruginosa* infection in patients with trauma, burn wounds, sepsis, or in patients requiring ventilation (McManus et al, 1985; Crouch Brewer et al, 1996; Vidal et al, 1996).

Previous studies have demonstrated that various natural lipids and lyso-lipids act as bactericidal agents in skin (Fischer et al, 2012). Among these is the sphingoid long chain base (LCB), sphingosine (SPH), which protects human skin from bacterial colonization (Bibel et al, 1992). SPH is generated by hydrolysis of ceramide via acid ceramidase (AC). We now demonstrate that tracheal and bronchial epithelial SPH levels play a vital role in preventing normal individuals from *P. aeruginosa* lung infection. SPH levels are significantly reduced in tracheal and bronchial epithelia of CF patients and of CF mice, due to reduced AC activity, and normalization of SPH levels reverses susceptibility to *P. aeruginosa*. These data suggest two novel paradigms, namely that epithelial SPH is a natural antibactericidal agent in airways and that patients susceptible to bacterial infection in the lung could be treated by administration of AC or of SPH analogs.
Results and Discussion

Immunohistochemical analysis using an anti-SPH antibody demonstrated that SPH was abundantly expressed on the luminal surface of human nasal epithelial cells obtained from healthy individuals, but was almost undetectable on the surface of nasal epithelial cells from CF patients (Fig 1A – for validation of the specificity of the antibody, see Supplementary Information, Supplementary Fig S1 and Fig 2B). The reduction in SPH levels was recapitulated in tracheal and bronchial cells from CF mice (Fig 1B and C) and in airway epithelial cells from ceramide synthase 2 (CerS2)-null mice (Fig 1C). CerS2-null mice also displayed increased ceramide levels specifically in tracheal and bronchial epithelial cells (Fig 1D and Supplementary Fig S2), reminiscent of the elevated ceramide levels in these cells in CF mice and in CF patients (Fig 1D) (Teichgräber et al., 2008; Becker et al., 2010; Brodlie et al., 2010; Ulrich et al., 2010; Bodas et al., 2011). Thus, CerS2-null mice display similar changes in ceramide and SPH levels as in CF mice despite their different genetic alterations. AC inhalation increased surface SPH in bronchial epithelial cells of CF and CerS2-null mice (Fig 1C) with a concomitant reduction in ceramide levels (Fig 1D).

To quantify SPH levels in the lung, we established a number of innovative methods, including: (i) mass spectrometry (MS) and enzymatic assays for SPH and ceramide using extracts from freshly isolated tracheal epithelial cells, which detects total SPH levels in these cells in CF mice and in CF patients (Fig 1D) (Teichgräber et al., 2008; Becker et al., 2010; Brodlie et al., 2010; Ulrich et al., 2010; Bodas et al., 2011). Thus, CerS2-null mice display similar changes in ceramide and SPH levels as in CF mice despite their different genetic alterations. AC inhalation increased surface SPH in bronchial epithelial cells of CF and CerS2-null mice (Fig 1C) with a concomitant reduction in ceramide levels (Fig 1D).

To determine the mechanism by which SPH levels are decreased on the surface of CF tracheal epithelial cells, AC activity was analyzed by loading trachea with [14C]C16-ceramide and its consumption in vivo was analyzed. Significantly lower levels of AC activity were detected in CF mice (Fig 2D). Cfr regulates the pH in secretory lysosomes (Barash et al., 1991; Teichgräber et al., 2008) and probably also regulates the pH in small domains on the surface of tracheal epithelial cells that express proton pumps (Xu et al., 2012) by providing Cl- counterions for H+, thus permitting continuous activity of protons pumps. Moreover, since alkalization results in situ kinase assay on the luminal surface of intact trachea, which also detects SPH exclusively on the luminal surface. First, freshly isolated tracheal epithelial cells were extracted and SPH assayed using MS and enzymatic assays for SPH, demonstrating an approximately 75% reduction in total SPH levels in CF mice (Fig 2A). Next, an in situ enzyme assay, performed by application of SPH kinase (SK) and [32P]γATP directly to the luminal side of the intact tracheal epithelial cell layer, revealed an approximately 75% reduction in SPH levels (Fig 2B). The reduced SPH on the tracheal surface was confirmed by SPH immunoprecipitation using the anti-SPH antibody coupled to protein L-agarose beads, followed by lipid extraction and an enzymatic assay for SPH (Fig 2B). Application of AC to the surface of isolated CF trachea prior to the in situ enzyme assay normalized SPH levels (Fig 2B). Incubation of the isolated tracheal surface with 10 μM cytochalasin B (an actin filament polymerization inhibitor) prevented P. aeruginosa internalization into tracheal epithelial cells, but did not alter the amount of SPH detected by the in situ enzyme assay for SK or by SPH immunoprecipitation, excluding the possibility that SK and/or antibody internalization occurs during the assay (Fig 2B). These results demonstrate that SPH is present on the surface of WT epithelial cells while almost completely absent on the surface of CF epithelia.

We next demonstrated that AC or SPH inhalation increased SPH levels in CF tracheal epithelial cells and on the surface of CF trachea in vivo (Fig 2A and B).

Moreover, significant accumulation of ceramide was detected by mass spectrometry (MS) (Fig 2C, left) in extracts of isolated CF epithelial cells and by in situ kinase assay on the luminal surface of these cells in trachea of CF mice (Fig 2C, right), which was corrected by inhalation of AC (Fig 2C). The specificity of the enzyme assay was confirmed by treating isolated trachea with AC in vitro (Fig 2C, right).

To determine the mechanism by which SPH levels are decreased on the surface of CF tracheal epithelial cells, AC activity was analyzed by loading trachea with [14C]C16-ceramide and its consumption in vivo was analyzed. Significantly lower levels of AC activity were detected in CF mice (Fig 2D). Cfr regulates the pH in secretory lysosomes (Barash et al., 1991; Teichgräber et al., 2008), we next demonstrated that surface acidification of the trachea restored AC activity, increased surface sphingosine and decreased ceramide levels (Fig 2D). To test whether acid-mediated hydrolysis of ceramide contributes to its consumption and the generation of sphingosine, we performed the experiment in the presence of the AC inhibitors, carmofur, or oleoylthanolamine. The inhibitors completely prevented sphingosine generation excluding significant acid-mediated hydrolysis of ceramide (Fig 2D). It is important to note that the pH of small microdomains on the cell membrane of CF cells is independent of the airway liquid surface pH, which is decreased from pH 7.2 to pH 6.8 in CF cells (Pezzulo et al., 2012), which is probably not enough to alter AC activity.

Next, we examined whether sphingosine plays a role in infections with P. aeruginosa. We used three different P. aeruginosa strains to exclude any strain-specific effects. Upon intranasal infection, CF and CerS2-null mice displayed a dramatically increased sensitivity to P. aeruginosa strains 762 (Fig 3A), PA14, and ATCC 27853 (Fig 3B), with 10- to 100-fold more bacteria in the lung 3–4 h after infection (Fig 3A and B), clinical signs of severe pneumonia (Supplementary Fig S3A and B), massive release of cytokines such as interleukin-1β (IL-1β) (Fig 3C) and TNF-α (Supplementary Fig S4) and influx of leukocytes (Supplementary Fig S5A–H). Inhalation of CF mice with SPH to a level similar to that found in WT mice (Fig 2B), of AC or of FTY720 (a SPH analog in clinical use for treating multiple sclerosis) 1 h prior to P. aeruginosa infection, protected CF and CerS2-null mice from infection (Fig 3A–C and Supplementary Fig S6).
Figure 1.
Figure 2.
Supplementary Figs S3, S4 and S5). To test whether sphingosine and FTY720 are also able to cure an existing pulmonary infection, mice were inhaled with SPH or FTY720 1 h after infection, a stage at which the mice show the first signs of infection, with both completely blocking the development of infection (Fig 3A–C, Supplementary Figs S3, S4 and S5). The low bacterial numbers and the lack of cytokines and leukocyte influx demonstrate that sphingosine and FTY720 directly and rapidly kill the bacteria, thus preventing further inflammation.

Controls on non-infected mice showed that inhalation of SPH, AC, or FTY720 had no influence on pulmonary cytokines nor did it induce neutrophil influx 4 h, 1, 7, or 14 days after inhalation (Fig 3C, Supplementary Figs S4, S5A, B, E, F, and S6A–C).

P. aeruginosa was next incubated in vitro with a number of natural and synthetic sphingoid LCBS. A number of LCBS inhibited bacterial growth with EC50 values of 0.3–2.2 μM, including the plant LCB, phytosphingosine, natural-sphingosine stereoisomers of SPH, and dihydrosphingosine (spinganine), as well as LCBS which have recently been discovered to occur at low levels in mammals (Pruett et al, 2008) (Table 1). A number of other LCBS also inhibited bacterial growth with a somewhat higher EC50 value, while other LCBS were without effect at concentrations as high as 50 μM (Table 1). These results demonstrate a notable specificity of the effect of LCBS on bacterial growth, providing structural information that could be used in development of novel LCBS-based drugs to treat lung infection. To investigate whether other bacteria that affect CF patients are also susceptible to LCBS treatment, we examined the effect of sphingosine on Acinetobacter baumannii, Haemophilus influenzae, Burkholderia cepacia, and Moraxella catarrhalis. Sphingosine inhibited growth of and/or killed Acinetobacter baumannii with an EC50 of 0.07 ± 0.05 μM, of Moraxella catarrhalis with an EC50 of 0.4 ± 0.004 μM of Haemophilus influenzae with an EC50 of 4.8 ± 0.49 μM and Burkholderia cepacia with an EC50 of 45 ± 6.3 μM. The relative resistance of Burkholderia cepacia might lie in its outer membrane unique composition (Cox & Wilkinson, 1991).

The results presented herein uncover a major innate defense mechanism of healthy airways, namely the bactericidal effect of lung and tracheal epithelial SPH. Upon loss of surface SPH, individuals become susceptible to P. aeruginosa infection, as exemplified in CF patients and mice, and in CerS2-null mice. Moreover, restoration of surface SPH by inhalation of SPH, of other LCBS or with AC, reverses susceptibility and cures existing P. aeruginosa infection, suggesting that LCB inhalation might provide a novel therapeutic option to counteract pulmonary infection by bacteria. This is of great importance since many P. aeruginosa strains are multi-resistant to antibiotics, rendering pulmonary infections difficult to treat. Acidification of the trachea, which increases AC activity, leads to a reduction in ceramide levels and an increase in sphingosine levels, consistent with the clinical use of hypertonic saline (pH 5.0–5.5) in treating CF patients (Elkins et al, 2006).

Ours is not the first study to identify SPH as an anti-bacterial agent (Bibel et al, 1992; Arikawa et al, 2002; Fischer et al, 2013). However, it is the first to show that LCBS are efficacious in acute lung infection. The mechanism of bacterial cell death may involve a direct effect of the LCBS on the bacteria (Fischer et al, 2012), or possibly up-regulation of porin-like proteins that are responsible for channel formation in the bacterial membrane (LaBauve & Wargo, 2014). Information obtained by comparing LCBS structures suggests that longer and more positively charged LCBS are more effective than shorter, negatively charged LCBS; moreover, modification of the C-1 position of the LCBS has variable effects on bacterial survival. Based on this structural information, we predict that it will be possible to generate LCBS that are highly efficacious in killing bacteria without causing effects on the host. If this notion is correct, then these putative novel LCBS analogs could pave the way for generation of drugs used for inhalation against P. aeruginosa infection and possibly infection by other bacteria.

Materials and Methods

Mice

CerS2-null and CF mice were generated as described (Charizopoulou et al, 2006; Pewzner-Jung et al, 2010). Two different CF mouse strains were used, Cfrm1104-Tg(CFTR), abbreviated CfrKO (mice that lack Cfr but express human CFTR in the gut under the control of a fatty acid binding protein promoter), obtained from Jackson Laboratories (Bar Harbor, ME, USA) and backcrossed for 10 generations onto a C57BL/6 background. All data shown in the current paper are from CfrKO mice. To confirm data obtained with this strain, we used an additional strain, that is B6.129P2(CF/3)-CfrTg(neo;m1H6K) (abbreviated CfrABH) congenic mice that were established by brother–sister mating from the original CfrTg(neom;m1H6K) mutant.
Figure 3.
mouse that was generated using insertional mutagenesis in Cfr
exon 10. Congenic Cfr<sup>ATM</sup> mice were generated by backcrossing the targeted mutation onto the B6 inbred background. The strain produces low levels of Cfr. Cfr-null mice were on an F1 background achieved by intercross of heterozygous Cfr<sup>ATM/+</sup>; C57BL/6 mice (GT = gene trap) and heterozygous Cfr<sup>ATM/+</sup>; 129S4/SvJae mice, to generate Cfr<sup>ATM/GT</sup> F1, and Cfr<sup>ATM</sup> F1 mice used in the experiments as Cfr-null and wild-type control, respectively.

Control mice were syngenic littermates (C57BL/6 for CF mice and C57BL/6;129S4/SvJae WT for Cfr-null mice). The cystic fibrosis and the corresponding wild-type mice were used at an age of 16–18 weeks. We used female CF and WT mice in the present study. The Cfr-deficient mice were both male and females and used at an age of 6–8 weeks. Mice were bred in a special pathogen free (SPF) facility at the Weizmann Institute of Science and in the University of Duisburg-Essen. Mice were handled according to protocols approved by the Weizmann Institute of Science and the University of Duisburg-Essen Animal Care Committee as per international guidelines.

**Immunohistochemical analysis of ceramide and SPH and light microscopy**

Animals were sacrificed, lungs or trachea immediately removed, fixed for 36 h in 4% paraformaldehyde (PFA) in phosphate buffer saline (PBS), embedded in paraffin, and trimmed to 6 μm. Sections were dewaxed, incubated for 30 min with Pepsin Digest All (Invitrogen Life Technologies, USA) at 37°C, washed, and blocked for 10 min with PBS, 0.05% Tween-20, and 5% fetal calf serum (FCS). Sections were subsequently immunostained for 45 min with an anti-ceramide antibody (clone 58S-9, Glycobiotech, Germany), an anti-SPH antibody (clone NHSPH, Alfresa Pharma Corporation, Japan), or an anti-Gr1-antibodies (BD, Germany). Anti-ceramide antibodies were diluted 1:100, anti-SPH antibodies 1:100, anti-Gr1 antibodies 1:200 in H/S (132 mM NaCl, 20 mM HEPES, pH 7.4, 5 mM KCl, 1 mM CaCl<sub>2</sub>, 0.7 mM MgCl<sub>2</sub>, 0.8 mM MgSO<sub>4</sub>) plus 1% FCS, washed three times in PBS plus 0.05% Tween-20, incubated for 45 min with Cy3-coupled donkey anti-mouse IgM F(ab)2 fragments or Cy3-coupled donkey anti-rat IgG (Jackson ImmunoResearch), washed again three times in PBS containing 0.05% Tween-20 and once in PBS, and embedded in coverslip mounting solution (Mowiol). Immunostained samples were analyzed on a confocal Leica TCS SL or SP5 microscope (Leica, Germany). Immunofluorescence was analyzed using Leica software, version 2.61, in 10–25 areas per sample, by measuring the fluorescence in the apical one-third of the bronchial or tracheal epithelial cells.

To exclude binding of the anti-SPH antibody to sphinganine, the trachea from WT or CF mice were removed, incubated in vitro with 10 μM sphinganine in 150 mM sodium acetate (pH 7.4) for 30 min, washed three times in 150 mM sodium acetate (pH 7.4), fixed for 36 h in 2% PFA (pH 7.3), and stained with the anti-SPH antibody. Hemalaun was used to stain lung sections for light microscopy.

**Mouse inhalation**

Inhalation was performed using a PARI Boy SX nebulizer (PARI GmbH, Starnberg, Germany), which generates a fine aerosol by pumping the fluid with an air jet. Mice were inhaled with the aerosol via a mask that is part of an oral inhalation device for children (LL-Nebulizer); the mask was clamped at the sides to cover only the nose and the surrounding part of the face. Mice were inhaled with 800 μl of 0.9% NaCl containing SPH (125 μM), AC (80 μg of purified protein), or FTY720 (125 μM). Approximately 10% of the volume that is applied to the mice is inhaled.

**Enzymatic assays to measure ceramide and SPH analysis in situ**

Mice were sacrificed and the trachea immediately removed. The trachea was carefully opened, washed in 150 mM sodium acetate (pH 7.4), placed on a 30°C pre-warmed plastic plate, and incubated with 0.01 units diacylglycerol (DAG) kinase (Biomol, Germany) to measure ceramide, or with 0.001 units SK (R&D Systems, Germany) to measure SPH, in 4 μl of 150 mM sodium acetate (titrated to pH 7.4), 1 mM adenosine triphosphate (ATP), and 10 μCi [<sup>32</sup>P]γ-ATP (for the buffer composition see also Supplementary Fig S7). Controls were incubated with the same buffer without SK or left untreated. We ensured that the kinase buffer was only added to the luminal surface of the trachea. The kinase reaction for ceramide was performed for 15 min at 30°C and terminated by transfer of the trachea into CHCl<sub>3</sub>/CH<sub>3</sub>OH:1N HCl (100:100:1, v/v/v) for 30 min and 30 μl of 100 mM EDTA. The SK reaction was terminated by placing the trachea in 100 μl H<sub>2</sub>O, followed by addition of 20 μl 1N HCl, 800 μl CHCl<sub>3</sub>/CH<sub>3</sub>OH:1N HCl (100:200:1, v/v/v), 240 μl CHCl<sub>3</sub>, and 2 M KCl. The lower phase was collected, dried, dissolved in 20 μl of CHCl<sub>3</sub>/CH<sub>3</sub>OH (1:1, v/v), and separated on silica G60 thin layer chromatography (TLC) plates using CHCl<sub>3</sub>/acetone/CH<sub>3</sub>OH/acetic acid/H<sub>2</sub>O (50:20:15:10:5, v/v/v/v/v) as developing solvent for ceramide and CHCl<sub>3</sub>/CH<sub>3</sub>OH/acetic acid/H<sub>2</sub>O (90:90:15:10:5, v/v/v/v/v) for SPH. The TLC plates were exposed to radiography films, spots were removed from the plates, and the incorporation of [<sup>32</sup>P] into ceramide measured by liquid scintillation counting. Ceramide and SPH were determined using a standard curve of C16-ceramides to C24-ceramides or C18-SPH. Surface pH was varied by adjusting the pH of the 150 mM sodium acetate buffer from 7.4 to 5.0 and incubation of the trachea for 20 min prior to the in situ kinase assay. To exclude acid-mediated lysis of ceramide, we pre-incubated the trachea for 15 min with 0.5 mM oleoylthanolamine (Calbiochem) or 1 μM carmofur (Sigma) prior to adjusting the pH.
Sphingosine prevents lung infection  Yael Peuzner-Jung et al

SPH analysis in extracts of freshly isolated tracheal epithelial cells

Epithelial cells were removed from the trachea by carefully scraping the inner surface of the trachea. Cells were extracted in CHCl₃/CH₃OH/1N HCl (100:200:1, v/v/v), and the lower phase was dried and resuspended in a detergent solution (7.5% [w/v] n-octyl glucopyranoside, 5 mM cardiolipin in 1 mM diethylenetriaminepentaacetic acid [DTPA]), and sonicated for 10 min. The kinase reaction was started by addition of 70 μl of a reaction mixture containing 2 μl SK in 50 mM HEPES (pH 7.4), 250 mM NaCl, 30 mM MgCl₂, 1 mM ATP, and 10 μCi [³²P]γATP. The kinase reaction was terminated after 30 min, and samples extracted and processed as above.

Mass spectrometry

Ceramides and SPH in isolated tracheal epithelial cells were extracted and quantified as described (Fayyaz et al., 2014). Sample analysis was carried out by rapid-resolution liquid chromatography-mass spectrometry (Agilent Technologies, Waldbronn, Germany) operating in the positive ESI mode. The precursor ions of SPH (m/z 300.289), C17-SPH (m/z 286.274), and ceramides (C16-ceramide (m/z 520.508), C17-ceramide (m/z 534.524)), C18-ceramide (m/z 548.540), C18:1-ceramide (m/z 546.524), C20-ceramide (m/z 576.571), C22-ceramide (m/z 604.602), C24-ceramide (m/z 632.634), and C24:1-ceramide (m/z 630.618)) were cleaved into the fragment ions of m/z 282.280, m/z 268.264, and m/z 264.270, respectively. Quantification was performed with Mass Hunter Software (Agilent Technologies).

Enzyme assays of SPH immunoprecipitates

The luminal epithelial surface of the trachea was incubated with 50 ng of the anti-SPH antibody, that was pre-coupled to protein L-agarose beads (Santa Cruz Inc., Heidelberg, Germany), at 4°C for 30 min in 150 mM sodium acetate (pH 7.4) plus 1% FCS. Alternatively, the antibody was bound to the surface of the trachea by incubation at 4°C for 20 min in 150 mM sodium acetate (pH 7.4) plus 1% FCS. Trachea was washed three times in 150 mM sodium acetate (pH 7.4), and L-agarose was added. Samples were incubated for an additional 20 min, washed six times with ice-cold H/S buffer, and lysed in 125 mM NaCl, 25 mM Tris–HCl (pH 7.4), 10 mM EDTA, 10 mM sodium pyrophosphate, 3% Nonidet P-40, and 10 μg/ml aprotinin and leupeptin for 10 min on ice. Insoluble tissue was removed, and the agarose beads washed five times in lysis buffer. The beads were pelleted, the supernatants discarded, the beads were resuspended in 200 μl H₂O and extracted using 600 μl CHCl₃/CH₃OH/1N HCl (100:200:1, v/v/v). The lower organic phase was collected, dried, resuspended in 20 μl of a detergent solution (7.5% [w/v] n-octyl glucopyranoside, 5 mM cardiolipin in 1 mM diethylenetriaminepentaacetic acid [DTPA]), and sonicated for 10 min. The kinase reaction was started by addition of 70 μl of a reaction mixture containing 2 μl SK in 50 mM HEPES (pH 7.4), 250 mM NaCl, 30 mM MgCl₂, 1 mM ATP, and 10 μCi [³²P]γATP. The kinase reaction was terminated after 30 min, and samples extracted and processed as above.

Cytochalasin B treatment

Trachea was incubated with 10 μM cytochalasin B for 20 min prior to the kinase assay or prior to SPH immunoprecipitation experiments. Surface SPH was measured by the in situ kinase assay or by immunoprecipitation as above. As controls, trachea was incubated with cytochalasin B prior to infection with 1 × 10⁶ CFU P. aeruginosa ATCC 27853. The trachea was washed after infection, incubated for 60 min with 100 μg/ml polymixin, washed again, lysed in 5 mg/ml saponin for 10 min, and centrifuged (10 min, 1,600 g). Pellets were resuspended in H/S buffer, aliquots were plated on TSA, and grown overnight prior to quantification of the number of intracellular bacteria.

AC activity assay

Mice were anesthetized with ketamine and xylazine and 4 μl [¹⁴C] C₁₆-ceramide (ARC0831, 55 mCi/mmol) injected into the lumen of the trachea. Prior to injection, [¹⁴C]C₁₆-ceramide was dried, resuspended in 0.5% OGP in 150 mM sodium acetate (pH 7.4 or 5.0), and bath sonicated for 10 min. After 20–30 min, mice were sacrificed, the trachea was carefully removed and extracted in 200 μl H₂O and CHCl₃/CH₃OH/HCl (100:100:1, v/v/v). The lower phase

Table 1. Effect of sphingoid LCBs on P. aeruginosa growth in vitro.

| Long chain base                          | EC₅₀ (μM) |
|------------------------------------------|-----------|
| Inhibitors                               |           |
| 1-erythro-C₂₀-sphingosine                 | 0.3 ± 0.1 |
| 1-threo-C₁₈-dihydroxyphospho-SPH(Safingol)| 0.4 ± 0.1 |
| 3-deoxy-1-erythro-C₁₈-sphingosine         | 0.5 ± 0.1 |
| 1-threo-C₁₈-sphingosine                   | 0.6 ± 0.1 |
| 1-erythro-C₁₈-dihydroxyphospho-SPH(Safingol)| 0.6 ± 0.2 |
| 1-deoxy-1-erythro-C₁₈-dihydroxyphospho-SPH(Safingol)| 0.7 ± 0.0 |
| 1-desoxymethyl-C₁₈-sphingosine            | 0.7 ± 0.1 |
| 1-erythro-C₁₈-sphingosine                 | 0.8 ± 0.1 |
| 1-erythro-C₁₈-sphingosine                 | 0.8 ± 0.1 |
| 1-deoxy-1-erythro-C₁₈-sphingosine         | 0.9 ± 0.1 |
| Phosphysphingosine                        | 0.9 ± 0.4 |
| 1-erythro-C₁₈-sphingosine                 | 1.0 ± 0.2 |
| FTY720                                   | 1.9 ± 0.4 |
| Monomethyl 1-erythro-C₁₈-sphingosine       | 1.9 ± 0.1 |
| Galactosyl 1-erythro-C₁₈-sphingosine (psychosine) | 2.2 ± 1.4 |

Non-inhibitors

| Long chain base                          | Value     |
|------------------------------------------|-----------|
| 1-erythro-C₁₂-sphingosine                 | > 50      |
| 1-erythro-C₁₄-sphingosine                 | > 50      |
| N-acetyl-1-erythro-C₁₈-dihydroxyphospho-SPH(Safingol)| > 50      |
| Fumonisin B1                              | > 50      |
| Sphingosine-1-phosphate                   | > 50      |
was dried, samples were resuspended in CHCl₃:CH₃OH (1:1, v/v) and separated by TLC using CHCl₃:CH₃OH:ammoniumhydroxide (90:20:0.5, v/v/v) as the developing solvent. The TLC plates were analyzed using a Fuji-Imager.

**Bacterial infection**

P. aeruginosa strains 762, PA14, and ATCC 27853 were grown for 14–14.5 h on fresh tryptic soy agar (TSA) plates (Becton Dickinson Biosciences, Germany). Bacteria were then transferred into 40 ml of pre-warmed, sterile tryptic soy broth (TSB) (Becton Dickinson Biosciences) in Erlenmeyer flasks. The OD was adjusted to 0.225, and the bacteria were grown for 60 min at 37°C with shaking at 125 rpm to obtain bacteria in the early log phase. Bacteria were centrifuged (10 min, 1,600 g), the supernatant removed, and bacteria resuspended in pre-warmed H/S buffer (132 mM NaCl, 20 mM HEPES, pH 7.4, 5 mM KCl, 1 mM CaCl₂, 0.7 mM MgCl₂, 0.8 mM MgSO₄). The OD was determined, and the bacteria diluted in pre-warmed H/S to a concentration of 1 × 10⁸ CFU per 20 µl. This dose was previously shown to induce a severe infection in CF-mice, but only a mild infection in C57BL/6 mice (Teichgräber et al., 2008). Mice were anesthetized for 10–15 s, and bacteria carefully injected into the nose with a 30-gauge 1-ml syringe. The needle was covered with a tightly fitting, smooth plastic tube so that nasal injuries were avoided. Lungs were subsequently removed and homogenized and lysed for 10 min in 5 mg/ml saponin to release intracellular bacteria. Samples were centrifuged (10 min, 1,600 g), washed once with sterile H/S solution, and homogenates cultured on TSA plates. The number of bacteria (colony forming units, CFU) was analyzed after 18 h.

**Incubation of bacteria with sphingolipids**

The clinical P. aeruginosa strain 762 or clinical strains of Acinetobacter baumannii, Moraxella catarrhalis, and Burkholderia cepacia were grown in LB medium at 37°C until the early logarithmic phase (OD₅₃₀ of 0.2). Bacteria were diluted in PBS (without Ca²⁺ and Mg²⁺) to a concentration of 1 × 10⁸ CFU/ml. Haemophilus influenzae was grown on chocolate agar overnight and directly added to H/S, since it was not viable in PBS for 2 h. Stock solutions of sphingolipids in 5 mM in 7.5% n-octyl-β-D-glucopyranoside (OGP) or in dimethyl sulfoxide (DMSO) were prepared by sonication and warming to 40°C. SLs were added to the bacteria (0.025–200 µM in 0.075% OGP or 1% DMSO). Bacteria were incubated with SLs for 2 h at 37°C, and 200 µl of a bacteria solution plated on LB agar plates, incubated overnight at 37°C, and colonies counted.

**Interleukin-1β (IL-1β) and TNF-α measurements**

Lungs were removed 4 h after infection of mice or from uninfected mice, shock-frozen, lysed in 125 mM NaCl, 25 mM Tris–HCl (pH 7.4), 10 mM EDTA and 1% NP-40, and homogenized using a tip sonicator. Aliquots were analyzed for IL-1β and TNF-α by commercial ELISA kits (R&D).

**Statistics**

Data are means ± s.d. unless otherwise indicated. All data were tested with the David-Pearson-Stephens-test for normal distribution. All exact P-values are indicated in the figures and were calculated using a two-tailed distribution with a two sample equal or unequal variance Student’s t-test (when the same sample or strains of mice were compared in different conditions or when different samples or strains of mice where compared in the same conditions, respectively). 0.05 was considered statistically significant.

**Supplementary information** for this article is available online: http://embomolmed.embopress.org

**Acknowledgements**

This study was supported by grant number 1105-69.11/2010 from the German-Israeli-Foundations to A.H.F. and E.G., DFG grant 335/16-2 to E.G., BMBF grant 0315827B to B.T. and E.G., and NIH grant DK25809 to E.S.A.H. Futerman is the Joseph Meyerhoff Professor of Biochemistry at the Weizmann Institute of Science.

**Author contributions**

STT performed the sphingolipid immunofluorescence experiments on human tissues and on some CF mouse tissues, KAB and HG performed confocal microscopy studies, TJ tested the effects of LCBs on P. aeruginosa growth in vitro, SL contributed human samples, BT contributed mice and P. aeruginosa strains, ES purified and provided human recombinant acid ceramidase, and KAB contributed to the in situ kinase assays. LJ and BK performed MS. MJÆ and ABL contributed to ECA measurements, JS characterized bacteria. YPJ and EG performed all other experiments. YPJ, EG, MJÆ, and AHF...
Sphingosine prevents lung infection

Yael Pewzner-jung et al

designed the study and wrote the manuscript. All authors commented on the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References

Arikawa J, Ishibashi M, Kawashima M, Takagi Y, Ichikawa Y, Imokawa G (2002) Decreased levels of sphingosine, a natural antimicrobial agent, may be associated with vulnerability of the stratum corneum from patients with atopic dermatitis to colonization by Staphylococcus aureus. J Invest Dermatol 119: 433 – 439

Barasch J, Kiss B, Prince A, Saiman L, Gruenert D, al-Awqati Q (2003) Ionizing groups in lipopolysaccharides of Pseudomonas aeruginosa bacteria in the murine lung. Nature 427: 1070 – 1073

Becker KA, Riethmüller J, Lüth A, Döring G, Kleuser B, Gulbins E (2001) Acidification of intracellular organelles in cystic fibrosis. Nature 412: 496 – 499

Bodas M, Min T, Mazur S, Vij N (2011) Critical modifier role of membrane-cytosolic ceramide transport conductance regulator-dependent ceramide signaling in lung injury and emphysema. J Immunol 186: 602 – 613

Brodie M, McKeen MC, Johnson CE, Gray J, Fisher AJ, Corris PA, Lordan JL, Ward C (2010) Ceramide is increased in the lower airway epithelium of people with advanced cystic fibrosis lung disease. Am J Respir Crit Care Med 182: 369 – 375

Charizopoulou N, Wilke M, Dorsch M, Bot A, Jorna H, Jansen S, Stanke F, al-Awqati Q, Wilke M, Bot A, Jorna H, Jansen S, Stanke F (2010) Spontaneous escape from cystic fibrosis in a mouse model. BMC Genet 11: 18

Cox AD, Wilkinson SG (1993) Ionizing groups in lipopolysaccharides of Pseudomonas cepacia in relation to antibiotic resistance. Mol Microbiol 5: 641 – 646

Crouch Brewer S, Wunderink RG, Jones CB, Leeper KV (2010) Ventilator-associated pneumonia due to Pseudomonas aeruginosa. Chest 137: 1009 – 1029

Cystic Fibrosis Foundation (2011) 2011 Patient Registry Annual Data Report. Bethesda, MD: Cystic Fibrosis Foundation

Elkins MR, Robinson M, Rose BR, Harbour C, Moriarty CP, Marks GB, Belousova EG, Xuan W, Bye PT National Hypertonic Saline in Cystic Fibrosis (NHSCF) Study Group (2006) A controlled trial of long-term inhaled hypertonic saline in patients with cystic fibrosis. N Engl J Med 354: 229 – 240

Fayyaz S, Henkel J, Japtok L, Krämer S, Damm G, Seehofer D, Puschel GP, Kleuser B (2014) Involvement of sphingosine 1-phosphate in palmitate-induced resistance of hepatocytes via the S1P2 receptor subtype. Diabetologia 57: 373 – 382

Fischer CL, Drake DR, Dawson DV, Blanchette DR, Brogden KA, Wertz PW (2012) Antibacterial activity of sphingoid bases and fatty acids against Gram-positive and Gram-negative bacteria. Antimicrob Agents Chemother 56: 1157 – 1161

Fischer CL, Walters KS, Drake DR, Blanchette DR, Dawson DV, Brogden KA, Wertz PW (2013) Sphingoid bases are taken up by Escherichia coli and Staphylococcus aureus and induce ultrastructural damage. Skin Pharmacol Physiol 26: 36 – 44

LaBauve AE, Wargo MJ (2014) Detection of host-derived sphingosine by Pseudomonas aeruginosa is important for survival in the murine lung. PLoS Pathog 10: e1003889

Li CM, Hong SB, Kopal G, He X, Linke T, Hou WS, Koch J, Gatt S, Sandhoff K, Schuchman EH (1998) Cloning and characterization of the full-length cDNA and genomic sequences encoding murine acid ceramidase. Genomics 50: 267 – 274

McManus AT, Mason AD, McManus WF, Pruitt BA (1985) Twenty-five year review of Pseudomonas aeruginosa bacteremia in a burn center. Eur J Clin Microbiol 4: 219 – 223

Pewzner-Jung Y, Park H, Laviad EL, Silva LC, Lahiri S, Stiban J, Erez-Roman R, Brugger B, Sachsenheimer T, Wieland F et al (2010) A critical role for ceramide synthase 2 in liver homeostasis: I. alterations in lipid metabolic pathways. J Biol Chem 285: 10902 – 10910

Pezzullo AA, Tang XX, Hoeegger MJ, Alaiwa MHA, Ramachandran S, Moninger TO, Karp PH, Wohlfird-Lenane CL, Haagsman HP, van Eijk M et al (2012) Reduced airway surface pH impairs bacterial killing in the porcine cystic fibrosis lung. Nature 487: 109 – 113

Pruett ST, Bushnev A, Hagedorn K, Adiga M, Haynes CA, Sullards MC, Liotta DC, Merrill AH (2008) Biodiversity of sphingoid bases (sphingosines) and related amino alcohols. J Lipid Res 49: 1621 – 1639

Rakhimova E, Wiehllman L, Brauer AL, Sethi S, Murphy TF, Tüümmler B (2009) Pseudomonas aeruginosa population biology in chronic obstructive pulmonary disease. J Infect Dis 200: 1928 – 1935

Teichgräber V, Ulrich M, Endlich N, Riethmüller J, Wilker B, De Oliveira-Munding CC, van Heeckeren AM, Barr ML, von Kurthy G, Schmid KW et al (2008) Ceramide accumulation mediates inflammation, cell death and infection susceptibility in cystic fibrosis. Nat Med 14: 382 – 391

Ullrich M, Worlitzsch D, Viggio S, Siegmund N, Iadarola P, Shute JK, Geiser M, Pier GB, Friedel G, Barr ML et al (2010) Alveolar inflammation in cystic fibrosis. J Cyst Fibros 9: 217 – 227

Vidal F, Mensa J, Almela J, Martínez JA, Marco F, Casals C, Gatell JM, Soriano E, Jimenez de Anta MT (1996) Epidemiology and outcome of Pseudomonas aeruginosa bacteremia, with special emphasis on the influence of antibiotic treatment. Analysis of 189 episodes. Arch Intern Med 156: 2121 – 2126

Xu M, Xia M, Li X-X, Han W-Q, Boini KM, Zhang F, Zhang Y, Ritter JK, Li P-L (2012) Requirement of translocated lysosomal V1, H(+)-ATPase for activation of membrane acid sphingomyelinase and raft clustering in coronary endothelial cells. Mol Biol Cell 23: 1546 – 1557

License: This is an open access article under the terms of the Creative Commons Attribution 4.0 License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.