Original Paper

Lack of Sphingosine Causes Susceptibility to Pulmonary *Staphylococcus Aureus* Infections in Cystic Fibrosis

Shaghayegh Tavakoli Tabazavareh^a Aaron Seitz^b Peter Jernigan^{a,b} Carolin Sehl^a
Simone Keitsch^a Stephan Lang^c Barbara C. Kahl^d Michael Edwards^b
Heike Grassmé^a Erich Gulbins^{a,b} Katrin Anne Becker^a

^aDepartment of Molecular Biology, University Hospital Essen, University of Duisburg-Essen, Essen, Germany; ^bDepartment of Surgery, University of Cincinnati, Cincinnati, USA; ^cDepartment of Otorhinolaryngology, University Hospital Essen, Essen, Germany; ^dInstitute of Medical Microbiology, University Hospital Münster, Münster, Germany

Key Words

Sphingosine • Ceramide • *Staphylococcus aureus* • Pneumonia • Cystic fibrosis

Abstract

Background: Pulmonary *Staphylococcus aureus* (*S. aureus*) infections occur early in a high percentage of cystic fibrosis (CF) patients and it is believed that these infections facilitate further colonization of CF lungs with *Pseudomonas aeruginosa* (*P. aeruginosa*). Previous studies demonstrated a marked reduction of sphingosine in tracheal and bronchial epithelial cells in CF compared to wild type mice, while ceramide is massively increased in CF mice. **Methods:** We investigated the effect of C18-sphingosine and C16-ceramide on *S. aureus in vitro*. Based on our results we performed pulmonary infections with *S. aureus* and tested the influence of sphingosine inhalation. **Results:** *In vitro* incubation of *S. aureus* with C18-sphingosine rapidly killed *S. aureus*, while C16-ceramide did not affect bacterial survival, but abrogated the effect of C18-sphingosine when applied together. The *in vivo* infection experiments revealed a high susceptibility of CF mice to pulmonary infection with *S. aureus*. Inhalation of C18-sphingosine rescued CF mice from pulmonary infections with different clinical *S. aureus* isolates, including a methicillin-resistant *S. aureus* (MRSA) strain. **Conclusions:** Our data indicate that the imbalance between ceramide and sphingosine in the CF respiratory tract prevents killing of *S. aureus* and causes the high susceptibility of CF mice to pulmonary *S. aureus* infections.

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Introduction

Cystic fibrosis (CF) is caused by mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) and is one of the most common autosomal recessive disorders in Western populations [1-3]. The genetic defect results in a multisystem disease, but chronic pulmonary inflammations and infections are the major cause of morbidity and are responsible for the reduced life expectancy of CF patients. The most common microorganisms in CF lungs are *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Haemophilus influenzae* (*H. influenzae*) and *Stenotrophomonas maltophilia* [4]. The cause of the high susceptibility of CF patients to pulmonary infections is still incompletely understood.

Several studies in recent years demonstrated that sphingolipids play a crucial role in the pathogenesis of CF [5-11]. We previously showed an age-dependent accumulation of ceramide in the respiratory tract of uninfected *Cftr*-deficient mice, which mediates inflammation and susceptibility to pulmonary *P. aeruginosa* infections [5, 12]. Analysis of human samples confirmed the accumulation of ceramide in the respiratory tract of CF patients, including bronchial epithelial cells, submucosal glands, and alveolar type II epithelial cells [5-10]. Further, we have previously demonstrated a marked reduction of sphingosine, a metabolite of ceramide, in the respiratory tract of CF mice compared to wild type (WT) mice [13]. Ceramide accumulation and deficiency of sphingosine in CF airways is due to an imbalance between ceramide formation and degradation, caused by alkalization of intracellular secretory lysosomes and small surface domains of CF cells [5, 13-15]. Deficiency of *Cftr* may result in a reduced flow of Cl⁻ ions into lysosomes and possibly small domains on the cell surface preventing accumulation of H⁺ and thereby resulting in alkalization of secretory lysosomes and surface domains in CF epithelial cells. This increase of pH almost completely prevents activity of acid ceramidase (responsible for ceramide hydrolysis), while the activity of the acid sphingomyelinase (responsible for ceramide formation) is only partly affected [5, 16]. The imbalance in the activities of these two enzymes ultimately results in ceramide accumulation and sphingosine deficiency in CF cells [13].

Our studies have demonstrated that the presence of sphingosine in the airway epithelium is required for the host defense against pulmonary *P. aeruginosa* infections [13]. This is consistent with previous studies that demonstrated antimicrobial activity of sphingosine in the skin and in the oral mucosa [17-19].

Here we investigated the bactericidal effect of sphingosine and its role in pulmonary infections with *S. aureus*. We analyzed the effect of sphingosine, ceramide or a combination of both lipids on bacterial survival. We confirmed these *in vitro* data *in vivo* by inhalation of C18-sphingosine prior to pulmonary infection with different *S. aureus* strains. The data show that the presence of sphingosine is critical for the pulmonary defense against *S. aureus* as its reconstitution in CF mice protects these mice from pulmonary infection with *S. aureus*.

Material and methods

Mice

We employed two CF mouse strains: *Cftr*^{tm1Unc-Tg^{FABP-CFTR}} (abbreviated *Cftr*^{KO}) and *B6.129P2(CF/3)-Cftr*^{TgH(neoim)Hgu} (abbreviated *Cftr*^{MHH}) generated as previously described [20-25]. All mice were bred and housed in a special pathogen free facility at the University of Duisburg-Essen, Germany. Their hygienic status was repeatedly tested against a panel of common murine pathogens according to the 2002 recommendations of the Federation of European Laboratory Animal Science Associations (FELASA). Mice were handled according to protocols approved by the University of Duisburg-Essen Animal Care Committee as per international guidelines (Az 84-02.04.2013.A282). All mice were used after 16 weeks of age to ensure full manifestation of CF disease.

Human samples

Polyyps were surgically removed from the paranasal sinuses of patients with and without CF after having given informed consent. Surgical interventions were performed at the Department of Otorhinolaryngology,

University Hospital Essen. The polyps were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich, Steinheim, Germany) in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 7 mM CaCl₂, 0.8 mM MgSO₄, 1.4 mM KH₂PO₄, and 6.5 mM Na₂HPO₄, pH 7.3) for 36 hrs and then embedded in paraffin.

Bacteria

In vivo and *in vitro* infections were performed with the clinical *S. aureus* strain E25 isolated from sputum of a patient with CF [26], a septic strain (*septic*) isolated from a patient with sepsis [27] and a clinical MRSA strain USA 300 isolated from a burn patient (Cincinnati).

All *S. aureus* strains were grown on fresh trypticase soy agar (TSA) plates supplemented with 5% sheep blood (Becton Dickinson Biosciences, Heidelberg, Germany). The plates were incubated for 16 hrs at 37°C. The next day, bacteria were transferred into 40 mL of pre-warmed, sterile trypticase soy broth (Becton Dickinson Biosciences). The optical density was adjusted to 0.25 (at 550 nm), and the bacteria were grown for 1 hr at 37°C with shaking at 125 rpm to obtain bacteria in the early logarithmic phase. Bacteria were centrifuged (10 min, 870 g), the supernatant removed, and bacteria were resuspended and diluted in pre-warmed airway surface liquid (ASL) buffer (consisting of 10 mM HEPES, 63 mM NaCl, 2.4 mM KCl, 1.2 mM CaCl₂, 1.9 mM MgSO₄, pH 6.6, 6.8, or 7.2, respectively) [28] or in Hepes/saline (H/S, consisting of 20 mM HEPES, 132 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 0.7 mM MgCl₂, 0.8 mM MgSO₄, pH 7.4).

Treatment of bacteria *in vitro*

To determine the effect of ceramide and sphingosine on *S. aureus* strain E25 *in vitro*, the bacteria were grown as above and diluted to 5 × 10³ colony forming units (CFU) in ASL buffer. Stock solutions of sphingolipids (C18-sphingosine, C18-sphingosine 1-phosphate and C16-ceramide, all from Avanti Polar Lipids, Inc., Alabama, USA) in Octyl β-D-glucopyranoside solution (OGP, Sigma-Aldrich, Steinheim, Germany) were prepared by sonication (to gain micelles) and added to the bacteria. The buffer or OGP alone served as controls. Bacteria were incubated with the different sphingolipids for 1 hr at 37°C, following which aliquots of the bacterial suspensions were plated on LB agar plates, incubated overnight at 37°C and colonies were counted.

Infection of mice

Mice were anesthetized with ether for 10–15 seconds and 2.5 × 10⁸ CFU of the *S. aureus* strain E25, or 1 × 10⁸ CFU of the *S. aureus* strains *septic* or MRSA in 20 μL H/S were 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.

Mice were infected once with *S. aureus* strains *septic* and MRSA. The infection with the strain E25 was repeated after 24 hrs. Animals were sacrificed by cervical dislocation 4 hrs after the final infection, and the lung was removed under sterile conditions, homogenized, lysed in 5 mg/mL saponin for 10 min at 37°C, washed in RPMI-1640 supplemented with 10 mM HEPES, resuspended in the same buffer and aliquots were plated on TSA plates for overnight growth.

Mouse inhalation

Inhalation was performed 30–40 min prior to infection using a PARI Boy SX nebulizer (PARI GmbH, Starnberg, Germany) as previously described [13]. Mice inhaled 800 μL of 0.9% NaCl containing C18-sphingosine (125 μM; dissolved in 0.6% OGP) or the corresponding amount of OGP as a control. C18-sphingosine and OGP were ultrasound treated in a sonication bath for 10 min directly before use.

Immunohistochemical analysis of sphingosine

Animals were sacrificed and lungs or trachea were removed immediately, fixed with PFA, embedded in paraffin and sections stained with anti-sphingosine antibody (clone NHSPH, Alfresa Pharma Corporation, Japan) as previously described [13]; human samples were digested 20 min with Pepsin Digest All (Invitrogen Life Technologies, Darmstadt, Germany), mouse lung and trachea for 30 min. The specificity of the anti-sphingosine antibody used has been previously described [13].

Statistics

Data are means ± SD. Statistical significance was evaluated using the GraphPad Prism 5 program performing ANOVA with Bonferroni as post-hoc-test. Values less than 0.05 were considered statistically significant.

Results and Discussion

Sphingosine in the respiratory tract

Immunohistochemical stainings using a previously characterized [13] sphingosine-specific antibody revealed an abundant expression of sphingosine in epithelial cells of airways. Sphingosine was highly expressed in apical membranes and cilia of human nasal epithelial cells obtained from healthy individuals or tracheal and bronchial epithelial cells from WT mice, while it was almost absent or greatly reduced at the luminal surface of nasal epithelial cells of CF patients or tracheal and bronchial cells of two different CF mouse strains (Fig. 1A,B). *Cftr^{MHH}* mice, which have a residual expression of *Cftr*, showed marginal sphingosine quantities in the trachea and lung, while sphingosine was almost undetectable in *Cftr^{KO}* mice completely lacking *Cftr* (Fig. 1B).

Sphingosine versus ceramide effects on S. aureus strain E25

In addition to reduced sphingosine levels in the respiratory tract (this work and [13]), human patients and mice with CF also show increased ceramide levels in nasal, tracheal and bronchial CF epithelial cells [5-8, 10]. We aimed to determine the effect of ceramide and sphingosine on the *S. aureus* strain E25, because this strain is a clinical isolate from sputum of a CF patient. Mimicking the milieu of the respiratory tract, we incubated the *S. aureus* strain with C18-sphingosine, C16-ceramide or a combination of both lipids in an airway surface liquid (ASL) buffer. This buffer contains a similar electrolyte composition as the ASL [28].

Since the pH of the ASL differs in healthy (pH 6.8-7.2) and CF (pH 6.6) individuals [29-33], the ASL buffer was tested at different pH values. ASL buffer alone showed a bactericidal

Fig. 1. Immunohistochemical analysis of sphingosine in human nasal epithelial cells and murine airways. Paraffin sections of the paranasal sinus mucosa, obtained from patients with or without CF (A), and of trachea and lung, obtained from WT and CF mice (B), were probed with a Cy3-coupled anti-sphingosine antibody and analyzed by confocal microscopy. The size scale at the bottom right is valid for the entire row. Shown are representative images ($n \geq 5$).

Fig. 2. Incubation of *S. aureus* E25 with different sphingolipids in airway surface liquid. *S. aureus* E25 was incubated with sphingolipid micelles diluted in airway surface liquid (ASL) buffer at one of three different pH values (6.6, 6.8, 7.2). After 1 hr incubation 10% of the suspension was cultured on agar plates for determination of CFU the next day. Survival of *S. aureus* was calculated as percentage of the initial amount of bacteria. Bacterial survival after incubation with ASL buffer alone or the solvent OGP served as controls. (SPH: sphingosine; CER: ceramide; S1P: sphingosine 1-phosphate). Shown are the mean $\pm$ SD, $n \geq 3$, * $p < 0.05$, ANOVA and Bonferroni post-hoc test.

Fig. 3. C18-sphingosine inhalation prevents pulmonary *S. aureus* infections in CF mice. WT and CF mice were infected intranasally with 2.5×10^8 CFU of *S. aureus* E25 twice within 24 hrs (A) or once with 1×10^8 CFU of the *S. aureus* strain *septic* or an MRSA strain (B). The bacterial load of lungs was determined 4 hrs after the final infection. Mice inhaled C18-sphingosine (125 μ M) or the solvent OGP (0.6%) 30-40 minutes prior to infection. Shown are the mean $\pm$ SD, each WT $n = 11$; Cfr^{MHH} $n = 6$; Cfr^{KO} $n = 5$, inhaled Cfr^{MHH} mice with $n = 4$ for C18-sphingosine and $n = 5$ for OGP in A, $n = 6$ for each Cfr^{MHH} group in B; * $p < 0.05$, ANOVA and Bonferroni post-hoc test.

effect, irrespective of the pH value (Fig. 2). The solvent OGP had no additional effect compared to buffer alone. C16-ceramide at different concentrations did not change the survival rate of the *S. aureus* strain E25. In contrast, addition of C18-sphingosine for 1 hr significantly reduced bacterial survival: 1 μ M micellar C18-sphingosine killed more than 90% of the bacteria and 5 μ M C18-sphingosine killed all *S. aureus* (Fig. 2). The antibacterial activity of micellar C18-sphingosine was independent of the pH value of ASL buffer between 6.6 and 7.2. C16-ceramide and C18-sphingosine 1-phosphate had no bactericidal effect (Fig. 2). Notably, the combination of sphingosine and ceramide (Fig. 2, second bar from right) did not kill *S. aureus* indicating that the ratio between ceramide and sphingosine determines killing of *S. aureus* by sphingosine in membranes.

It is unknown how ceramide reduces or even abolishes the bactericidal effect of sphingosine. Further, the exact mechanism of the antibacterial activity of sphingoid bases is still unclear. Electron microscopy of sphingosine-treated *S. aureus* revealed multiple lesions of cell wall, membrane evaginations, loss of ribosomes and leakage of cellular debris through

Fig. 4. The ratio between ceramide and sphingosine determines the bactericidal defense of the upper airways. The respiratory tract of CF is characterized by sphingosine deficiency and ceramide accumulation. This imbalance prevents effective pathogen clearance and causes a high susceptibility to pulmonary infections with *S. aureus*. Inhalation of C18-sphingosine corrects this high susceptibility of CF mice.

gaps in the wall [34, 35]. In addition Fischer and colleagues (2013) demonstrated extensive uptake of sphingoid bases by bacteria and detected internal inclusion bodies, likely associated with cell death, but it is unknown whether such an uptake of sphingosine by the pathogen occurs *in vivo* after contact of the pathogen with mammalian membranes [35].

Sphingosine might act in bacterial membranes after transfer from the mammalian to the bacterial membrane and this transfer process might be inhibited by ceramide. Alternatively, sphingosine might act after aggregation to small domains in the mammalian membrane. These domains may interfere with the bacterial membrane or surface proteins to kill the pathogen. Higher concentrations of ceramide might interfere with the formation of these domains and thus block the bactericidal effect of sphingosine. Finally, sphingosine may bind to bacterial proteins to kill the pathogen and ceramide might either interfere with that binding or actively prevent the function of these proteins in the bacteria.

It is suspected that the positive charge of the nitrogen atom of sphingoid bases is relevant for the antibacterial effect of sphingosine [13, 17, 35]. The following observations support this hypothesis: First, an increase of the pH value (pH > 8) and consequent deprotonation of the sphingoid base lead to loss of antibacterial activity [17]. Second, structural comparison revealed that longer and more positively charged sphingoid bases are more effective than shorter, negatively charged ones [13]. However, within the relevant pH range for airway surface liquid, we did not detect a significant impact of the pH on the anti-bactericidal effect of sphingosine.

Sphingosine inhalation prevents in vivo infections with several S. aureus strains, including MRSA

Infection of CF mice with the CF *S. aureus* strain E25 revealed a very high susceptibility of CF mice to *S. aureus* compared to WT mice (Fig. 3A). The two CF mouse strains showed high numbers of *S. aureus* in the lung, while WT rapidly cleared the infection. To analyze the relevance of the antibacterial activity of C18-sphingosine for pulmonary infections *in vivo*, *Cfr*^{MHH} mice were treated with C18-sphingosine via inhalation 30-40 min prior to infection. C18-sphingosine inhalation protected CF mice from infection with *S. aureus* strain E25 (Fig. 3A).

In addition to more toxic strains of *S. aureus*, infections with resistant *S. aureus* strains are a serious clinical problem [36]. Therefore, we tested whether sphingosine also kills a septic and a clinical MRSA isolate. Infection of CF mice with these two strains resulted in severe pneumonia (Fig. 3B). Inhalation of sphingosine by CF mice prior to the nasal infection prevented the development of pneumonia and killed the pathogen in both cases (Fig. 3B).

Here we show for the first time that sphingosine in airways acts against *S. aureus* and prevents infections with this pathogen. The antibacterial activity of this sphingoid base is not limited to *S. aureus* and it has been demonstrated against various Gram-positive and Gram-negative bacteria [13, 17, 19]. Specifically, sphingosine demonstrates antibacterial

activity against numerous CF-relevant pathogens including *P. aeruginosa*, *H. influenzae*, *Acinetobacter baumannii*, *Moraxella catarrhalis* and *Burkholderia cepacia* [13].

Consistent with previous findings, CF mice in the present study show a much higher infection susceptibility to pulmonary *S. aureus* infections than WT mice [37]. This susceptibility to *S. aureus* correlates with decreased expression of sphingosine in the respiratory epithelium. These data suggest that the absence of sphingosine in the airways of CF patients and mice results in the high susceptibility to pulmonary *S. aureus* infections. Inhalation of C18-sphingosine corrects these defects, reconstitutes sphingosine to a level similar to that in healthy controls, and confers resistance to *S. aureus* strains including a MRSA strain. Moreover, we provide evidence that the ratio between ceramide and sphingosine in the airways determines the bactericidal effects (Fig. 4). Thus, therapies that combine a normalization of ceramide in CF airways [38, 39] with the application of sphingosine might be most effective to prevent or treat an existing bacterial infection in these patients. Preventing early manifestation of chronic pulmonary *S. aureus* infections by inhaling sphingosine during the early childhood would be a great chance to minimize the risk for later infections with *P. aeruginosa* of CF patients.

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Disclosure Statement

The authors have nothing to disclose.

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