Original Paper

Neutrophils Kill Reactive Oxygen Species-Resistant *Pseudomonas aeruginosa* by Sphingosine

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Key Words

Reactive oxygen species • Sphingosine • Neutrophils • *Pseudomonas aeruginosa* • Pneumonia

Abstract

Background/Aims: Cystic fibrosis (CF) is dominated by chronic inflammation and infection of the lung resulting in lung destruction and early death of patients. The lungs of CF patients are characterized by a massive accumulation of neutrophils. It requires definition why these massive numbers of neutrophils fail to eliminate typical CF pathogens like *Staphylococcus aureus* and *Pseudomonas aeruginosa* (*P. aeruginosa*) in CF lungs. **Methods:** We determined ceramide, sphingosine and reactive oxygen species (ROS) in neutrophils from wildtype and CF mice and determined the effect of sphingosine and ROS alone or in combination on killing of different *P. aeruginosa* strains. **Results:** We demonstrate that wildtype neutrophils are able to kill non-mucoid and mucoid clinical *P. aeruginosa* strains, while neutrophils from CF mice are insufficient to kill these *P. aeruginosa* strains, although both types of neutrophils infected with *P. aeruginosa* produce comparable levels of superoxide. All three analyzed *P. aeruginosa* strains are resistant to reactive oxygen species. The inability of CF neutrophils to kill *P. aeruginosa* is caused by a marked decrease of surface sphingosine levels in CF neutrophils. Wildtype neutrophils contain much higher concentrations of surface sphingosine than CF neutrophils. Further, wildtype neutrophils, but not CF neutrophils, release sphingosine, most likely as microparticles, upon infection. Sphingosine kills *P. aeruginosa* *in vitro* at low micromolar concentrations. Reconstitution of sphingosine in CF neutrophils restores their ability to kill these pathogens, demonstrating the significance of sphingosine for bacterial killing. **Conclusion:** The data provide evidence for a new paradigm explaining how neutrophils kill ROS-resistant *P. aeruginosa*, i.e. by sphingosine that kills *P. aeruginosa* at low concentrations. This mechanism is defective in CF neutrophils.

© 2017 The Author(s)
Published by S. Karger AG, Basel

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Introduction

Cystic fibrosis (CF) is caused by mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) [1]. With 1 of 2,500 births, the disease is the most common autosomal recessive disorder in the EU and the USA, with approximately 80,000 persons affected [2]. At present, the most important CF-related clinical problems are pulmonary; these problems determine life expectancy, whereas the gastrointestinal problems associated with CF are relatively well controlled [2]. Pulmonary problems include chronic inflammation, fibrosis, and recurrent and chronic bacterial infections with *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Burkholderia cepacia*, *Haemophilis influenzae*, and others [3]. The molecular mechanisms that lead to CF patients' high infection susceptibility are not completely known.

Because CFTR exhibits chloride channel activity, it was speculated, on the basis of *in vitro* studies, that water absorption from the mucus present on the epithelial cells of the respiratory tract may be altered in CF, and that these alterations may reduce mucociliary clearance and the ability to eliminate *P. aeruginosa* [4, 5]. The increased viscosity of the mucus may also affect the ability of neutrophils to migrate to and kill bacteria in the respiratory tract [5]. However, *in vivo* studies involving CF mice and CF patients failed to demonstrate a significant and uniform reduction in mucociliary clearance [6]. Furthermore, CF patients do not seem to exhibit a general defect of neutrophil function as a potential explanation for the high susceptibility to infection, although some distinct changes of neutrophil were reported [7, 8].

A recent study demonstrated that CF mice lack the ATP12A channel [9] compared to pigs and patients with CF. The ATP1A1 gene encodes the ATP12A protein, which is the catalytic subunit of the ouabain-sensitive H^+/K^+ -ATPase [10]. The protein catalyzes the hydrolysis of ATP and thereby allows the transport of H^+ and K^+ over membranes. It is expressed in various tissues and also named H^+/K^+ transporting, nongastric, alpha polypeptide. Expression of ATP12A in CF pigs and patients resulted in airway acidification and therefore a higher susceptibility to chronic bacterial infections of these species compared to CF mice. Thus, while the lungs of CF mice contained approximately 100 colony forming units (CFU) 3 days after exposure to bacteria, they contained 10,000 CFU if ATP12A was expressed in the lung by adenoviral infection [9].

While these findings explain at least in part the reduced susceptibility of CF mice to chronic bacterial infections, they do not explain the very early phase of a pulmonary infection and the defense mechanisms in the lung that allow the bacteria to initiate the infection, or vice versa how the host organism prevents bacterial colonization.

We and others have previously shown that ceramide concentrations are increased in bronchial and tracheal epithelial cells and in CF alveolar macrophages [11-21]. A recent study indicated that specific ceramide species, i.e. C16 ceramide, which seem to be pro-inflammatory, are increased in CF lungs, while other ceramide species that might be anti-inflammatory, in particular C24 ceramide, are decreased [22]. The various studies used biochemical techniques, fluorescence microscopy, mass spectrometry and *in situ* surface kinase assays of mouse and human tissues to show that ceramide accumulates in the trachea, bronchi, and nasal epithelial cells of CF patients and various *Cftr*-deficient mouse strains [11-21]. Partial inhibition of acid sphingomyelinase (Asm) activity [23, 24], achieved genetically or pharmacologically, normalized tracheal and bronchial ceramide levels and prevented bacterial infections of CF mice [11, 12, 19, 21]. These studies demonstrated that increased concentrations of ceramide in bronchial epithelial cells of *Cftr*-deficient mice mediate the high susceptibility of *Cftr*-deficient mice to acute pulmonary infections with *P. aeruginosa*, and trigger chronic pulmonary inflammation and deposition of DNA in bronchi. Normalization of ceramide concentrations by genetic or pharmacological means also normalizes these changes. Several studies indicated that increased ceramide concentrations in the plasma membrane of CF epithelial cells alters the biophysical properties of these membranes and results in the formation of ceramide enriched membrane platforms [21,

25-31]. These domains trap $\beta 1$ -integrin molecules, which results in a downregulation of acid ceramidase and a further increase of ceramide and a decrease of sphingosine in CF bronchial epithelial cells [19-21, 30, 31]. The decrease of sphingosine in an environment of increased ceramide concentrations facilitates acute infections in CF lungs [19-21].

In addition, we have recently demonstrated that sphingosine is abundantly expressed on the luminal surface of human nasal epithelial cells obtained from healthy persons and on the trachea and large bronchi of wildtype mice, whereas it is almost undetectable on the surface of nasal epithelial cells from CF patients and in tracheal and bronchial cells from CF mice [19-21]. Inhalation of ceramidase, which degrades ceramide and produces sphingosine, or of sphingosine not only restored sphingosine levels on the surface of CF tracheal epithelial cells, but also prevented or eliminated existing pulmonary infections of CF mice with several *P. aeruginosa* or *S. aureus* strains [19-21].

The role of sphingolipids for the response of neutrophils to *P. aeruginosa* is unknown. It is also unknown whether these lipids are altered in CF neutrophils and whether a change in these lipids contributes to the failure of CF neutrophils to kill pathogens in the lung. We therefore investigated whether both reactive oxygen species, that are typically released by activated neutrophils, and sphingolipids are required to mediate bactericidal effects of CF neutrophils. Here, we demonstrate that the release of superoxide from neutrophils is not altered by *Cftr*-deficiency, while the levels of sphingosine are greatly reduced in these cells. Neutrophils release sphingosine, most likely in sphingosine containing microparticles, upon infection with *P. aeruginosa*. Sphingosine kills several clinical *P. aeruginosa* strains at low micromolar concentrations, while reactive oxygen species are largely ineffective at least in the clinical strains used in the present study. The reduction of sphingosine causes the reduced bacterial killing by CF neutrophils, even if normal amounts of reactive oxygen species are formed. Reconstitution of sphingosine in CF neutrophils restores bacterial killing.

Materials and Methods

Human samples

Biopsies from explanted CF or donor lungs were fixed in 4% PFA, serially dehydrated with an ethanol to xylol gradient and embedded in paraffin. The specimen were sectioned at 7 μ m, dewaxed and rehydrated. The specimens were then stained with hemalaun for 60 sec. The samples were finally embedded in Mowiol and analyzed by a Leica fluorescence microscope SP5. The studies were approved by the local ethics committees, all patients gave informed consent.

Mice

We used *Cftr*^{tm1Unc-Tg(FABPCFTR)} mice (*Cftr*^{-/-}; Jackson Laboratory, Bar Harbor, ME, USA) backcrossed for more than 10 generations onto the C57BL/6 background. These mice are deficient in *Cftr* in all organs except the intestine, where they express human CFTR under the control of a fatty acid binding protein (FABP) promoter. Expression of intestinal CFTR prevents intestinal obstruction and allows feeding of the mice with a normal diet. Littermates were used as controls.

Neutrophils

Bone marrow-derived neutrophils were obtained by flushing the long bones of the legs from wildtype and CF mice using PBS. The cells were then overlaid on a Percoll/Ficoll gradient, centrifuged for 20 min at 800xg (no brake) and the cells in the intermediate phase were collected. Cells were washed twice, counted and resuspended in RPMI-1640 supplemented with 10 mM HEPES (pH 7.4, Carl Roth GmbH, Karlsruhe, Germany), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μ M nonessential amino acids, 100 U/mL penicillin, 100 μ g/mL streptomycin (all from Invitrogen) and 10 % fetal calf serum. Cells were allowed to rest for 60 min, washed once in HEPES/saline (H/S, consisting of 20 mM HEPES, 132 mM NaCl, 5mM KCl, 1 mM CaCl₂, 0,7 mM MgCl₂, 0,8 mM MgSO₄, pH 7,4) and then used for experiments.

Bacteria

We used three clinical *P. aeruginosa* isolates, named 762 [25, 32], 1242 and 1245 [21]. The strain 762 was from a patient with *P. aeruginosa* sepsis, 1242 and 1245 from CF patients. 1242 and 1245 are

multidrug-resistant mucoid *P. aeruginosa* strains. The strain 762 is a multidrug-resistant non-mucoid strain. Bacteria were plated from frozen stocks on fresh tryptic soy agar plates (TSA; Becton Dickinson), grown at 37°C for 14 to 16 hours. The *P. aeruginosa* strain 762 was then resuspended in 40 mL tryptic soy broth (Becton Dickinson), warmed to 37°C, to an optical density of 0.225 at 550 nm. The bacterial suspension was incubated at 37°C for 1 hr with shaking at 125 rpm to obtain bacteria in the early logarithmic growth phase. Bacteria were washed twice and resuspended in warmed 10 mM HEPES, 63 mM NaCl, 2.4 mM KCl, 1.2 mM CaCl₂, 1.9 mM MgSO₄, pH 6.8), which is similar to the ion concentration in the airway surface liquid (named ASL-buffer) [33]. The final concentration of bacteria was quantified by photospectrometry. The mucoid strains 1242 and 1245 were directly taken from the agar plate, resuspended in ASL-buffer and used at the appropriate concentration.

Superoxide measurements

Superoxide production was determined by electron spin resonance (ESR), as previously described [34]. Neutrophils were infected with 100 bacteria per cell for the indicated times and shock frozen in liquid nitrogen. Proteins were isolated and resuspended in modified Krebs-HEPES buffer containing deferoxamine (100 µM, Sigma) and diethyldithiocarbamate (5 µM, Sigma). A spin trap, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH; Noxygen Science Transfer & Diagnostics, Elzach, Germany; 1 mM final concentration), was then added to the mixture in the presence or absence of manganese-dependent superoxide dismutase (SOD, 200 U/mL; Sigma-Aldrich). The latter served to calibrate the system. O₂^{•−} production in the mixture was then kinetically analyzed over a period of 10 min using a Miniscope MS400. The ESR settings were as follows: biofield, 3, 350; field sweep, 60 G; microwave frequency, 9.78 GHz; microwave power, 20 mW; modulation amplitude, 3 G; points of resolution, 4, 096; receiver gain, 100; and kinetic time, 10 min.

Measurement of total cellular sphingosine

Neutrophils (2 × 10⁶ cells) were resuspended in 200 µL H/S and infected at 37°C with *P. aeruginosa* strain 762 at a multiplicity of infection (MOI) of 1 cell per 100 bacteria or left uninfected. In a first set of experiments the total samples were extracted in 600 µL CHCl₃/CH₃OH/1N HCl (100:100:1, v/v/v). Second, after the infection time, the samples were centrifuged for 7 min at 150×g at 4°C, the supernatant was collected and the pellet was extracted in 200 µL water and 600 µL CHCl₃/CH₃OH/1N HCl (100:100:1, v/v/v). The supernatant was extracted in 600 µL CHCl₃/CH₃OH/1N HCl (100:100:1, v/v/v). This fraction was considered as microparticle and bacteria fraction. After extraction, 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). The kinase reaction was initiated by adding 0.004 units sphingosine kinase 1 (R&D) in 50 mM HEPES (pH 7.4), 250 mM NaCl, 30 mM MgCl₂, 1 µM ATP, and 5 µCi [³²P]γATP. Samples were incubated for 1 hr at 37°C with shaking (350 rpm) and then extracted in 20 µL 1N HCl, 800 µL CHCl₃/CH₃OH/1N HCl (100:200:1, v/v/v), 240 µL CHCl₃, and 2 M KCl. The lower phase was collected, dried, dissolved in 20 µL of CHCl₃:CH₃OH (1:1, v/v), and separated by Silica G60 thin-layer chromatography (TLC) using CHCl₃/CH₃OH/acetic acid/H₂O (90:90:15:5, v/v/v/v). The TLC plates were analyzed with a phosphorimager.

Measurement of surface sphingosine

Neutrophils were prepared as above, rested for 1 hr in RPMI-1640 supplemented as above and washed once in H/S. The pellets were resuspended in 50 µL 50 mM HEPES (pH 7.4), 250 mM NaCl, 30 mM MgCl₂, 1 µM ATP, and 5 µCi [³²P]γATP containing 0.004 units sphingosine kinase. Samples were incubated for 15 min at 37°C with shaking at 300 rpm. Controls were incubated with the same buffer without sphingosine kinase or were left untreated. The sphingosine kinase reaction was terminated by centrifugation of the samples at 240 × g for 5 min, removal of the supernatant and addition of 70 µL H₂O, followed by the addition of 20 µL 1N HCl, 800 µL CHCl₃:CH₃OH:1N HCl (100:200:1, v:v:v), and 240 µL each of CHCl₃ and 2 M KCl. The lower phase was collected, dried, dissolved in 20 µL CHCl₃:CH₃OH (1:1, v/v), and separated on Silica G60 TLC plates with CHCl₃:CH₃OH:acetic acid:H₂O (90:90:15:5, v:v:v:v) as developing solvent. The TLC plates were analyzed with a phosphorimager. Surface sphingosine levels were determined with a standard curve of C18-sphingosine. This assay analyzes sphingosine on the cell surface and, if released, also in large particles that are pelleted by the slow centrifugation, while exosomes or small microparticles are not pelleted and excluded from the analysis.

Ceramide measurements

Neutrophils were infected with *P. aeruginosa* strain 762 at an MOI of 1 cell per 100 bacteria or left uninfected. The incubations (200 µl volume) were terminated by extraction in CHCl₃:CH₃OH:1N HCl (100:100:1; v/v/v). The lower phase was collected, dried and resuspended in 20 µl of a detergent solution (7.5 % (w/v) n-octylglucopyranoside, 5 mM cardiolipin in 1 mM diethylenetriaminepentaacetic acid), sonicated for 10 min and 70 µl of a reaction mixture containing 10 µl diacylglycerol kinase (GE Healthcare Europe, München, Germany), 0.1 M imidazole/HCl (pH 6.6), 0.2 mM diethylenetriaminepentaacetic acid (pH 6.6), 70 mM NaCl, 17 mM MgCl₂ and 1.4 mM EGTA, 2 mM DTT, 1 µM ATP and 10 µCi [³²P]ATP were added. The kinase reaction was performed for 1 hr at room temperature and terminated by addition of 1 ml CHCl₃:CH₃OH:1N HCl (100:100:1; v/v/v), 170 µl buffered saline solution (135 mM NaCl, 1.5 mM CaCl₂, 0.5 mM MgCl₂, 5.6 mM glucose, 10 mM HEPES, pH 7.2) and 30 µl of a 100 mM EDTA-solution. The lower phase was collected, dried and separated on Silica G60 TLC plates employing chloroform/acetone/methanol/acetic acid/H₂O (50:20:15:10:5, v/v/v/v/v). Ceramide amounts were determined by comparison with a standard curve using C16 ceramide as substrate. The results are the mean ± SD from 4 independent experiments. The TLC plates were analyzed with a phosphorimager.

Neutrophil - *P. aeruginosa* co-incubation experiments

10⁴ neutrophils were resuspended in 20 µl H/S and infected with 5,000 CFU *P. aeruginosa* strains 762, 1242 or 1245. The bacteria were added in a volume of 2 µl. The samples were incubated for 1 hr at 37°C. Aliquots were then plated on LB plates and colony forming units (CFU) were counted after o/n growth at 37°C. To restore sphingosine, we incubated CF and, as controls, also wildtype neutrophils with 10 µM, 25 µM or 50 µM sphingosine or 15 µg/ml acid ceramidase for 45 min at 37°C. The cells were washed twice in H/S and then infected as above.

Sphingosine- and ROS-mediated killing of *P. aeruginosa*

D-erythro-sphingosine (d18:1) was purchased from Avanti Polar Lipids, USA, H₂O₂ from Merck, Germany. Sphingosine was dissolved in 10% octylglucopyranoside (Sigma) at a concentration of 2 mM. The stock was sonicated in a bath sonicator for 10 min immediately prior to addition to the bacteria. Sonication was performed to achieve the formation of micelles. Sphingosine was then immediately added to 1,000 CFU of the *P. aeruginosa* strains 762, 1242 or 1245 resuspended in ASL-buffer. H₂O₂ was diluted in ALS prior to addition to the bacteria. All samples were incubated for 1 hr at 37°C. An aliquot was then plated on LB plates, cultured o/n and the CFUs were counted.

Sphingosine stainings of neutrophils and *P. aeruginosa*

Neutrophils were infected for the indicated times as above or left uninfected. Cells were fixed with 1% PFA in PBS (pH 7.2) for 15 min, washed, permeabilised with 0.1 % Triton in PBS for 15 min, washed, blocked with a buffer containing H/S and 5 % fetal calf serum (FCS) for 15 min and stained with anti-sphingosine antibodies (clone NHSPH, Alfresa Pharma Corporation, Japan) [19] for 45 min at room temperature. The antibodies were diluted 1:1000 in H/S containing 5% FCS. Cells were then washed twice in H/S followed by a 45 min incubation with Cy3-coupled anti-mouse IgM antibodies (1:250 dilution, Jackson ImmunoResearch). The cells were washed again twice in H/S and mounted in Mowiol. The samples were analyzed with a Leica confocal microscope DMIRE2. The magnification was 400-fold. Shown are representative results of at least 4 independent experiments.

Statistical Methods

Data are expressed as arithmetic means ± SD. For the comparison of two independent groups we used Student's t-test. For more than two groups we used ANOVA followed by a post-hoc t-test for all pairwise comparisons applying Bonferroni correction for multiple testing. *P* values of 0.05 or less (two-tailed) were considered indicative of statistical significance. All values were normally distributed.

Results

Human CF lungs contain massive amounts of neutrophils (Fig. 1A, B) that are ineffective at eliminating *S. aureus* and *P. aeruginosa* and other typical CF pathogens. Thus, a high number of bacteria are observed in the bronchial debris in CF lungs (Fig. 1A, B).

Fig. 1. Neutrophils and bacteria accumulate in the bronchial lumen of lungs from cystic fibrosis patients. Sections of lung specimen from cystic fibrosis (CF) or donor (healthy) patients were stained with hemalaun and analyzed by light microscopy. A large number of mononuclear cells and rod-like bacteria were detected in the bronchial lumen of CF lungs, while the lumen of the donor lung is free of cells. Panel A shows donor and CF patient lungs at a 400-fold magnification, panel B shows the debris in the airways of CF patients with 1000-fold magnification. The rod-like bacteria are clearly visible in the debris and among the mononuclear cells. Shown is a representative study of 5 samples each.

Fig. 2. Wildtype and cystic fibrosis neutrophils release similar amounts of superoxide, but only wildtype neutrophils kill *P. aeruginosa*. (A) Wildtype and CF neutrophils were infected with 100 bacteria per cell for the indicated times or left uninfected, shock frozen in liquid nitrogen and the release of superoxide was determined by electron spin resonance. The release of superoxide is given in arbitrary units. Shown is the mean $\pm$ SD of 4 independent experiments. * $p < 0.05$ compared to the untreated control of the same genotype, # $p < 0.05$ compared to wildtype at the same time, ANOVA and post hoc t-test. (B) Wildtype and CF neutrophils were infected with *P. aeruginosa* at a multiplicity of infection of 1 cell per 0.5 bacteria for 1 hr at 37°C. The samples were then spotted on agar plates, cultured overnight and the number of the bacteria was determined. Wildtype neutrophils kill the different *P. aeruginosa* strains, while CF neutrophils fail to kill the pathogens. Given is the mean $\pm$ SD of 4 independent experiments. * $p < 0.05$ compared to wildtype, t-test. (C) Incubation of *P. aeruginosa* strains 762, 1242 or 1245 with increasing concentrations of H_2O_2 reveals the high resistance of these strains to reactive oxygen species. Shown is the mean $\pm$ SD of 3 independent experiments. * $p < 0.05$ compared to untreated samples, ANOVA and post hoc t-test.

The release of reactive oxygen species is a prototypical defense mechanism by which neutrophils kill pathogens. We therefore tested whether *Cftr*-deficiency impairs this defense

mechanism. However, our results demonstrated that CF and wildtype neutrophils released comparable amounts of superoxide prior to or after infection with *P. aeruginosa* (Fig. 2A).

Despite the release of similar amounts of superoxide from CF and wildtype neutrophils, only wildtype neutrophils were able to efficiently kill the clinical non-mucoid and mucoid *P. aeruginosa* strains 762, 1242 and 1245 (Fig. 2B), indicating that reactive oxygen species alone are insufficient to kill pathogenic *P. aeruginosa* by neutrophils. In addition, all three *P. aeruginosa* strains were rather resistant to reactive oxygen species and only high concentrations H_2O_2 at least partly killed the bacteria (Fig. 2C).

We have previously shown that ceramide and sphingosine in epithelial cells of the respiratory tract play an important role in the defense against pathogens [11, 19-21].

Fig. 3. Ceramide is increased and sphingosine decreased in cystic fibrosis neutrophils. (A) Ceramide was measured in wildtype and cystic fibrosis (CF) neutrophils prior and after infection with *P. aeruginosa* 762 employing a ceramide-kinase assay. Ceramide was extracted and the phosphorylation in the presence of [^{32} P] γ ATP and a ceramide kinase was measured. Samples were separated by thin layer chromatography and analyzed using a phosphoimager. Ceramide is markedly increased in CF neutrophils compared to wildtype neutrophils. (B) Sphingosine kinase assays of wildtype and CF neutrophils reveal reduced levels of sphingosine in CF compared to wildtype neutrophils. Sphingosine was quantified by phosphorylation in the presence of [^{32} P] γ ATP and sphingosine kinase, separation by thin layer chromatography and analysis by a phosphoimager. (C) Wildtype neutrophils release much higher levels of sphingosine into the cell culture supernatant than CF neutrophils. Infection does not change the sphingosine levels in the cell pellet and the supernatants. Sphingosine in the supernatant and post-centrifugation pellets was determined as above. Panels A-C display the mean $\pm$ SD of 4 independent experiments. * $p < 0.05$ compared to wildtype neutrophils with the same treatment, ANOVA and post hoc t-test. (D) Surface sphingosine was quantified by incubation of intact neutrophils freshly isolated from wildtype and CF mice with sphingosine kinase in the presence of [^{32} P] γ ATP for 15 min at 37°C. Cells were then extracted, lipids were separated by thin layer chromatography and radioactive sphingosine 1-phosphate was quantified. The ratio between radioactive and non-radioactive ATP present in the kinase buffer was taken into account to calculate the total amount of surface sphingosine. Shown is the mean $\pm$ SD from 3 independent samples. * $p < 0.05$ compared to wildtype, t-test. (E) Intact, non-permeabilized wildtype or cystic fibrosis (CF) neutrophils were stained with Cy3-coupled anti-sphingosine antibodies. Confocal microscopy studies revealed a higher sphingosine expression in the outer leaflet of the plasma membrane of wildtype neutrophils than in CF neutrophils. Shown are representative studies from 4 independent experiments.

Sphingosine very rapidly and efficiently kills *P. aeruginosa* and *S. aureus* *in vitro* and *in vivo* in the lung [19-21, 30]. We have also shown that sphingosine levels are greatly reduced in the epithelial cells of the upper airways in CF, and that this lack of sphingosine results in the failure of upper airway epithelial cells in CF to kill invading pathogens before they reach the lung [21]. However, a potential role of ceramide or sphingosine in neutrophils and their response to *P. aeruginosa* is presently unknown. We therefore investigated the expression pattern of ceramide and sphingosine in wildtype and CF neutrophils. Here, we demonstrate a marked increase of ceramide in CF neutrophils compared to wildtype neutrophils (Fig. 3A). Infection with *P. aeruginosa* strain 762 did not further increase ceramide levels.

Total cellular sphingosine was markedly reduced in CF compared to wildtype neutrophils (Fig. 3B). Moreover, wildtype neutrophils released a high amount of sphingosine in the culture supernatant (Fig. 3C). The constitutive release of sphingosine into the culture supernatant was much lower in cultures from CF neutrophils (Fig. 3C). Infection did not significantly

Fig. 4. Sphingosine accumulates in *P. aeruginosa* after infection of wildtype neutrophils. Confocal microscopy studies demonstrate the presence of sphingosine in *P. aeruginosa* strain 762 20 min after infection of wildtype neutrophils, while incubation of the bacteria with cystic fibrosis (CF) neutrophils results in much lower sphingosine incorporation in *P. aeruginosa*. Shown are representative Figures from 4 independent experiments.

Fig. 5. Killing of *P. aeruginosa* by sphingosine and H_2O_2 *in vitro*. Low micromolar concentrations of sphingosine kill *P. aeruginosa* strains 762 (A), 1242 (B) and 1245 (C). These strains are rather resistant to reactive oxygen species (A-C). Reactive oxygen species and sphingosine did not synergize in killing *P. aeruginosa*. 1000 CFU of the *P. aeruginosa* strains were incubated for 60 min at 37°C with the indicated concentration of sphingosine or H_2O_2 or both, the samples were plated on agar, grown overnight and the CFU were counted. Shown is the mean $\pm$ SD of 4 independent experiments. ** $p < 0.01$, *** $p < 0.001$ compared to untreated controls, ANOVA and post hoc t-test.

change the cellular concentration or the release of sphingosine into the culture supernatant (Fig. 3C).

Since the concentration of sphingosine on the cell surface determines the effect of cellular sphingosine on pathogens, we determined the surface concentration of sphingosine by a kinase assay and by immunofluorescence studies. In the surface kinase assays intact neutrophils are incubated with sphingosine kinase in the presence of [³²P]γATP. The kinase reaction results in the conversion of surface sphingosine to radioactive sphingosine 1-phosphate. Extraction of lipids then allows quantification of surface sphingosine. In the immunofluorescence studies, intact neutrophils were stained with Cy3-labelled anti-sphingosine antibodies and analyzed by confocal microscopy. Both methods demonstrated a marked down-regulation of surface sphingosine in CF neutrophils compared to wildtype neutrophils (Fig. 3D and E).

The confocal microscopy studies also revealed the presence of sphingosine in *P. aeruginosa* 20 min after infection of wildtype neutrophils, indicating a direct interaction of sphingosine with *P. aeruginosa* upon the release from neutrophils (Fig. 4). The incorporation of sphingosine by *P. aeruginosa* was much lower after infection of CF neutrophils (Fig. 4).

To gain insight into whether sphingosine in neutrophils contributes to killing of bacteria by these cells, we tested whether exogenously added sphingosine kills *P. aeruginosa*. These studies revealed killing of the *P. aeruginosa* strains 762, 1242 and 1245 by sphingosine

Table 1. Cell death of *P. aeruginosa* after treatment with sphingosine and H₂O₂; Experimental vs summed values. Listed is the percentage of killing of *P. aeruginosa* induced by the combination of sphingosine and H₂O₂ as observed in the experiments described in Fig. 5 and the theoretical value of killing if the effects of sphingosine and H₂O₂ as single treatments were summed. This allows us to determine additive, synergistic or antagonistic effects of sphingosine and H₂O₂ at least at concentrations, which do not induce almost 100% bacterial killing anyway. The data reveal a slight antagonistic effect of H₂O₂ on sphingosine

	5 μM Sph + 50 μM H ₂ O ₂	5 μM Sph + 100 μM H ₂ O ₂	5 μM Sph + 250 μM H ₂ O ₂	5 μM Sph + 500 μM H ₂ O ₂	10 μM Sph + 50 μM H ₂ O ₂	10 μM Sph + 100 μM H ₂ O ₂	10 μM Sph + 250 μM H ₂ O ₂	10 μM Sph + 500 μM H ₂ O ₂	20 μM Sph + 50 μM H ₂ O ₂	20 μM Sph + 100 μM H ₂ O ₂	20 μM Sph + 250 μM H ₂ O ₂	20 μM Sph + 500 μM H ₂ O ₂
762 exp.	6.87 ± 17.36 %	10.26 ± 17.71 %	-2.37 ± 14.43 %	39.19 ± 8.77 %	44.04 ± 2.27 %	45.13 ± 8.09 %	37.02 ± 31.69 %	72.70 ± 5.67 %	99.75 ± 0.43 %	99.75 ± 0.43 %	99.75 ± 0.43 %	99.20 ± 0.84 %
762 sum	5.80 ± 5.04 %	3.17 ± 3.91 %	13.90 ± 4.98 %	37.52 ± 2.72 %	46.76 ± 2.89 %	43.76 ± 3.16 %	54.85 ± 2.67 %	78.48 ± 3.16 %	114.65 ± 2.37 %	114.65 ± 2.37 %	114.65 ± 2.37 %	123.53 ± 2.38 %
1242 exp.	8.69 ± 1.78 %	53.29 ± 5.17 %	88.95 ± 1.57 %	99.93 ± 0.06 %	57.42 ± 1.81 %	65.40 ± 1.21 %	65.40 ± 1.21 %	99.96 ± 0.06 %	99.96 ± 0.06 %	99.96 ± 0.06 %	99.93 ± 0.06 %	99.96 ± 0.06 %
1242 sum	56.82 ± 6.72 %	43.32 ± 33.67 %	80.79 ± 7.85 %	100.63 ± 6.14 %	107.74 ± 0.65 %	118.24 ± 2.64 %	131.71 ± 1.94 %	151.55 ± 0.65 %	166.65 ± 3.20 %	166.65 ± 3.20 %	193.74 ± 10.85 %	199.96 ± 0.06 %
1245 exp.	65.33 ± 2.48 %	73.01 ± 3.21 %	86.42 ± 1.00 %	92.86 ± 1.10 %	67.29 ± 9.13 %	74.94 ± 2.56 %	90.68 ± 0.38 %	97.06 ± 0.48 %	100 ± 0 %	100 ± 0 %	99.97 ± 0.05 %	99.97 ± 0.05 %
1245 sum	97.75 ± 23.41 %	100.45 ± 65.38 %	146.52 ± 9.77 %	151.96 ± 14.44 %	119.99 ± 15.07 %	147.88 ± 12.94 %	168.76 ± 1.51 %	174.20 ± 4.94 %	137.70 ± 11.02 %	165.59 ± 8.90 %	191.89 ± 1.07 %	191.92 ± 1.02 %

at low micromolar concentrations (Fig. 5A-C). Finally, we investigated whether reactive oxygen species and sphingosine synergize. However, the results revealed that reactive oxygen species and sphingosine did not synergize to kill *P. aeruginosa* (Fig. 5A-C). Table 1 shows comparison between hypothetical and actual additive effects of sphingosine and H₂O₂. The data reveal that sphingosine and H₂O₂ are not synergistic and even slightly antagonistic, at least for intermediate H₂O₂ concentrations.

Fig. 6. Reconstitution of sphingosine in cystic fibrosis neutrophils restores killing of *P. aeruginosa*. Addition of sphingosine to cystic fibrosis (CF) neutrophils or generation of endogenous sphingosine restores the bactericidal capacity of CF neutrophils and killing of the clinical *P. aeruginosa* strains 762 (A), 1245 (B) and 1242 (C). CF or wildtype neutrophils were treated with 10 μ M, 25 μ M or 50 μ M sphingosine (SPH) or 250 μ g/ml acid ceramidase (AC) for 45 min at 37°C, extensively washed, infected and the number of bacteria was determined after overnight growth on agar plates. Shown is the mean $\pm$ SD of each 4 independent experiments. * $p < 0.05$ compared to untreated of the same genotype, # $p < 0.05$ compared between wildtype and CF neutrophils with the same treatment, ANOVA and post hoc t-test.

To prove a role of sphingosine in neutrophils for killing of *P. aeruginosa*, we reconstituted sphingosine in CF neutrophils by treatment with sphingosine or acid ceramidase, which generates sphingosine from ceramide [35]. These treatments completely restored the bactericidal capacity of CF neutrophils, which were now able to kill the clinical *P. aeruginosa* strains 762, 1242 and 1245 as efficient as wildtype neutrophils (Fig. 6A-C).

Discussion

In the present study we provide evidence for a novel mechanism of how neutrophils kill at least some clinical strains of *P. aeruginosa*. Although CF and wildtype neutrophils release similar amounts of superoxide, CF neutrophils have a reduced capacity to kill *P. aeruginosa* indicating that this mechanism alone is insufficient to kill these bacteria. We also demonstrate that these clinical isolates of *P. aeruginosa* are relatively resistant to reactive oxygen species. Wildtype neutrophils overcome this resistance by sphingosine-mediated killing of *P. aeruginosa*. In contrast, sphingosine is markedly reduced in CF neutrophils, resulting in a failure of these cells to kill *P. aeruginosa*. Reconstitution of sphingosine in CF neutrophils is sufficient to restore the ability of CF neutrophils to kill *P. aeruginosa*. In fact, addition of acid ceramidase to CF neutrophils restored killing of *P. aeruginosa* by these cells even over the level of wildtype neutrophils. We assume that this is caused by a high formation of sphingosine from ceramide, which is accumulated in CF neutrophils.

It is important to note that we investigated the role of sphingosine in neutrophils on killing of planktonic and mucoid *P. aeruginosa*, representing the clinically most relevant forms of this pathogen inducing acute and chronic pneumonia.

It is surprising that all three tested *P. aeruginosa* strains are resistant to rather high concentrations of reactive oxygen species. However, *P. aeruginosa* is a very versatile pathogen and has developed multiple pathways to deal with potentially toxic exogenous compounds. For instance, it has been shown that *P. aeruginosa* neutralizes reactive oxygen species by tryptophan metabolites [36], expression of catalases [37], superoxide dismutase [38], etc. In this context, it is interesting to note that all three *P. aeruginosa* strains are sensitive to sphingosine.

At present, the mechanism(s) underlying killing of *P. aeruginosa* by sphingosine are unknown. At least *P. aeruginosa* PAO-1 expresses sphingosine response elements, i.e. sphA, which are specifically induced by sphingosine via a sphingosine-specific transcription factor (PA5324), a member of the AraC-family transcription factor, named SphR [39]. Deletion of the SphR transcription factor resulted in reduced bacterial survival during mouse lung infection and increased sensitivity of the pathogen to the antimicrobial effects of sphingosine. Thus, *P. aeruginosa* seems to be able to express sphingosine-binding molecules and factors that protect the pathogen from sphingosine effects, at least partially.

Lower concentrations of free sphingosine (in the nanomolar range) activate the transcriptional regulator SphR in *P. aeruginosa*, which results in secretion of a bacterial ceramidase [40]. If the cells release only small amounts of sphingosine or if the released sphingosine binds to proteins that reduce the free concentration of sphingosine, the concentration of free sphingosine would be too low to kill the bacteria immediately and instead would activate SphR. Bacterial ceramidase may induce sphingosine on the surface of mammalian cells. However, our data argue against such a scenario, since the concentration of ceramide on the cell surface of wildtype cells was low.

Thus, from the point of view of neutrophils it is important to kill *P. aeruginosa* very rapidly with a high concentration of sphingosine delivered to the bacteria without neutralization by proteins and prior to the release of ceramidase and damage to the host cells.

Our studies demonstrate that a small increase of sphingosine concentration from 5 μM to 20 μM is already sufficient to kill 100% of the bacteria. It seems unlikely that classical biochemical pathways mediate the sharp increase of the effects of sphingosine, and we assume that the bacterio-toxic effects of sphingosine are mainly mediated by biophysical mechanisms, such as integration into bacterial membranes and disruption of their function. However, at present it is unknown how sphingosine kills pathogens.

We also investigated whether reactive oxygen species and sphingosine may act synergistically. The data do not provide any synergistic effect of the two mediators on bacterial growth. In fact, higher concentrations of H_2O_2 seem to reduce the effects of sphingosine on bacterial killing. It is possible that oxidation of the double bond in sphingosine might at least partly inactivate the molecule. This assumption is consistent with the previous observation that sphinganine, lacking a double bond compared to sphingosine, is much less bactericidal than sphingosine [19], although sphinganine is not an oxidation product of sphingosine.

Thus, it is tempting to speculate that the high number of neutrophils continuously present in the lungs of CF patients produce high concentrations of reactive oxygen species that oxidize sphingosine, which is already reduced in these cells, and thereby further reducing the bactericidal effects of neutrophils and also the effects of epithelial cell-derived sphingosine on invading pathogens. This would result in a vicious cycle in the lungs of CF patients with more neutrophils, a lack of sphingosine in these cells, and further inactivation of the remaining sphingosine by reaction with reactive oxygen species finally resulting in the lack of killing of *P. aeruginosa*. This vicious cycle could be interrupted by inhalation of CF patients with acid ceramidase and/or direct application of sphingosine into the lung. In addition, the continuous release of reactive oxygen species from CF neutrophils in the lung may also damage the lung tissue and contribute to the typical development of CF.

Our studies demonstrate that sphingosine is present in cell culture supernatants and also indicate that sphingosine is incorporated into the bacteria and can be visualized by staining with a sphingosine-specific antibody [19]. Since sphingosine is insoluble in water, it might be present in microvesicles or exosomes [41-43]. These microparticles might be

shed from the surface of neutrophils [44] and/or released from multivesicular bodies [45], and fuse with bacterial membranes resulting in incorporation of sphingosine in the bacterial membrane and killing of the pathogen. However, it might be also possible that sphingosine is transferred from the neutrophil membrane after contact with bacterial membranes, although this would require a higher affinity of the bacterial membrane for sphingosine than the mammalian counterpart.

We have previously shown that sphingosine expressed in airway epithelial cells plays an important role in the prevention and defense of *P. aeruginosa* infections in CF mice and after burn-injury [19-21, 42]. These studies revealed that sphingosine is expressed predominantly in upper airway epithelial cells and reduced in CF mice or in wildtype mice subjected to burn-injury. Reconstitution of sphingosine in epithelial cells prevented acute pulmonary infections with *P. aeruginosa*. *In vitro* studies demonstrated that sphingosine kills many bacterial species, for instance *P. aeruginosa*, *Staphylococcus aureus*, *Moraxella catarrhalis*, *Haemophilus influenzae*, Burkholderia species and *Escherichia coli* [19, 46, 47]. While these studies investigated the effect of sphingosine in airway epithelial cells on infections, they did not study the expression and role in neutrophils or other classical cells of the innate immune system for their response to pathogens.

Our studies demonstrate that sphingosine plays an important anti-bacterial role in neutrophils. However, they certainly do not exclude that other mechanisms such as the release of proteases [43] or NO-radicals [48] are also involved in killing of pathogens. Further, although clinically-relevant *P. aeruginosa* strains are resistant to reactive oxygen species, these radicals might be the predominant mechanisms in killing of other bacterial species.

Conclusion

We demonstrate that several clinical *P. aeruginosa* strains are resistant to reactive oxygen species. Although neutrophils from wildtype or CF mice produce similar amounts of superoxide, only wildtype neutrophils are able to kill these *P. aeruginosa* strains, while CF neutrophils fail to do so. Wildtype neutrophils kill *P. aeruginosa* at least in part by expression and release of sphingosine, and reconstitution of sphingosine in CF neutrophils restores their capability to kill *P. aeruginosa* strains. These data establish a novel paradigm for killing of *P. aeruginosa* by neutrophils, but also provide novel options for the reconstitution of antibacterial functions of CF neutrophils, for instance by application of acid ceramidase, which consumes increased ceramide in these neutrophils to sphingosine.

Acknowledgements

The study was supported by DFG-grant Gu 335/35-1 to EG and GRK 2098 to KAB and EG.

Disclosure Statement

The authors have no conflicts of interest to disclose.

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