Sphingosine kills bacteria by binding to cardiolipin

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Abstract

Sphingosine is a long-chain sphingoid base that has been shown to have bactericidal activity against many pathogens, including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*. We have previously demonstrated that sphingosine is present in nasal, tracheal, and bronchial epithelial cells and constitutes a central element of the defense of the airways against bacterial pathogens. Here, using assorted lipid-binding and cell biology assays, we demonstrate that exposing *P. aeruginosa* and *S. aureus* cells to sphingosine results in a very rapid, i.e. within minutes, permeabilization of the bacterial plasma membrane, resulting in leakiness of the bacterial cells, loss of ATP, and loss of bacterial metabolic activity. These alterations rapidly induced bacterial death. Mechanistically, we demonstrate that the presence of the protonated NH$_2$ group in sphingosine, which is an amino-alcohol, is required for sphingosine’s bactericidal activity. We also show that the protonated NH$_2$ group of sphingosine binds to the highly negatively charged lipid cardiolipin in bacterial plasma membranes. Of note, this binding was required for bacterial killing by sphingosine, as revealed by genetic experiments indicating that *E. coli* or *P. aeruginosa* strains that lack cardiolipin synthase are resistant to sphingosine, both in vitro and in vivo. We propose that binding of sphingosine to cardiolipin clusters cardiolipin molecules in the plasma membrane of bacteria. This clustering results in the formation of gel- or even crystal-like structures in the bacterial plasma membrane and thereby promoting rapid permeabilization of the plasma membrane and bacterial cell death.
**Introduction**

*P. aeruginosa* (*P. aeruginosa*) is an ubiquitous and opportunistic pathogen that causes severe respiratory tract and systemic infections, especially among patients with cystic fibrosis (CF) and chronic obstructive pulmonary disorder (COPD), previous viral infections, burn wounds, trauma, or sepsis, and those requiring mechanical ventilation (1-6). Most important are *P. aeruginosa* and *Staphylococcus aureus* (*S. aureus*) infections among patients with CF. CF, which is caused by mutations of the cystic fibrosis transmembrane conductance regulator gene (human, CFTR; murine, Cftr) (7,8), is the most common recessively inherited disorder in North America and Europe, with more than 80,000 CF patients in the EU and the USA alone (9). The most frequent cause of morbidity and mortality among CF patients is chronic pulmonary infection with bacterial pathogens, in particular *P. aeruginosa* and *S. aureus* (e.g., 10,11). Approximately 80% of all CF patients are colonized with *P. aeruginosa* by the age of 25. At present, no treatment to eradicate these bacterial infections is available.

Many *P. aeruginosa* and *S. aureus* strains are highly resistant to existing antibiotics, and attempts to eradicate pulmonary *P. aeruginosa* or *S. aureus* among CF or COPD patients usually fail. Thus, it is important to develop novel strategies for treating pulmonary infections with *P. aeruginosa* and *S. aureus*.

Sphingolipids are a class of lipids that share an amino alcohol (sphingoid base) backbone. We have previously demonstrated that sphingosine efficiently kills many bacterial species *in vitro* and *in vivo*, including *P. aeruginosa*, *S. aureus* (even MRSA), *Acinetobacter baumannii*, *Haemophilus influenzae*, *Burkholderia cepacia*, and *Moraxella catarrhalis* (12,13). Other groups have shown that sphingosine also kills *Escherichia coli* (*E. coli*) and *Porphyromonas gingivalis* (14-17). Our previous studies also demonstrated that sphingosine is an abundant constituent of the luminal surface of human nasal, tracheal and bronchial epithelial cells obtained from healthy subjects and from epithelial cells of trachea and conducting bronchi of wild-type mice, whereas it is almost undetectable on the surface of nasal, tracheal and bronchial epithelial cells from CF patients and on tracheal and
bronchial cells from CF mice (12,13,18). Treating CF mice with inhaled sphingosine eliminated existing acute and chronic pulmonary *P. aeruginosa* infections and prevented new *P. aeruginosa* or *S. aureus* infections in these mice (12,13,18), a finding demonstrating that sphingosine plays a key role in the innate and immediate defense of the upper respiratory tract.

Likewise, the inhalation of recombinant human acid ceramidase by CF mice restored epithelial airway sphingosine levels and reversed acute and chronic infection with *P. aeruginosa* (12,18). These studies demonstrate a central role of sphingosine in the defense against bacterial pathogens in pulmonary infections.

Electron microscopy studies by Fischer et al. indicated that killing of pathogens by sphingosine does not result in simple lysis of pathogens such as gram-positive *S. aureus* and gram-negative *E. coli* (15).

These studies clearly indicate that sphingosine is bactericidal for a variety of pathogens, but the molecular mechanisms of sphingosine-mediated killing of bacteria remain to be defined.

Here, we demonstrate that sphingosine binds to cardiolipin in the bacterial plasma membrane resulting in rapid permeabilization of the bacterial membrane and death. Bacterial mutants that lack cardiolipin are resistant to sphingosine-mediated toxicity *in vitro* and *in vivo*. Thus, the interaction of sphingosine with cardiolipin is required for its bactericidal effects. Sphingosine most likely acts by inducing very rigid cardiolipin-membrane domains, a process that results in leakiness of the membrane and bacterial death. This notion is consistent with biophysical experiments measuring membrane fluidity in liposomes after treatment with sphingosine.
Results

To define molecular mechanisms that mediate the bactericidal effects of sphingosine, we incubated two *P. aeruginosa* strains, i.e. the clinical isolate 762 and the laboratory strain ATCC 27853, and a clinical isolate of *S. aureus*, named DH, with 1 M or 10 M sphingosine or the corresponding concentrations of octylglucopyranoside, the detergent used to solubilize sphingosine. The bacteria were incubated with sphingosine for 5 min. We then determined the membrane permeability of the bacteria by staining with TO-PRO-3 iodide. Viable cells are impermeable to TO-PRO 3, which binds to chromosomes after an increase in membrane permeability. This can be visualized by fluorescence microscopy or flow cytometry. The data show that incubation of *P. aeruginosa* or *S. aureus* with sphingosine results in a rapid increase in bacterial permeability (Fig. 1A, B). An additional time-course study showed that sphingosine mediates almost complete cell permeability already within 5 min, resulting in increased staining of the bacteria with TO-PRO-3 (not shown).

To confirm the rapid effect of sphingosine on viability of the bacteria, we determined the release of ATP into the medium upon incubation of *P. aeruginosa* and *S. aureus* with sphingosine. The results showed a rapid release of adenosine triphosphate (ATP) into the medium (Fig. 1C, D).

In accordance with the massive increase of membrane permeability upon incubation of the bacteria with sphingosine, we also observed a dramatic decrease in metabolic activity in *P. aeruginosa* or *S. aureus* upon incubation with sphingosine (Fig. 2).

Next, we aimed to further define the time course of sphingosine-mediated killing of *P. aeruginosa*. To this end, we incubated the *P. aeruginosa* strains 762 or ATCC 27853 with 1 M or 10 M sphingosine for 15 or 60 min, washed and re-cultured the bacteria. The results of these studies demonstrate that sphingosine killed the bacteria within 15 min (Fig. 3), a finding consistent with the very rapid action of sphingosine on membrane integrity/permeability.

Sphingosine contains an NH$_2$ group and an OH group. At neutral or slightly acidic pH, as found in airways and on many epithelial cell surfaces (19), the NH$_2$ group will be protonated and,
thus, positively charged. Therefore, we first tested whether the NH$_2$ group is important and, second, whether protonation of this group is required for the bactericidal effects of sphingosine. First, to test whether the amine group mediates the bactericidal effects of sphingosine, we incubated *P. aeruginosa* with 1 µM or 10 µM stearylamine, which is structurally very similar to sphingosine but lacks the OH group. The results showed that stearylamine kills *P. aeruginosa* as efficient as sphingosine (Fig. 4A). Second, to test whether the pH determines the effects of sphingosine on *P. aeruginosa*, we measured whether an increase of the pH from 6.0 to pH 8.0 alters the bactericidal effects of sphingosine. The results show that the effects of sphingosine on *P. aeruginosa* are greatly reduced at pH 8.0 (Fig. 4B). This finding indicates that protonation of the NH$_2$ group is necessary for the antibacterial effects of sphingosine.

To further confirm the notion of an interaction of sphingosine with cardiolipin, we tested whether sphingosine induces a release of cytochrome c from isolated mitochondria. The data (Fig. 4C) show that sphingosine induces a release of cytochrome C from mitochondria, which is prevented by alkalization of the samples.

Given (i) the structure of sphingosine, which is an amino alcohol, (ii) the obvious significance of the NH$_2$ group for sphingosine-mediated killing of bacteria, and (iii) the observation that bacteria are much more sensitive to sphingosine than mammalian cells (12, 13, 18), we hypothesized that the NH$_2$ group in sphingosine becomes protonated at a neutral or slightly acidic pH and binds to negatively charged molecules in the plasma membrane of bacteria. One candidate meeting the characteristics of such a molecule is cardiolipin, a negatively charged lipid that is very important for respiration. Cardiolipin is present in the plasma membrane of bacteria, but is absent from the plasma membrane of mammalian cells, in which it is present exclusively in mitochondrial membranes (consistent with the endosymbiont hypothesis).

To test this hypothesis, we determined whether sphingosine binds to cardiolipin *in vitro*. To this end, we co-incubated cardiolipin that was immobilized to agarose beads with soluble sphingosine and determined
binding of sphingosine. The results of these co-precipitation experiments show that sphingosine binds cardiolipin (Fig. 5A), most efficiently at low pH. Sphingosine showed some residual binding to control lipids, i.e. phosphatidylethanolamine or phosphatidylcholine, which was removed by addition of 0.1% NP40 to reduce hydrophobic interactions between the acyl chains (Fig. 5A).

Next, we incubated sphingosine immobilized to beads with soluble cardiolipin. The results confirm the interaction of sphingosine with cardiolipin (Fig. 5B).

The binding of sphingosine to cardiolipin depends on the pH and is maximal at pH 6.0 and greatly reduced at pH 8.0. This supports the notion that protonation of the NH₂ group in sphingosine is required for binding to cardiolipin. The binding assays show an almost quantitative binding of the two lipids at low or physiological pH indicating a strong binding affinity (Fig. 5A, B).

To gain insight into potential mechanisms how sphingosine kills bacteria upon binding to cardiolipin, we generated cardiolipin-containing liposomes and tested binding of sphingosine. These studies demonstrate that cardiolipin-containing liposomes bind sphingosine. Binding of sphingosine to cardiolipin results in a marked decrease of membrane fluidity (Fig. 5C). This is consistent with the hypothesis that sphingosine induces rigid, gel like cardiolipin-enriched domains in bacteria in vivo.

To demonstrate the role of cardiolipin in sphingosine-mediated killing of bacteria, we used an E. coli strain that lacks cardiolipin synthase A. Although E. coli expresses three cardiolipin synthases, only cardiolipin synthase A is expressed under logarithmic growth conditions (20). The results show that the E. coli strain lacking cardiolipin synthase A is almost completely resistant to sphingosine, whereas the wild-type strain is rapidly killed (Fig. 6A).

These studies were recapitulated with two transposon mutants of P. aeruginosa strain PAO-1 that also lack cardiolipin synthase. These strains were resistant to sphingosine-mediated killing in vitro (Fig. 6B). We also infected mice deficient for Cftr (22) with the wild-type and the two mutant P. aeruginosa strains and determined the effect of an inhalation of sphingosine on pulmonary P. aeruginosa counts. These studies reveal that the cardiolipin synthase-deficient P. aeruginosa strains are resistant to
sphingosine inhalation, while the parental strain was killed by inhalation of sphingosine (Fig. 6C,D).

In addition, we wanted to exclude the possibility that sphingosine kills bacteria by interfering with the respiratory process only. Therefore, we tested sphingosine efficiency under hypoxic conditions. Hypoxia reduced, but did not abrogate the bactericidal effects of sphingosine on *P. aeruginosa* (Fig. 6E).
Discussion

Our studies demonstrate that sphingosine induces a very rapid death of *P. aeruginosa* and *S. aureus* that is obviously caused by a massive increase of membrane permeability, loss of important metabolites such as ATP and a loss of metabolic activity. These findings are consistent with a previous study that showed rapid membrane permeabilization of *S. aureus* upon incubation with certain fatty acids (23). This study also tested the effects of sphingosine, which is not a fatty acid but rather a sphingoid base, on bacterial killing and reported a MIC of 7.8 µM for sphingosine (23), which is very similar to the observations in the present manuscript. However, the molecular mechanism of sphingosine-mediated killing of bacteria was not addressed in this study. Additional electron microscopy studies demonstrated that incubation of *S. aureus* and *E. coli* with 5 µM sphingosine induces a disruption and even loss of the cell wall of *S. aureus* (15). In contrast, the cytoplasmic membrane and the outer envelope in gram negative *E. coli* appeared to be intact after sphingosine incubation. This indicates that sphingosine does not simply lyse bacteria, in accordance with the conclusion of the present manuscript. Here, we demonstrate that the NH$_2$-group in sphingosine is critical for mediating bacterial killing and that sphingosine is inactive at alkaline pH. This strongly suggests that the NH$_2$-group of sphingosine must be protonated to mediate killing of pathogens. We have previously shown that sphingosine is mainly present on the surface of epithelial cells in the nose, trachea and bronchi (12,13,18) and, in fact, the pH on these epithelial cell layers is slightly acidic (19). Thus, sphingosine will be protonated in its natural environment. These findings let us to speculate that sphingosine binds to negatively-charged lipids in the plasma membrane of bacteria. Further, we noted that inhalation of mice or mini-pigs with even high doses of sphingosine (up to 3 µmol total) twice daily over 14 days did not result in any damage or inflammation in the trachea, bronchi, or lungs (24,25). Thus, we further speculated that sphingosine targets a highly negatively-charged lipid in bacterial membranes that is not present in mammalian plasma membranes. Cardiolipin fulfills these criteria, since it is highly negatively charged and present in the plasma
membrane of bacteria but absent from the plasma membrane of mammalian cells, in which it is present exclusively in mitochondrial membranes. Further, cardiolipin is critical for respiration and interference with cardiolipin might also interfere with respiration, although our data show that sphingosine also kills pathogens under hypoxic conditions, suggesting that an interaction of sphingosine with cardiolipin does not kill bacteria by blocking respiration. Our studies demonstrate that sphingosine binds to cardiolipin and, further, they show that the presence of cardiolipin is required for sphingosine-mediated bacterial killing. How does the interaction of sphingosine with cardiolipin might mediate death of bacteria? Cardiolipin contains 2 phosphatidyl-residues connected by a glycerol bridge and four associated fatty acyl chains, which are characterized by a high degree of symmetry and unsaturation. This structure results in a relatively small cross-section of its headgroup relative to the large cross-section of its four large tail groups. Therefore, the incorporation of cardiolipin into membranes results in a large negative curvature (26). Binding of positively charged sphingosine may trigger an aggregation of negatively charged cardiolipin molecules. The aggregation of cardiolipin may result in the formation of very rigid, gel-like membrane domains in the otherwise more fluid membrane. The disturbance of the structure of the membrane may ultimately result in membrane permeabilization and thereby in rapid bacterial death. These notions are supported by our studies on liposomes that indicate a marked decrease of membrane fluidity upon binding of sphingosine. Previous studies have shown that the aminoglycoside antibiotic 3',6-dinonyl neamine interacts with cardiolipin in bacterial membranes and mediates a clustering of cardiolipin in the bacterial membrane (27). This resulted in decreased fluidity and increased permeability. These data strongly support the notion that binding of positively charged, amphiphilic amines to bacterial membranes results in killing of bacteria by changing the biophysical properties of the bacterial plasma membrane by clustering cardiolipin (27).

Consistent with the notion that positively charged sphingosine binds to negatively charged membrane lipids, we noted that higher doses (>20 µM) of sphingosine also killed the E. coli strain that was deficient in cardiolipin.
synthase-A (not shown). This finding suggests that cardiolipin is the primary target of sphingosine and that, at lower concentrations of sphingosine, the clustering of cardiolipin is sufficient and required to alter bacterial membranes and to kill bacteria. However, sphingosine is also able to cluster smaller negatively charged lipids, but higher concentrations of sphingosine are necessary for producing large rigid domains that disrupt the membrane structure in the absence of cardiolipin. The binding of sphingosine to cardiolipin in bacterial membranes also provides an explanation for the finding that inhalation of sphingosine even up to concentrations of 1 mM does not induce any toxicity in epithelial cells: Because mammalian cells contain cardiolipin exclusively in mitochondria, much higher concentrations and internalization of exogenously added sphingosine are required to reach cardiolipin within mitochondria and to affect the viability of mammalian cells. However, since extracellular sphingosine is either integrated into the plasma membrane or endocytosed and, thus reaches endosomes, multivesicular bodies and lysosomes, mitochondria are not easily targeted by extracellular-applied sphingosine.

In summary, our data demonstrate that sphingosine very rapidly kills *P. aeruginosa* and *S. aureus* at low micromolar concentrations. Mechanistically, we demonstrate that sphingosine binds to cardiolipin in bacterial membranes and exerts thereby its bactericidal effects. These studies provide a mechanism how sphingosine kills pathogens and justify the further development of sphingosine as a novel antibacterial drug.
Experimental procedures

Bacterial strains
We used the *P. aeruginosa* strains 762 and American Type Culture Collection (ATCC) 27853, the septic *S. aureus* strain DH and the *E. coli* strains BW25113 JW1241-5. The *P. aeruginosa* strain 762 and the *S. aureus* strain DH are clinical isolates (13,21), the *P. aeruginosa* strain ATCC 27853 is a laboratory strain. The *E. coli* strain BW25113 is a wildtype strain, the JW1241-5 strain has a deletion of cardiolipin synthase A (Δcls). The *E. coli* strains were obtained from the *E. coli* stock center of Yale University, USA. Two cardiolipin synthase (Δcls) - deficient *P. aeruginosa* strains that were generated from PAO-1 by transposon-mediated mutagenesis were obtained from the TANSPOSON MUTANT COLLECTION of the University of Washington, Seattle, USA. All *P. aeruginosa* strains and *E. coli* strains BW25113 and JW1241-5 were grown overnight on tryptic soy agar (TSA; Becton Dickinson Biosciences, Heidelberg, Germany). *S. aureus* DH was grown overnight on blood agar plates. The bacteria were then transferred to tryptic soy broth (TSB, Becton Dickinson Biosciences), the density was adjusted to an OD$_{550nm}$ of 0.2 - 0.25 and the bacteria were grown for 1 h at 37°C with 125 rpm shaking to reach the early logarithmic phase and provide reproducible growth conditions. Bacteria were then centrifuged at 1710 x g (2800 rpm) (*P. aeruginosa* and *E. coli*) or 2240 x g (3000 rpm) (*S. aureus*) for 10 min, washed once in sterile HEPES/Saline (H/S; 132 mM NaCl, 20 mM HEPES [pH 7.4], 5 mM KCl, 1 mM CaCl$_2$, 0.7 mM MgCl$_2$, 0.8 mM MgSO$_4$) and resuspended in H/S for the following assays.

Sphingosine
Sphingosine (Avanti Polar Lipids, Alabaster, USA) was resuspended as a 20 mM stock solution in distilled water containing 10% octyl glucopyranoside (Sigma, Deisenhofen, Germany). Prior to use, the sphingosine stock was sonicated in a bath sonicator for 10 min to promote the formation of micelles.

Cell permeability
Each 10$^5$ colony forming units (CFU) of *P. aeruginosa* strains 762 and ATCC 27853 or *S. aureus* strain DH were incubated in H/S for 5 min with sphingosine at a concentration of 1 µM or 10 µM. Octylglucopyranoside, the solvent of sphingosine, was added at the same concentrations as in the
samples with sphingosine. If indicated, either 0.1% Triton or 80 µg/mL Nisin (Sigma, Deisenhofen, Germany) were added as positive controls for membrane permeabilization. Bacteria were then incubated with 100 nM TO-PRO-3 iodide (Life Technologies Inc.) at room temperature for 5 min. TO-PRO-3 is a monomeric carbocyanine, staining double stranded DNA and thereby detecting permeabilization of the bacteria. Bacteria were pelleted and analyzed by flow cytometry using a FACSCalibur (BD Biosciences) counting 20,000 bacteria/sample.

**ATP measurements**

Each $10^5$ CFU *P. aeruginosa* strains 762 and ATCC 27853 or *S. aureus* strain DH were incubated in H/S for 60 min with sphingosine at a concentration of 1 µM or 10 µM. Octylglucopyranoside was added at the same concentrations as in the samples with sphingosine. Bacteria were pelleted by centrifugation for 10 min at 2240 x g and the release of adenosine triphosphate (ATP) from the bacteria into the supernatant/medium and the remaining ATP in the resuspended pellet was measured with the BacTiter-Glo reagent (Promega) according to the instructions of the manufacturer. The pellet was resuspended in PBS and treated with 0.1% Triton to permeabilize bacteria. The samples were incubated for 2 min, 250 rpm horizontal shaking and the luminescence was measured using a luminescence reader. Either 0.1% Triton or 80 µg/mL Nisin were added as positive controls for membrane permeabilization and ATP release into the medium. ATP concentration was determined using a standard curve of ATP. Protein concentrations were determined in aliquots of the incubations prior to centrifugation using a commercial Bradford assay (BioRad).

**Metabolic activity of *P. aeruginosa***

Each $10^5$ CFU *P. aeruginosa* strains 762 and ATCC 27853 or *S. aureus* strain DH were incubated in H/S for 15 min with sphingosine at a concentration of 1 µM or 10 µM. Octylglucopyranoside was added at the same concentrations as in the samples with sphingosine. Metabolic activity was determined using the Vybrant Cell Metabolic Assay Kit (Life Technologies Inc., Darmstadt, Germany). To this end, bacteria were incubated with 1 µM C<sub>12</sub>-resazurin for an additional 15 min, washed and analyzed by flow cytometry. The assay employs the conversion (reduction) of
the nonfluorescent C₁₂-resazurin to fluorescent C₁₂-resorufin by viable cells. Either 0.1% Triton or 80 µg/mL Nisin were added as a positive controls for membrane permeabilization.

**Interaction of cardiolipin, phosphatidylcholine and phosphatidylethanolamine with sphingosine and measurements of sphingosine by sphingosine kinase assay**

Agarose-bound cardiolipin, phosphatidylcholine, phosphatidylethanolamine or control beads were purchased from Echelon Biosciences (Salt Lake City, USA). Aliquots of 30 µL were co-incubated with 1 nmol of either sphingosine or octylglucopyranoside in 100 µL H/S for 60 min at room temperature. The beads were extensively washed 6-times in H/S and extracted in CHCl₃/CH₃OH/1N HCl (100:200:1, v/v/v). Samples were dried and then resuspended in 50 mM HEPES (pH 7.4), 250 mM NaCl, 30 mM MgCl₂, 1 mM adenosine triphosphate (ATP), 10 µCi [³²P]γATP and 0.01 u/mL sphingosine kinase 1 (R&D). Samples were incubated for 30 min at 37°C with shaking (350 rpm). The sphingosine kinase reaction was terminated by adding 100 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 thin-layer chromatography (TLC) plates (Merck) with CHCl₃:CH₃OH:acetic acid:H₂O (90:90:15:5, v/v/v/v) as developing solvent. The TLC plates were analyzed with a phosphoimager, and sphingosine was quantified with a standard curve. The solvent octylglucopyranoside (used as a control) showed no background binding to cardiolipin in the kinase assays.

**Interaction of sphingosine with cardiolipin**

Agarose-bound sphingosine or control beads were purchased from Echelon Biosciences. Aliquots of 20 µL were co-incubated with 2 nmol of either cardiolipin or, as control, dimethyl sulfoxide in 200 µL H/S at different pH values (pH 5.0, 6.0, 7.0, 8.0 and 9.0) for 30 min at room temperature. The beads were extensively washed 6-times in H/S and extracted in CHCl₃:CH₃OH:1N HCl (100:100:1, v/v/v). Samples were dried and then resuspended in 10 µM Cardiolipin probe (BioVision, Biozol Diagnostica, Eching, Germany), a fluorescent
probe for the detection and quantification of cardiolipin. The samples were incubated for 5 min, 250 rpm shaking and the fluorescence was measured using a fluorescence reader. Cardiolipin concentration was determined using a standard curve of cardiolipin.

**Bacterial killing assays**
Each 10,000 CFU *P. aeruginosa* strains 762, ATCC 27853, PAO-1 or of the two cardiolipin synthase (Δcls) deficient PAO-1 mutants or *S. aureus* strain DH or 5,000 CFU *E. coli* strains BW25113 or JW1241-5 that were grown to the early logarithmic phase as described above, were incubated with 1 μM, 5 μM or 10 μM stearylamine or sphingosine or with the corresponding concentrations of the solvent octylglucopyranoside in H/S or phosphate-buffered saline (PBS) for the indicated times. If indicated the pH of the PBS was varied from pH 6.0 to pH 7.0 and pH 8.0. The bacteria were then washed, aliquots were plated on trypticase soy broth (TSB) plates, and CFU were counted after growth overnight.

**Liposome studies**
Membrane fluidity of different liposomes prior and after addition of 10 μM sphingosine was evaluated by measuring the fluorescence intensity of 1,6-diphenyl-1,3,5-hexatriene (DPH; Sigma-Aldrich) incorporated in the liposome membrane. Liposomes were prepared by high-pressure extrusion immediately before use. Briefly, to achieve the desired composition of the different liposomes (see figure 5C; lipid composition is indicated in the figure in mol%), stock solutions of the indicated lipids (L-α-phosphatidylcholine, Avanti Polar Lipids; Cholesterol, Echelon Biosciences; Sphingomyelin, Avanti Polar Lipids; Cardiolipin, Avanti Polar Lipids) (dissolved in chloroform; 10 mM) were mixed accordingly and dried by rotary evaporation under vacuum. After the solvent removal, dried lipid mixtures were hydrated in 10 mM HEPES/100 mM NaCl buffer (pH 7.4) at 50°C with periodic agitation on a thermal mixer. The liposome solution was frozen at -80°C and thawed at 50°C. This freeze-thaw cycle was performed 5 times. The resulting multilamellar vesicles (MLVs) were used to prepare large uni-lamellar vesicles (LUVs) by passing the MLV suspension 11 times through 100 nm polycarbonate filters in an extruder device (Avanti Mini-Extruder; Avanti Polar Lipids, Alabaster, USA). Indicated LUVs were incubated with 10
µM sphingosine for 1 h at 40°C with periodic agitation on a thermal mixer. A 1 mM stock solution of the fluorescent probe DPH was prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and a working solution of 10 µM (in PBS) was added to the LUV suspension in a ratio of 200/1 lipid/DPH. Fluorescence intensity of DPH was measured using a fluorescence reader (FLUOstar Omega; BMG Labtech) after incubation at 40°C for 1 h, 250 rpm shaking in the dark.

**Isolation and treatment of mitochondria**

To isolate mitochondria, cells were incubated for 30 min at 4°C in 0.3 M sucrose, 10 mM TES (pH 7.4), and 0.5 mM EGTA. Cells were then Dounce-homogenized and nuclei and unbroken cells were pelleted by centrifugation for 5 min at 600xg at 4°C. Supernatants were collected and centrifuged at 6000xg for 10 min at 4°C. The pellets were resuspended and mitochondria were purified employing a 10 min 60%, 30%, 18% Percoll gradient centrifugation at 8500xg at 4°C. Mitochondria at the interface between the 30% and 60% layers were collected, washed twice, and resuspended in 50 mM PIPES-KOH (pH 7.0 or pH 8.0), 50 mM KCl, 2 mM MgCl₂, 2 mM EGTA, 10 µg/ml A/L, 2 mM ATP, 10 mM phosphocreatine, 5 mM succinate, and 50 µg/ml creatine kinase (buffer 1). Mitochondria (corresponding to 1x10⁶ cells) were incubated on ice for 30 min with 0.5 µM sphingosine at pH 7.0 or pH 8.0 or left untreated. The mitochondria were then centrifuged, supernatants were discarded, the mitochondria were resuspended in 37°C prewarmed buffer 1 and incubated for 10 min at 37°C to permit release of cytochrome c. The reaction was terminated by addition of one volume ice-cold buffer 1, centrifugation at 20 800 x g at 4°C and addition of the supernatant to 5x SDS-sample buffer. The samples were analyzed for cytochrome c release by Western blotting. Western blots were analyzed using a monoclonal mouse anti-cytochrome c antibody (clone 7H8.2C12, BD Pharmingen, San Diego, USA) and an ECL system.

**Hypoxia experiments**

*P. aeruginosa 762* was grown under hypoxic or normoxic conditions in a BD Gas Pak chamber (Becton Dickinson) for 24 h. 10⁵ colony forming units (CFU) were then exposed to 10 µM sphingosine for 1 h. Hypoxia or normoxia was maintained during the incubation period. CFU were then
determined to measure the survival of the bacteria.

**Mouse infections**

B6.129P2(CF/3)-Cfr

\(Tg^H(neoim)Hgu\) (abbreviated \(Cfr^{MHH}\)) mice were used in the present study (22). These mice express low levels of Cftr allowing feeding of a normal diet as previously described. Mice were housed in the Central Laboratory Animal Facility of the University of Duisburg-Essen, Germany. We used female and male mice with an age of at least 16 weeks and a weight between 25 and 35 g. Mice were divided into cages of equal size (usually 3-4 mice) by animal unit technical staff with no involvement in study design. Cages were randomly assigned to an experimental group. The investigators were blinded to the group allocation during the experiment and/or when assessing the outcome. PAO-1 or of the two \(\Delta\)cls-PAO-1 mutants were grown to the early logarithmic phase as described above and resuspended in RPMI 1640 plus 10 mM HEPES to a final concentration of \(5 \times 10^6\) CFU in 20 \(\mu\)L medium. To infect mice with the \(P.\ aeruginosa\) strains, mice were lightly anesthetized with diethyl ether, which does not affect ciliary function (22, 28). The mice were then inoculated intranasally with \(5 \times 10^7\) CFU of each \(P.\ aeruginosa\) strain employing a plastic-coated 30-gauge needle, which was inserted 2 mm into the nose. Mice were inhaled with 800 \(\mu\)L of a 125 \(\mu\)M sphingosine suspension in 0.9% NaCl 1 hr after the infection (the mice inhale approximately 10% of the inhalation volume). We determined the mouse health status 5 h after infection using the following score: Sickness score 0, unaffected (healthy appearance); score 1, slightly affected (ruffled fur); score 2, moderately affected (ruffled fur, breathing slightly impaired, normal body temperature); score 3, severely affected (ruffled fur, heavy breathing, lower body temperature). Bacterial numbers were then determined in the mouse lungs 5 h after infection. To this end, mice were sacrificed, the lungs were removed, homogenized, and lysed in 5 mg/mL saponin to release intracellular bacteria. Bacteria were then pelleted at 2240 \(x\) g, washed once in sterile PBS, diluted, plated and grown in duplicate on TSA plates for 12 hours. Bacterial numbers were counted. Infection experiments were approved by the Bezirksregierung Duesseldorf, Duesseldorf, Germany, under permission number 81-02.04.2019.A134. The actual care and treatment of the animals was
performed and/or overseen by veterinarians of the Central Animal Facility of the University Hospital Essen, Essen, Germany.

**Statistics**
Data are expressed as arithmetic means ± SD. In order to compare more than two groups we used one-way ANOVA followed by post-hoc Student’s t-tests for all pairwise comparisons applying Bonferroni correction for multiple testing. The p-values for the pairwise comparisons were calculated after Bonferroni correction. If only two groups were compared we used Student’s t-test for.

All values were tested for normal distribution. The sample size planning for the continuous variables in vivo infection experiments was based on two-sided Wilcoxon-Mann-Whitney tests (software: G*Power Version 3.1.7 of the University of Duesseldorf, Germany). Investigators were blinded for histology experiments and animal identity.

**Data Availability**
All the data are given in the manuscript.
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Conflicts of Interest
The authors declare to have no competing financial interests.

Author contributions
RV, KAB and EG performed the experiments. KAB, MJE and EG designed the studies. All authors reviewed the manuscript.

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Figure Legends

**Fig. 1: Sphingosine induces a rapid increase in the permeability of *P. aeruginosa***

(A, B) *P. aeruginosa* (P.a.) strains 762 and American Type Culture Collection (ATCC) 27853 or *S. aureus* (S.a.) strain DH were incubated with 100 nM TO-PRO-3 iodide. Bacteria were then treated for 5 min with 1 µM or 10 µM sphingosine (SPH) or the corresponding concentration of octyl glucopyranoside (OGP), the solvent of sphingosine. The bacteria were analyzed by flow cytometry. Panel A shows the quantitative analysis of the means of the fluorescence intensity obtained in the flow cytometry studies (given in arbitrary units; a.u.), panel B shows representative flow cytometry stainings from 4 independent experiments. Either Triton X-100 or Nisin was added as a positive control for membrane permeabilization.

(C, D) Intracellular ATP in *P. aeruginosa* (P.a.) strains 762 and ATCC 27853 or *S. aureus* (S.a.) strain DH (C) and the release of ATP (D) from the bacteria was measured with the BacTiter-Glo reagent. Sphingosine (SPH) results in a very rapid release of ATP from *P. aeruginosa* or *S. aureus* into the supernatant. Octyl glucopyranoside (OGP), the solvent of sphingosine, added at the same concentrations as in the samples with sphingosine, exerted no effect. Either Triton or Nisin was added as a positive control for membrane permeabilization and ATP release into the medium.

Displayed are the means ± SD of 4 independent experiments each in A, C and D. ***p<0.001, ANOVA.

**Fig. 2: Sphingosine abrogates the metabolic activity of *P. aeruginosa* and *S. aureus***

*P. aeruginosa* (P.a.) strains 762 and American Type Culture Collection (ATCC) 27853 or *S. aureus* (S.a.) strain DH were treated with 1 µM or 10 µM sphingosine (SPH) or octyl glucopyranoside (OGP), the solvent of sphingosine, at the corresponding concentrations. Samples were then incubated with the oxidized, non-fluorescent form of C12-resazurin and the metabolic activity was determined with flow cytometry by measuring the generation of fluorescent C12-resorufin. The studies showed that sphingosine results in a very rapid decrease in bacterial metabolic activity. Either Triton or Nisin was added as a positive control for membrane permeabilization.
permeabilization and disruption of bacterial metabolism. Fluorescence intensity is given in arbitrary units (a.u.). Displayed are the means ± SD of 4 independent experiments each. ***p<0.001, ANOVA.

Fig. 3: Sphingosine mediates killing of bacteria within minutes
Each 10 000 colony-forming units (CFU) of *P. aeruginosa* (*P.a.*) strains 762 and American Type Culture Collection ATCC 27853 were incubated in phosphate-buffered saline (PBS, pH 7.0) with 1 µM or 10 µM sphingosine (SPH) or the corresponding concentrations of the solvent octyl glucopyranoside (OGP) for 15 min or 60 min. Samples were washed, aliquots were plated on trypticase soy broth plates, and CFU were counted after growth overnight. Shown are the means ± SD of 4 independent experiments each. ***p<0.001, ANOVA.

Fig. 4: The NH₂-group and protonation of this group is required for the bactericidal effects of sphingosine on *P. aeruginosa* (A) Each 10 000 colony-forming units (CFU) of *P. aeruginosa* (*P.a.*) strains 762 and American Type Culture Collection ATCC 27853 were incubated with 1 µM, 5 µM, or 10 µM stearylamine or sphingosine (SPH) or 0.0005, 0.0025 or 0.005% of the solvent octyl glucopyranoside (OGP) in phosphate-buffered saline (PBS) adjusted to pH 7.0. After 60 min the bacteria were washed, aliquots were plated on trypticase soy broth plates, and CFU were counted after growth overnight. Shown are the means ± SD of 4 independent experiments each. *p<0.05, **p<0.01, ***p<0.001, ANOVA.
(B) Each 10 000 colony-forming units (CFU) of *P. aeruginosa* (*P.a.*) strains 762 and American Type Culture Collection ATCC 27853 were incubated with 10 µM sphingosine (SPH) in phosphate-buffered saline (PBS) adjusted to a pH of 6.0, 7.0, or 8.0 for 60 min and then washed. Controls were incubated with the corresponding concentrations of the solvent octyl glucopyranoside (OGP). Aliquots were then plated on trypticase soy broth plates, and CFU were counted after growth overnight. Shown are the means ± SD of 4 independent experiments each. ***p<0.001, ANOVA.
(C) Isolated mitochondria were incubated with 0.5 µM sphingosine at pH 7.0 or pH 8.0 for 30 minutes on ice or left untreated. The results indicate a release of cytochrome c from isolated mitochondria at pH 7.0, while sphingosine is inactive at pH 8.0. Shown is a representative western blot demonstrating the release of
cytochrome c into the supernatant and the amount of cytochrome c in the corresponding mitochondrial pellets from 4 independent experiments and the quantitative analysis using Photoshop® for measuring the density of the western blot signals. Given are the means ± SD, n=4, ***p<0.001; t-test.

**Fig. 5: Sphingosine binds to cardiolipin**

(A) Agarose-bound cardiolipin or control phosphatidylethanolamine (PE) and phosphatidylcholine (PC) beads were co-incubated with 1 nmol of sphingosine (SPH) at the indicated pH, extensively washed, extracted and the amount of bound sphingosine was determined by a sphingosine kinase assay. The solvent octyl glucopyranoside (used as a control; not shown) showed no background binding to cardiolipin in the kinase assays. Addition of 0.1% NP40 removed the residual binding of sphingosine to PE and PC beads. Shown are the means ± SD from 4 independent experiments each of the percentage of the added 1 nmol SPH that was bound to cardiolipin; ***p<0.001; ANOVA.

(B) Agarose-bound sphingosine or control beads were co-incubated with 2 nmol of cardiolipin (CLP), extensively washed, and the amount of bound cardiolipin was measured with a fluorescence assay. Sphingosine beads showed no background in the assay. Shown are the means ± SD from 5 experiments each of the percentage of the added 2 nmol CLP that was bound to cardiolipin.

(C) Membrane fluidity of different liposomes prior and after addition of sphingosine was measured by a fluorescence assay using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH). The lipid composition is indicated in the figure in mol%. Shown are means ± SD from 5 independent experiments each, *** p < 0.001, t-test.

**Fig. 6: Sphingosine mediated killing of bacteria requires cardiolipin**

(A) Each 5 000 colony-forming units (CFU) of *Escherichia coli* (E. coli) strain JW1241-5 (with a deletion of cardiolipin synthase A (ΔclsA) or the parental wild-type strain (wt) BW25113 were incubated with 5 or 10 µM sphingosine (SPH) or with the corresponding concentration of the solvent octyl glucopyranoside (OGP), or were left untreated in phosphate-buffered saline (PBS, pH 7.0) for 60 min. The bacteria were then washed, aliquots were plated on trypticase soy broth (TSB) plates, and CFU were counted after growth overnight. Shown are the means ± SD of 4 independent experiments each, **p<0.01, ***p<0.001, ANOVA.
(B) Wild-type or cardiolipin synthase-deficient *P. aeruginosa* PAO-1 were treated with 10 or 20 \( \mu \text{M} \) sphingosine for 1 h, plated, grown overnight and CFU were counted. Displayed are the means ± SD of 4 independent experiments each, ***p<0.001; ANOVA.

(C, D) Wild-type mice or CF mice (CF\(^{\text{MHhH}}\)) were intranasally infected with \( 5 \times 10^7 \) CFU of PAO-1 or the cardiolipin synthase-deficient mutants of PAO-1, inhaled with 800 \( \mu \text{L} \) of a 125 \( \mu \text{M} \) sphingosine (SPH) suspension in 0.9% NaCl 1 hr after the infection and the number of the bacteria in the lung (C) and the sickness score (D) were determined 5 hrs after the infection. Inhalation with the solvent, i.e. 0.0625% OGP in 0.9% NaCl served as controls. Shown are the means ± SD of 4 independent experiments each, ***p<0.001; t-test.

(E) *P. aeruginosa* 762 (\( 10^5 \) CFU) was grown and treated with 10 \( \mu \text{M} \) sphingosine for 1 h under hypoxic or normoxic conditions in a BD Gas Pak chamber (Becton Dickinson), bacteria were plated and CFU were determined after overnight growth. Displayed are the means ± SD of 4 independent experiments each, ***p<0.001; ANOVA.
Untreated

1 μM SPH

10 μM SPH

0.0005% OGP

0.005% OGP

Triton X-100

Metabolic activity

Fluorescence Signal [a.u.]

2000 - 8000

Pa. 762

ATCC 27853

Nisin

Fig. 2
Untreated

1 μM SPH, 15 min
10 μM SPH, 15 min
1 μM SPH, 60 min
10 μM SPH, 60 min
0.005% OGP, 60 min
0.0005% OGP, 60 min
0.005% OGP, 15 min
0.0005% OGP, 15 min

No CFU detected
No CFU detected

***
***

P.a. 762
P.a. ATCC 27853
Untreated, pH 7.0
10 μM SPH, pH 7.0
10 μM SPH, pH 8.0
0.005% OGP, pH 8.0
0.005% OGP, pH 7.0
0.005% OGP, pH 6.0

Pellet Supernatant
untreated
SPH, pH 8.0
SPH, pH 7.0
untreated
SPH, pH 8.0
SPH, pH 7.0

Cytochrome C
18 kDa
Sphingosine kills bacteria by binding to cardiolipin
Rabea Verhaegh, Katrin Anne Becker, Michael J. Edwards and Erich Gulbins

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