Sphingosine prevents binding of SARS-CoV-2 spike to its cellular receptor ACE2

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Abstract

Sphingosine has been shown to prevent and eliminate bacterial infections of the respiratory tract, but it is unknown whether sphingosine can be also employed to prevent viral infections. To test this hypothesis, we analyzed whether sphingosine regulates the infection of cultured and freshly isolated ex vivo human epithelial cells with pseudoviral particles expressing SARS-CoV-2 spike (pp-VSV-SARS-CoV-2 spike) that served as a bona fide system mimicking SARS-CoV-2 infection. We demonstrate that exogenously applied sphingosine suspended in 0.9% NaCl prevents cellular infection with pp-SARS-CoV-2 spike. Pre-treatment of cultured Vero epithelial cells or freshly isolated human nasal epithelial cells with low concentrations of sphingosine prevented adhesion of and infection with pp-VSV-SARS-CoV-2 spike. Mechanistically, we demonstrate that sphingosine binds to ACE2, the cellular receptor of SARS-CoV-2, and prevents the interaction of the receptor binding domain of the viral spike protein with ACE2. These data indicate that sphingosine prevents at least some viral infections by interfering with the interaction of the virus with its receptor. Our data also suggest that further preclinical and finally clinical examination of sphingosine is warranted for potential use as a prophylactic or early treatment for coronavirus disease-19.

Introduction

Infections with a novel member of the Coronaviridae family, named severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), are a serious global health problem and are responsible for the coronavirus disease 2019 (COVID-19) pandemic. Many details of the infection with SARS-CoV-2 remain to be defined, but it is now clear that a substantial number of patients develop severe symptoms and pneumonia upon infection, eventually requiring intensive care and ventilator treatment with a high mortality rate (1). COVID-19 seems to be a high risk for certain groups of individuals, for instance elderly, those with high blood pressure or overweight, but even healthy and young individuals develop severe disease (2). It is therefore of outstanding interest to develop a prophylactic treatment to prevent SARS-CoV-2 infections or to prevent at least a severe course of the disease after diagnosis.
SARS-CoV-2 infects cells by the initial interaction of the surface unit S1 of the viral spike glycoprotein with its cellular receptor angiotensin-converting enzyme 2 (ACE2) (3-6). The interaction of the two proteins allows viral entry and cellular infection. Studies employing antibodies and mutations of the S1 protein proved that abrogating the interaction between the S1 unit of the viral spike protein and ACE2 prevents infection with SARS-CoV-2 (3, 7).

Previous studies have used replication-deficient vesicular stomatitis virus (VSV) pseudoviral particles (pp-VSV) presenting coronavirus spike protein on their surface, abbreviated pp-VSV-SARS-CoV-2 spike (3). The studies showed that these particles accurately reflect key aspects of the entry of coronavirus into host cells (3), in particular they bind to ACE2 for infectious entry, which was inhibited by anti-ACE2 antibodies. Thus, this system allows a safe and easy method to study principle biomedical questions of SARS-CoV-2 infections. We used VSV pseudoviral particles carrying the spike protein of SARS-2 on their surface and recombinant proteins of spike and ACE2 to test whether sphingosine has an impact on the infection of human epithelial cells with SARS-CoV-2.

Sphingolipids localize into cellular membranes. They determine biophysical membrane properties and are involved in diverse cellular processes, including proliferation, cellular differentiation, apoptosis, signal transduction, and membrane trafficking (8-10). We and others have shown that ceramide often promotes bacterial and viral infections, for instance by mediating bacterial or viral adhesion and internalization (11-17). In contrast, sphingosine (2-amino-4-trans-octadecene-1,3-diol), which is released from ceramide by the activity of neutral, acid, or alkaline ceramidases (18), kills many bacterial pathogens and is an important component of the defense against bacterial pathogens in the respiratory tract (15, 19-24). We also showed an anti-viral function of endogenous sphingosine, which traps viruses in endosomes and thereby shuttles them to lysosomal degradation (25).

However, it is presently unknown whether exogenous sphingosine could be also exploited as an antiviral compound.
The studies reported here examined whether exogenously applied sphingosine prevents pp-SARS-CoV-2 spike infection of epithelial cells. We found that treatment of Vero epithelial or freshly isolated human nasal epithelial cells with sphingosine prevents infection with pp-SARS-CoV-2 spike. Sphingosine binds to ACE2, the cellular receptor for SARS-CoV-2, and blocks the interaction of the viral spike protein with ACE2. Sphingosine had no adverse effects on the epithelial cells. A panel of other lipids was without effect on infection of cells with pp-SARS-CoV-2 spike and the interaction of ACE2 with the viral spike protein. Thus, sphingosine might be a novel drug for preventing and treating infections with SARS-CoV-2 in the respiratory tract.
Results

Exogenous sphingosine prevents infection of epithelial cells with pp-VSV-SARS-CoV-2

To investigate whether exogenous sphingosine prevents the infection of epithelial cells with SARS-CoV-2, we treated Vero epithelial cells with 0.25 µM to 5 µM sphingosine for 30 min and then infected the cells with pp-VSV-SARS-CoV-2 spike. The results show that sphingosine dose-dependently prevented the infection with pp-VSV-SARS-CoV-2 spike (Fig. 1A). Already concentrations of 1 µM sphingosine reduced the infection of Vero epithelial cells with pp-VSV-SARS-CoV-2 by more than 90% (Fig. 1A). As specificity controls, we tested several other lipids, i.e. phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingomyelin, C16-ceramide, sphingosine 1-phosphate, lactosylceramide, cardiolipin, and octylglucopyranoside. None of them inhibited the infection of Vero cells with pp-VSV-SARS-CoV-2 spike (Fig. 1B). Sphingosine at concentrations of 0.25 µM to 5 µM was without adverse effects on cultured Vero epithelial cells (Fig. 1C, D).

Next, we tested whether exogenous sphingosine also prevented infection of freshly isolated human nasal epithelial cells with pp-VSV-SARS-CoV-2. The studies revealed that 1 or 2 µM sphingosine also prevented infection of freshly isolated human nasal epithelial cells (Fig. 2A, B). Sphingosine had no toxic effects on nasal epithelial cells (Fig. 2C, D).

Exogenous sphingosine binds to ACE2 and blocks the interaction of the viral spike protein with ACE2

Sphingosine is a small lipid that is positively charged at neutral or slightly acidic pH. The pH on nasal, tracheal or bronchial epithelial cell surfaces is slightly acidic (26), and thus, sphingosine will be positively charged when acting on these surfaces. The binding domain of the spike protein contains many positively charged amino acids that bind to negatively charged amino acids in the binding pocket of the ACE2 receptor, but also hydrophobic interactions between the receptor binding domain of the viral spike protein and ACE2 are required for viral binding (27). Thus, we speculated that sphingosine, which is an amphiphilic molecule that is positively charged, may bind to ACE2 and prevent the interaction of the spike protein with ACE2, thereby blocking infection. We tested this hypothesis by
incubation of sphingosine immobilized to agarose with recombinant ACE2 protein or lysates from Vero cells and determined by western blotting whether ACE2 directly binds to sphingosine. The results confirmed the hypothesis and showed binding of cellular or recombinant ACE2 to sphingosine beads (Fig. 3A), while we did not detect a binding of the recombinant receptor-binding domain (RBD) of spike to sphingosine beads (not shown). Addition of soluble sphingosine prevented binding of ACE2 to immobilized sphingosine supporting the notion of a specific interaction between ACE2 and sphingosine (Fig. 3A).

Likewise, incubation of immobilized recombinant ACE2 protein with sphingosine revealed a binding of sphingosine to ACE2 (Fig. 3B). We also tested the binding of ACE2 to agarose beads coupled to sphingosine, phosphatidylcholine, phosphatidyl-ethanolamine, sphingomyelin, ceramide, sphingosine 1-phosphate or cardiolipin. Control beads were not coupled to any lipid. The results show that only sphingosine-coupled beads bound recombinant ACE2 (Fig. 3C).

Next, we tested whether sphingosine interferes with the binding of viral spike protein with ACE2. To this end, immobilized recombinant Fc-ACE2 was incubated with 2 µM sphingosine or left untreated, washed and then the recombinant receptor-binding domain of spike S1 was added. Pre-incubation of ACE2 with sphingosine reduced binding of the recombinant RBD of spike S1 to ACE2 (Fig. 3D).

Controls show that several other lipids such as phosphatidylcholine, phosphatidylserine, sphingomyelin, C16-ceramide, sphingosine 1-phosphate and cardiolipin did not affect binding of recombinant receptor-binding domain of spike S1 to immobilized recombinant ACE2 (Fig. 3E).

We then immobilized a recombinant protein of the receptor-binding domain of the viral spike protein (His tagged) on Ni²⁺-agarose and added recombinant Fc-ACE2 in the presence or absence of 2 µM sphingosine. The results show that sphingosine abolished the interaction of the receptor-binding domain of spike protein with ACE2 (Fig. 3F). A panel of other lipids, i.e. phosphatidylcholine, phosphatidylserine, phosphatidyl-ethanolamine, sphingomyelin, C16-ceramide, sphingosine 1-phosphate, lactosylceramide, cardiolipin or octyl-glucopyranoside did not affect the
interaction of immobilized recombinant Spike-protein with ACE2 (Fig. 3G).

Next, we tested whether sphingosine also interferes with the binding of pp-VSV-SARS-CoV-2 to recombinant ACE2. The studies revealed that 1 or 2 µM sphingosine abrogated binding of intact pp-VSV-SARS-CoV-2 to immobilized recombinant ACE2 resulting in effective pseudo-infection of Vero cells with the remaining pp-VSV-SARS-CoV-2 particles (Fig. 4A). Finally, we performed adhesion studies of pp-VSV-SARS-CoV-2 on Vero cells in the presence or absence of sphingosine: We infected Vero cells with pp-VSV-SARS-CoV-2 for 20 min in the presence or absence of sphingosine, phosphatidylcholine sphingomyelin, C16-ceramide, sphingosine 1-phosphate, lactosyl-ceramide or cardiolipin, stained with anti-spike S1 antibodies and determined binding of the virus to the cells by flow cytometry. The results (Fig. 4B) show that sphingosine prevented binding of pp-VSV-SARS-CoV-2 to the cell surface, while none of the other lipids had an effect on the interaction of the virus with the cells.
**Discussion**

Our results demonstrate that exogenous sphingosine prevents infection of cultured epithelial cells and, most importantly, also freshly isolated human nasal epithelial cells with ppVSV-SARS-CoV-2. Sphingosine inhibited infection of epithelial cells already at concentrations as low as 0.25 $\mu$M and an almost complete inhibition of infection was achieved with 2 $\mu$M sphingosine. Sphingosine had no side or toxic effects on human nasal epithelial cells or cultured Vero cells at these concentrations. In a series of biochemical and cellular studies, we demonstrate that the binding of recombinant ACE2 to viral spike protein and the adhesion of virus to cells is abrogated by co-incubation with sphingosine already at low concentrations. Sphingosine specifically interacted with ACE2, while we did not detect binding of sphingosine to the receptor-binding domain of the viral spike protein. At present it is unknown how sphingosine prevents binding of spike to ACE2. It might be possible that the amphiphilic sphingosine molecule binds to polar and hydrophobic amino acids in the pocket of ACE2 that interact with the receptor binding domain of the viral spike protein (27), but further, detailed structural studies are required to clarify the details of the interaction of sphingosine with ACE2. Although our data based on cellular systems and recombinant proteins strongly suggest that sphingosine directly acts on ACE2 to prevent binding of spike, our data do not prove that sphingosine directly interacts with the ACE2 domain that interacts with the receptor-binding domain of spike. It is also possible that sphingosine interacts with other domains of ACE2 outside of the interaction site between ACE2 and the spike protein. The interaction of sphingosine with ACE2 might result in a conformational change of the spike-binding domain within ACE2 thereby preventing binding of viral spike protein.

Sphingosine has been previously shown to kill bacterial pathogens at concentrations between 0.5 and 10 $\mu$M (15, 19-24). Mechanistically, it was demonstrated that the positively charged sphingosine interacts with negatively charged large lipids such as cardiolipin in bacterial membranes, clusters these lipids to large and rigid membranes and thereby induce a leakiness of the bacterial membrane (28). Here, using recombinant proteins, we demonstrate that sphingosine
directly interacts with cellular receptors such as ACE2 and thereby prevents interaction of the viral spike protein with its receptor excluding an indirect effect of sphingosine on ACE2 via membrane lipids. However, in vivo it might be possible that sphingosine also interacts with other lipids in the cell membrane, which may influence the distribution and conformation of ACE2 and this effect might contribute to the sphingosine-mediated inhibition of spike binding to ACE2.

We have previously shown that endogenous sphingosine also has an anti-viral function (25). These studies revealed that sphingosine binds to herpes simplex virus and thereby traps the virus within endosomes allowing the cell to degrade the virus particle within lysosomes upon fusion of endo- and lysosomes. Whether endogenous sphingosine plays a role in the cellular defense against SARS-CoV-2 remains to be determined, but it is possible that exogenous sphingosine also binds to complete SARS-CoV-2 and thereby targets the virus to lysosomal degradation, in addition to blocking the interaction of the virus with its receptor. Thus, sphingosine may have a dual function in the defense against SARS-CoV-2 acting on both, ACE2 and possibly also SARS-CoV-2.

Previous studies applying sphingosine to the airways of mice and minipigs via inhalation of aerosolized sphingosine demonstrated that even high concentrations of sphingosine do not induce any cell death or any other toxicity in airway epithelial cells (21, 22, 24, 29). We also did not observe a pro-inflammatory effect of sphingosine inhalation or nasal application, which could be induced by phosphorylation of exogenous sphingosine into sphingosine 1-phosphate and subsequent chemotaxis of immune cells. Further, even high concentrations of sphingosine applied to the epithelial cells upon coating of endo-tracheal tubes with sphingosine did not result in any measurable damage of epithelial cells or disruption of the integrity of the epithelial cell layer (24). These studies suggest that intranasal application of sphingosine or inhalation of sphingosine into the lower airways should be safe.

Our data demonstrate that application of exogenous sphingosine prevents infection with pp-VSV-SARS-CoV-2. These particles represent a bona fide system to study early events that
mediate cellular infection with SARS-CoV-2. Thus, our data suggest that a nasal spray of sphingosine or application of aerosolized sphingosine might be a novel approach to prevent or treat infection with SARS-CoV-2.
**Material and Methods**

**Pseudoviral particles**

Pseudotyped viral particles were based on a replication-deficient vesicular stomatitis virus as previously described (30). The particles encode for enhanced green fluorescent protein (eGFP) and firefly luciferase instead of parental VSV-G. VSV*ΔG-FLuc was kindly provided by Dr. Gert Zimmer (Institute of Virology and Immunology Mittelhäusern, Switzerland) (31). HEK-293T cells were transiently transfected for 24 h using the calcium-phosphate method to express either SARS-2-Spike or VSV-G. Cells were then inoculated with VSV-G-trans-complemented VSV*ΔG-FLuc for 1 h at 37°C and 5% CO₂. The inoculum was then removed, cells were washed with PBS and cultured in fresh medium at 37°C and 5% CO₂ for 16 h. In case of SARS-2-Spike-expressing cells, the culture medium was supplemented with anti-VSV-G antibody (11, mouse hybridoma supernatant from CRL-2700; ATCC) to inactivate residual VSV-G-trans-complemented VSV*ΔG-FLuc. Culture supernatants were finally harvested and cellular debris was pelleted by centrifugation (4 000 x g, 4°C, 10 min). The clarified supernatants, containing pp-VSV-SARS-CoV-2 spike, were used for the experiments.

**Sphingosine and other lipids**

Sphingosine was suspended in 0.9% NaCl and sonicated in a bath sonicator (Bandelin Sonorex) until we obtained a homogenous suspension. Sphingosine was stored at -20°C, thawed and bath sonicated for 10 min prior any experiment. The same volume of 0.9% NaCl as used for the sphingosine-treated samples was added to all control samples. The other lipids were also prepared as suspension in 0.9% NaCl and sonicated prior to use.

**Infection of human epithelial cells**

Human nasal epithelial cells were obtained from healthy volunteers by nasal brushings with a small brush. Cells were suspended immediately in HEPES/Saline (H/S; 132 mM NaCl, 20 mM HEPES [pH 7.4], 5 mM KCl, 1 mM CaCl₂, 0.7 mM MgCl₂, 0.8 mM MgSO₄), washed once and resuspended in H/S. Cells were treated with 0.25 µM, 0.5 µM, 1 µM, 2 µM or 5 µM sphingosine (Avanti Polar Lipids, #860490) or left untreated for 30 min, pelleted and resuspended in MEM supplemented with 10% FCS containing pp-VSV-SARS-CoV-2 spike and the same
concentration of sphingosine as used in the pre-incubation period. Cells were then infected for 60 min, washed once in H/S and cultured for 24 h in MEM supplemented with 10% FCS to allow expression of the eGFP encoded by the particles. Infection was analyzed on a Leica TCS-SP5 confocal microscope by counting the percentage of eGFP-positive epithelial cells in at least 500 epithelial cells per sample in randomly chosen microscopic fields.

The local ethics committee of the University Hospital Essen approved the experiments under the permission number 20-9348-BO. The studies were performed in accordance with the Declaration of Helsinki principles.

Infection of Vero cells

Vero cells (ATCC® CCL-81™; monkey kidney epithelial cells) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 mM HEPES (pH 7.4; Carl Roth GmbH, Karlsruhe, Germany), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 µM nonessential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin (all from Invitrogen), and 10% FCS (PAA Laboratories GmbH, Coelbe, Germany). The cells were grown to sub-confluency on glass cover slips in a 24-well plate for 24 h prior to the experiments. Cells were then washed once with H/S and incubated with 0.25 µM, 0.5 µM, 1 µM, 2 µM or 5 µM sphingosine or left untreated for 30 min in H/S. As further controls we incubated the cells with each 5 µM phosphatidylcholine (Sigma, #P7443), phosphatidylserine (Sigma, #P7769), phosphatidylethanolamine (Sigma, #P7693), sphingomyelin (Sigma, #P85615), C16-ceramide (Avanti-Polar Lipids, #860516), sphingosine 1-phosphate (Sigma, #S9666), lactosylceramide (Avanti-Polar Lipids, #860576), cardiolipin (Avanti-Polar Lipids, #710333) or octylglucopyranoside (Sigma, #O8001) for 30 min prior to the infection. The supernatant was removed and cells were infected with pp-VSV-SARS-CoV-2 spike in the presence of the same concentration of sphingosine or the above-described control lipids as used during the pre-incubation period. Infection was terminated after 60 min, the medium removed and the cells cultured for additional 24 h in DMEM medium supplemented as above to allow expression of eGFP. The medium was removed; cells were washed once in H/S, fixed in 1% PFA buffered with PBS (pH 7.3) for 10 min, washed,
embedded in Mowiol, and analyzed with a Leica TCS-SL confocal microscope equipped with a 40× lens and Leica LCS software version 2.61. We counted eGFP-positive cells in 2000 cells/sample in randomly chosen microscopic fields. Infected cells show a homogenous and strong expression of eGFP.

**FITC-Annexin V binding**
Human nasal epithelial or Vero epithelial cells were treated as above with sphingosine for 30 min in H/S or left untreated, washed and incubated in DMEM with sphingosine for 60 min or left untreated. Cells were washed again and incubation was then continued for 24 h in DMEM containing 10% FCS. Cells were trypsinized, washed in H/S, stained for 15 min with FITC-Annexin V (Roche), and analyzed by flow cytometry. Controls were permeabilized for 5 min with 0.1% Triton X-100 at room temperature before incubation with FITC-Annexin V.

**TUNEL studies**
Human nasal epithelial cells or Vero epithelial cells were treated with sphingosine or left untreated as described for FITC-Annexin V staining. Cells were trypsinized, washed with PBS, fixed in 1% PFA buffered in PBS (pH 7.4) for 10 min, washed in PBS, permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 5 min at room temperature, and washed. The TUNEL reaction was performed following exactly the instructions of the vendor (Roche) using recombinant terminal deoxynucleotidyl transferase (rTdT; EC 2.7.7.31) and tetramethyl rhodamine (TMR) red-labeled nucleotides. Positive controls were incubated with recombinant micrococcal nuclease or DNase I (3000 U/ml) for 10 min at 22°C before the TUNEL reaction. Samples were analyzed by flow cytometry.

**Binding of sphingosine to immobilized recombinant ACE2**
To determine binding of sphingosine to ACE2, 2 µg Fc-ACE2 (Abcam, # ab273687) were immobilized by binding to protein A/G agarose beads (30 µL/sample; Santa Cruz Inc., # sc2003) in 500 µL H/S supplemented with 10 µg/mL each aprotinin and leupeptin (H/S+A/L) at 4°C for 60 min. The complexes were washed 3-times in H/S, resuspended in 1 mL H/S+A/L and sphingosine was added at a concentration of 2 µM. To this end, sphingosine was suspended at 2.5 mM in 0.9% NaCl, sonicated for 10 min in a bath sonicator and then diluted into the
samples. The immobilized ACE2 samples were incubated with sphingosine for 120 min and washed 6-times in H/S+A/L. Controls were protein A/G agarose beads only that were incubated with sphingosine. Samples were then extracted in CHCl₃:CH₃OH:1N HCl (100:100:1, v/v/v) and sphingosine was quantified by a kinase assay.

**Quantitative sphingosine measurement by kinase assay**

Cells were extracted 200 µL H₂O and 800 µL CHCl₃:CH₃OH:1N HCl (100:200:1, v/v/v). The lower phase was dried and resuspended in a detergent solution (7.5% [w/v] n-octyl glucopyranoside, 5 mM cardiolipin in 1 mM diethylenetriaminepentaacetic acid [DTPA]). The kinase reaction was initiated by addition of 0.001 units sphingosine kinase in 50 mM HEPES (pH 7.4), 250 mM NaCl, 30 mM MgCl₂, 1 mM ATP and 10 µCi [³²P]γATP. Samples were incubated for 60 min at 37°C with shaking (350 rpm). The reaction was terminated by adding 100 µL H₂O, 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. Samples were vortexed between additions. Phases were separated, 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. Sphingosine levels were determined with a standard curve of C18-sphingosine.

**Binding of human recombinant ACE2 to immobilized lipids**

The following lipids were immobilized to agarose: Sphingosine (Sphingobeads, Echelon, #S-6100), phosphatidylcholine (Echelon, #P-BOPC), phosphatidylethanolamine (Echelon, #P-BOPE), sphingomyelin (Echelon, #P-BOSM), ceramide (Echelon, #P-BCER), sphingosine 1-phosphate (Echelon, #S6110), cardiolipin (Echelon, #P-BCLP), or unloaded control beads (Echelon, #P-8000). Lipid-coated beads (each 30 µL) were incubated with 2 µg Fc-ACE2 in 500 µL H/S+A/L at 4°C for 120 min. Complexes were washed 6-times in H/S+A/L, eluted in 1x SDS-sample buffer, boiled for 5 min at 94°C and centrifuged at 20 800 x g for 5 min. The supernatants were separated on 7.5% SDS-PAGE, blotted onto nitrocellulose membranes and blocked in Starting Block (Pierce, #37542). The
membranes were washed twice, each 5 min, in 20 mM Tris base, 150 mM NaCl (pH 8.0) supplemented with 0.1% Tween 20 (TBS/Tw), incubated for 45 min with anti-ACE2-antibodies (1:1000, R&D, #AF933), washed 6-times, each 10 min, in Tris-buffered saline supplemented with 0.05% Tween 20 (TBS/Tw), incubated for 45 min with alkaline phosphatase (AP)-coupled anti-goat antibodies (1:50 000, Abcam, #ab97107), washed 6-times and developed using an ECL kit from G&E Healthcare.

**Binding of cellular ACE2 to immobilized sphingosine**

Vero cells were lysed in 25 mM HEPES, 3% NP40, 0.1% Triton X-100, 10 mM EDTA, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 125 mM NaCl, and 10 µg/mL aprotinin/leupeptin with a concentration of 5 x 10⁶ cells/mL for 5 min on ice, centrifuged at 20 800 × g for 5 min at 4°C and the supernatants, equivalents of 2.5 x 10⁶ cells were diluted 1:10 in H/S+A/L and incubated with 30 µL sphingosine immobilized to agarose (Sphingobeads) or control beads for 60 min at 4°C. Samples were then analyzed by Western blotting for ACE2 as above. To prove specificity of the binding between immobilized sphingosine and ACE2, we also added 2 µM suspended sphingosine to some samples.

**Blocking of the binding of human recombinant ACE2 to pp-VSV-SARS-CoV-2-spike by sphingosine**

To test whether sphingosine blocks binding of viral spike to human ACE2, Fc-ACE2 (2 µg) was immobilized on 30 µL protein A/G agarose for 60 min in 500 µL H/S+A/L at 4°C. The complexes were washed twice in H/S+A/L, 2 µg sphingosine was added or the complexes were left untreated. Samples were incubated at 4°C for 60 min. Complexes were washed twice in H/S+A/L and 50 µL pp-VSV-SARS-CoV-2-spike was added. Samples were incubated for 60 min at 4°C, pelleted by centrifugation for 2 min at 20 800 × g and the supernatants were used to infect Vero cells as a measurement how much functional virus was depleted from the supernatant as a consequence of binding to immobilized Fc-ACE2.

**Binding of human recombinant ACE2 to recombinant RBD spike in the presence of sphingosine or other lipids**

a.) To test whether sphingosine blocks binding of viral spike to human ACE2,
we immobilized 2 µg Fc-ACE2 on 30 µL protein A/G agarose beads in 500 µL H/S+A/L at 4°C for 120 min in the presence or absence of each 2 µM sphingosine, phosphatidylcholine, phosphatidylserine, sphingomyelin, C16-ceramide, sphingosine 1-phosphate, cardiolipin or no lipid. Controls were protein A/G agarose beads. Samples were then washed 3-times in H/S+A/L, resuspended in 500 µL H/S+A/L and incubated with 2 µg recombinant receptor binding domain of spike (RBD-spike, Sino Biologicals, #40150-R007) for 60 min at 4°C. Samples were washed 6 times in H/S+A/L, eluted in 1x SDS-sample buffer and analyzed by western blotting for ACE2 as above using anti-ACE2 antibodies.

**Adhesion studies**

Vero-cells were incubated for 30 min with each 2 µM sphingosine, phosphatidylcholine, sphingomyelin, C16-ceramide, sphingosine 1-phosphate, lactosylceramide, cardiolipin, or were left untreated, washed, pp-VSV-SARS-CoV 2 spike were added, the lipids were reconstituted to the same concentration as before and the cells were infected for 20 min. The supernatants were discarded, the cells were then removed from the plates with cell dissociation buffer (Gibco, #13151-014), washed twice with H/S, incubated with anti-spike S1 antibodies (1:100, monoclonal rabbit antibody) for 45 min at 4°C, washed once in H/S.
and stained with F(ab)_2 donkey anti-rabbit IgG (Jackson ImmunoResearch, #711-096-152) for 45 min at 4°C. Samples were washed in H/S and analyzed by flow cytometry using a BD FACS-Calibur (Becton Dickinson).

**Quantification and Statistical Analysis**

Data are expressed as arithmetic means ± SD. For the comparison of continuous variables from independent groups we used one-way ANOVA followed by post hoc Student’s t-tests for all pairwise comparisons and the Bonferroni correction for multiple testing. The p values for the pairwise comparisons were calculated after Bonferroni correction. All values were normally distributed. Statistical significance was set at the level of p ≤ 0.05 (two-tailed). Sample size planning for the continuous variable was based on two-sided Wilcoxon-Mann-Whitney tests (free software: G*Power Version 3.1.7, University of Duesseldorf, Germany). Investigators were blinded to the identity of the samples in all microscopy experiments.

**Data availability statements**

All data are available on request from the authors. Authors confirm that all data are included in the manuscript.

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**Author contributions**

EG initiated the studies. MJE, KAB, BG, SHP, GCW, SW, KF, SAA and AC contributed to the planning of the studies. MH and SP prepared and provided pseudotyped VSV particles for the infection experiments. EC, AG, AC, KAB, BG and EG performed the studies on human epithelial cells. BW, MS and SK did the western blots, TUNEL, Annexin-V and actin stainings. EG did all other experiments. All authors read the manuscript and approved it.

**Conflict of interest**

MJE and EG have patented the use of sphingosine to treat viral infections.
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Figure legends

Fig. 1: Exogenous sphingosine prevents infection of Vero cells with pp-VSV-SARS-CoV-2

(A) Vero cells were treated with 0.25 µM to 5 µM sphingosine or left untreated for 30 min, infected with pp-VSV-SARS-CoV-2 spike for 1 h and infection was quantified after 24 h. Displayed are the mean ± SD of the percentage of infected cells from 8 independent experiments. ***p< 0.001, ANOVA followed by post hoc Student’s t-tests.

(B) A panel of other lipids added to the cells at each 5 µM concentration for 30 min did not change infection with pp-VSV-SARS-CoV-2 spike. Given are the mean ± SD of the percentage of infected cells from 6 independent experiments. ***p< 0.001 compared to sphingosine, ANOVA followed by post hoc Student’s t-tests.

(C, D) Adverse effects of sphingosine on Vero cells were assessed by FITC-Annexin V (C) and TUNEL (D). TUNEL and Annexin V stainings were analyzed by flow cytometry. DNase-treated cells served as positive controls for TUNEL assays. Permeabilized cells served as positive controls for the FITC-Annexin V staining. Shown are the mean ± SD, n=5, of the fluorescence (in arbitrary units, a.u.) of the flow cytometry studies.

Fig. 2: Treatment of freshly isolated nasal epithelial cells with sphingosine prevents infection with pp-VSV-SARS-spike without adverse effects on the cells

(A, B) Nasal epithelial cells were freshly isolated from volunteers using a small brush, treated with 0.25 µM to 2 µM sphingosine or left untreated for 30 min, infected with pp-VSV-SARS-CoV-2 for 1 h, washed and infected cells were quantified after 24 h. Displayed are the mean ± SD of infected cells from 6 independent experiments (A) and a representative fluorescence microscopy study from infected cells pretreated with 2 µM sphingosine or left untreated (n=6) (B). Infection of cells is indicated by intense, homogenous green fluorescence in these cells due to the expression of eGFP (B). *p<0.05 ***p< 0.001, ANOVA followed by post hoc Student’s t-tests.

(C, D) Adverse effects of sphingosine on nasal epithelial cells were assessed by FITC-Annexin V (C) and TUNEL stainings (D) as above. Shown are the mean ± SD
of the fluorescence in flow cytometry studies (n=5); ***p< 0.001, ANOVA followed by post hoc Student’s t-tests. Results are presented in arbitrary units (a.u.).

Fig. 3: Sphingosine binds to ACE2 and thereby blocks the interaction of ACE2 with the viral spike protein

(A) Sphingosine-coated beads (SPH-beads) or control (Ctr) beads were incubated with recombinant (rec.) Fc-ACE2 protein (left panel) or with lysates obtained from Vero cells (right panel), extensively washed and eluted in 1x SDS-sample buffer. As indicated, suspended sphingosine (SPH) was added (2 µM) to prevent binding of ACE2 to immobilized sphingosine. Samples were separated by 7.5% SDS PAGE electrophoresis and blotted with anti-ACE2 antibodies. The Fc-ACE2 protein has a molecular weight of approximately 180 kDa, the endogenous ACE2 protein of approximately 120 kDa. Shown are representative results from 5 independent experiments.

(B) Recombinant (rec.) Fc-ACE2 protein was immobilized on protein A/G agarose, washed and incubated with 2 µM sphingosine. Controls consisted of protein A/G agarose only, incubated with 2 µM sphingosine. Samples were washed, extracted in CHCl₃:CH₃OH:1N HCl (100:100:1, v/v/v) and sphingosine was quantified employing a kinase assay. Given are the mean ± SD of the sphingosine concentrations from 6 independent experiments. ***p< 0.001, ANOVA followed by post hoc Student’s t-tests.

(C) A panel of lipid-coated beads was used to test for the specificity of ACE2-binding to sphingosine (SPH) -coupled beads. Samples were prepared as above. Shown is a representative result from 4 independent experiments. SPH = sphingosine, PC = phosphatidyl-choline, PE = phosphatidylethanolamine, SM = sphingomyelin, CER = ceramide, S1P = sphingosine 1-phosphate, Clp = cardiolipin.

(D) Recombinant Fc-ACE2 protein was immobilized on protein A/G agarose, washed, incubated with 2 µM sphingosine (SPH) or left untreated, washed again and incubated with the recombinant receptor-binding domain of the spike protein. Samples were washed extensively, eluted, separated by 7.5% SDS-PAGE electrophoresis, blotted and developed with anti-spike antibodies. The recombinant receptor-binding domain of the spike protein is His-tagged and has a molecular weight of approximately 27 kDa. Protein A/G agarose beads without Fc-ACE2 served as control. Shown are representative results from 5 independent experiments.
(E) A variety of other lipids were added as indicated to immobilized recombinant (rec.) human Fc-ACE2. The effects of these lipids on binding of recombinant receptor-binding domain of the spike protein were determined by western blotting. Shown is a representative result from 4 independent studies.

(F) Recombinant (rec.) His-tagged receptor-binding domain (RBD) of spike was immobilized on Ni$^{2+}$-agarose, washed, incubated with 2 $\mu$M sphingosine or left untreated, recombinant human Fc-ACE2 was added and the samples were incubated for 60 min. Beads without addition of RBD-spike served as control. Samples were washed extensively, eluted, separated by 7.5% SDS-PAGE electrophoresis, blotted and developed with anti-ACE2 antibodies. Shown are representative results from 5 independent experiments.

(G) A variety of other lipids were added as indicated to immobilized recombinant (rec.) RBD-spike protein. The effects of these lipids on binding of recombinant human Fc-ACE2 were determined by western blotting. Shown is a representative result from 4 independent studies. SPH = sphingosine, PC = phosphatidyl-choline, PS = phosphatidylyserine, PE = phosphatidylethanolamine, SM = sphingomyelin, C16-CER = C16-ceramide, S1P = sphingosine 1-phosphate, LC = lactosylceramide, Clp = cardiolipin, OGP = octylglucopyranoside.

**Fig. 4: Sphingosine binds to ACE2 and prevents binding of pp-VSV-SARS-CoV-2 spike**

(A) Recombinant (rec.) Fc-ACE2 protein was immobilized (imm.) on protein A/G agarose, washed, incubated with 1 or 2 $\mu$M sphingosine (SPH) or left untreated, washed to remove unbound sphingosine and incubated with pp-VSV-SARS-CoV-2 spike. A/G agarose without recombinant Fc-ACE2 served as control. Samples were pelleted, the supernatants containing unbound virus were collected and Vero cells were infected with the supernatants for 24 h. The results show that pp-VSV-SARS-CoV-2 was bound to recombinant Fc-ACE2, which was prevented by sphingosine. Displayed are the means ± SD of the percentage of infected cells from 8 independent experiments. ***p< 0.001, ANOVA followed by post hoc Student’s t-tests.

(B) Vero-cells were incubated for 30 min with each 2 $\mu$M sphingosine, phosphatidylcholine, sphingomyelin, C16-ceramide, sphingosine 1-phosphate, lactosylceramide, cardio-lipin or were left untreated, washed, pp-VSV-SARS-CoV-2 spike were added, the lipids were reconstituted to the same concentration as before.
Cells were incubated for 20 min. The cells were then removed from the plate, stained with FITC-coupled anti-spike antibodies and analyzed by flow cytometry. Shown is a representative result from 4 independent experiments and the quantitative analysis of the fluorescence intensity; ***p< 0.001, ANOVA followed by post hoc Student’s t-tests. Fluorescence is given in arbitrary units (a.u.).
Fig. 1

A

Infection [% eGFP-positive cells]

Sphingosine [concentration]

pp-VSV-SARS-CoV-2 spike

B

Infection [% eGFP-positive cells]

Lipid treatment

pp-VSV-SARS-CoV-2 spike

C

FITC-Annexin V

Mean fluorescence [a.u.]

Sphingosine [concentration]

Permeabilized

D

TUNEL

Mean fluorescence [a.u.]

Sphingosine [concentration]

DNase treatment
Fig. 2

A

Infection [% eGFP-positive cells]

Sphingosine concentration

Infection pp-VSV-SARS-CoV-2 spike

0.25 μM
0.5 μM
1 μM
2 μM

B

pp-VSV-SARS-CoV-2 spike + sphingosine

C

FITC-Annexin V Mean fluorescence [a.u.]

Sphingosine concentration

Permeabilized

D

TUNEL Mean fluorescence [a.u.]

Sphingosine concentration

DNase treatment
Fig. 3

A) Immobilized sphingosine ± SPH + rec. ACE2

Beads SPH rec. Fc-ACE2

Beads SPH rec. Fc-ACE2

SPH binding on beads [pmol]

C) Immobilized sphingosine ± SPH + rec. ACE2

Beads SPH rec. Fc-ACE2

Bead-immobilized lipids ± SPH + soluble rec. ACE2

D) Immobilized Fc-ACE2 ± SPH + soluble rec. RBD-spike

Beads SPH rec. RBD-spike

E) Immobilized rec. RBD-spike ± different Lipids + soluble rec. Fc-ACE2

Lipid-treatment SPH rec. RBD-spike

F) Immobilized rec. Fc-ACE2 ± different lipids + soluble rec. Fc-ACE2

G) Immobilized rec. RBD-spike ± different lipids + soluble rec. Fc-ACE2
Infection with supernatants from precipitates [% eGFP-positive cells]

+ pp-VSV-SARS-CoV-2 spike

Imm. rec. Fc-ACE2 + 1 μM SPH

Imm. rec. Fc-ACE2 + 2 μM SPH

Control beads

Fig. 4

A

B

Mean fluorescence [a.u.]

Counts [n]

FITC-anti-Spike S1 (Fluorescence, a.u.)

Red: no pretreatment, not infected
Orange: Sphingosine pretreatment, not infected
Blue: no pretreatment, infected with pp-SARS-CoV-2 spike
Green: Sphingosine pretreatment, infected with pp-SARS-CoV-2 spike
Sphingosine prevents binding of SARS-CoV-2 spike to its cellular receptor ACE2
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