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Sphingosine rescues aged mice from pulmonary pseudomonas infection

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ABSTRACT

Background: Bacterial lung infection is a leading cause of death for those 65 y or older, often requiring intensive care unit admission and mechanical ventilation, which consumes considerable health care resources. Although administration of antibiotics is the standard of care for bacterial pneumonia, its overuse has led to the emergence of multidrug resistant organisms. Therefore, alternative strategies to help minimize the effects of bacterial pneumonia in the elderly are necessary. As studies have shown that sphingosine (SPH) has inherent bacterial killing properties, our goal was to assess whether it could act as a prophylactic treatment to protect aged mice from pulmonary infection by *Pseudomonas aeruginosa*.

Methods: Aged (51 wk) and young (8 wk) C57Bl/6 mice were used in this study. Pulmonary SPH levels were determined by histology. SPH content of microparticles was quantified using a SPH kinase assay. Pneumonia was induced by intranasally treating mice with 10^6 Colony Forming Unit (CFU) *P aeruginosa*. Microparticles were isolated from young mice, whereas some were further incubated with SPH.

Results: We observed that SPH levels are reduced in the bronchial epithelial cells as well as the bronchoalveolar lavage microparticles isolated from aged mice, which correlates with a susceptibility to infection. We demonstrate that SPH or microparticle treatment can protect aged mice from pulmonary *P aeruginosa* infection. Finally, we observed that enriching microparticles with SPH before treatment eliminated the bacterial load in *P aeruginosa*-infected aged mice.

Conclusions: These data suggest that prophylactic treatment with SPH could reduce lung bacterial infections for the at-risk elderly population.

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Introduction

Elderly individuals suffering from pneumonia make up almost half of the intensive care unit admissions.[1,2] Pneumonia caused by *P aeruginosa* (PA), in particular, continues to be a major cause of morbidity and mortality in all hospitalized patients.[3-5] Mechanically ventilated patients are particularly susceptible to PA, which is thought to be caused by the endotracheal tube bypassing the innate immune responses of the upper respiratory tract.[6,7] Given that antibiotic resistance to PA is increasingly problematic, it is essential that alternative mechanisms to treat PA infection are identified.

The enzyme acid sphingomyelinase (Asm) catalyzes the hydrolysis of sphingomyelin to ceramide. Peak activity of sphingomyelinases depends on pH, and subtypes acid, neutral, and alkaline have been classified. *In vitro*, the optimum pH for acid sphingomyelinase activity is 4.5-5.0.[8] Originally, this indicated that the enzyme was solely lysosomal. However, more recent data indicate that the lipid composition of the membrane alters the Km of the enzyme, thus permitting Asm activity at higher pH.[9,10] Further, recent studies demonstrate that acidic microenvironments exist not only in lysosomes but also in domains on the outer leaflet of plasma membrane, where acid sphingomyelinase localizes after certain stimuli.[11] Ceramide is further metabolized to sphingosine (SPH) by acid ceramidase (Ac). SPH has been reported to have the ability to enhance killing of mycobacteria[12] as well as to kill a variety of Gram-positive and Gram-negative bacteria.[13]

Microparticles (MPs) are products of cell membranes, which are released in response to cellular stress.[14-16] These MPs can contain a mixture of lipids, RNA, and proteins. Specifically, it has been shown that microvesicles do contain ceramide,[17] but nothing is currently reported concerning SPH content. Thus, it is unclear whether MP can curb lung bacterial infections, and if the SPH content could be of influence.

The role of SPH and MPs in the response to PA pneumonia in the elderly has yet to be investigated. Given our previous data on cystic fibrosis and after burn,[4] we hypothesize that SPH expression is reduced in the lungs of aged mice and this decreases susceptibility to bacterial infection. Restoration of the SPH in the elderly may therefore have a protective mechanism toward infection. Furthermore, utilization of MPs to deliver SPH may be a novel therapy for management of pulmonary infection in the elderly.

Material and methods

Mice

Eight and 51-week-old male C57Bl/6 mice were purchased from Taconic Farms (Cambridge City, IN) and allowed to acclimate 1 wk before conducting experiments. Mice were fed a standard pellet diet and water ad libitum. All mouse experiments were performed using protocols approved by the Institution Animal Care and Use Committee (Protocol number: 08-09-19-01) of the University of Cincinnati.

Bacterial preparation

PA 762 strain was grown for 14 h on a tryptic soy agar plate (BD Biosciences, Franklin Lakes, NJ) and was transferred to an Erlenmeyer flask containing 40 mL of tryptic soy broth (BD Biosciences). Bacteria were incubated for 60 min at 37°C and 125 rpm to allow bacteria to be in the logarithmic phase of growth. Bacteria were centrifuged at 2800 rpm for 10 min at 21°C. The bacteria were then resuspended in prewarmed 40 mL of PBS (Thermo Fischer Scientific; Waltham, MA). An optical density (O.D.) of 0.04 was obtained using a spectrophotometer, and the concentration of the bacteria was determined based on a standard absorption curve. Mice were infected with 10^6 Colony Forming Unit (CFU)/20 μL of bacteria. Before the inoculation, mice were anesthetized with 3% isoflurane in oxygen. Using a 31-gauge needle on a 1-mL syringe, each mouse was intranasally inoculated with 20 μL of PA.

SPH immunohistochemical analysis

Lungs were harvested from young and aged mice, fixed in 10% neutral buffered formalin (Thermo Fisher Scientific), and stained with Cy3-coupled anti-SPH antibodies (clone NHSPH; Alfresa Pharma Corporation, Osaka, Japan). ImageJ software was used to analyze the immunofluorescence via histology comparison of 10-12 sections in the apical third of tracheal epithelial cells.

MP isolation and SPH content analysis

BAL fluid was collected from aged and young mice and centrifuged at 450 × g for 10 min to remove cells. The supernatant was collected and centrifuged at 10,000 × g for 10 min to remove any residual cells. The supernatant was collected and centrifuged at 25,000 × g for 30 min to pellet the MPs. MP pellets were resuspended and Nanoparticle Tracking Analysis (Nanosight, Malvern Instruments Ltd, Worcestershire, UK) used to enumerate MPs. The SPH content of the MPs was quantified using an SPH kinase assay as previously described.[6]

SPH and MP administration

Before treatment, SPH (D-erythro-sphingosine [d18:1]) was prepared by sonicating for 10 min. For SPH treatment, mice inhaled 1 mL of 125-μM SPH (Avanti Polar Lipids, Inc, Alabaster, AL) in normal saline vehicle using a Vios Compressor and Nebulizer (Model 310B0003; PARI Respiratory Equipment, Midlothian, VA) 30 min before inoculation with PA. This dose has been shown to be well tolerated and nontoxic to the lungs of mice.[18] For SPH-enriched MP treatment, 125-μM SPH was added to MPs isolated from BAL fluid harvested from young mice and incubated for 3 h at 37°C. MPs were isolated into a pellet by centrifuging at 25,000 × g for 30 min and resuspended in normal saline. Mice inhaled 1×10^9 of these SPH-enriched MPs via nebulizer 30 min before inoculation with PA.