Inhibition of Neutrophil Apoptosis via Sphingolipid Signaling in Acute Lung Injury

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ABSTRACT

Acute lung injury (ALI) is characterized by lung inflammation and diffuse infiltration of neutrophils into the alveolar space. The inhibition of alveolar neutrophil apoptosis has been implicated in the pathogenesis of ALI. Although sphingolipids may regulate cell apoptosis, the role of sphingolipids in activated neutrophils during ALI is not clear. In this study, we test the hypothesis that sphingolipids would attenuate neutrophil apoptosis that contributes to the development of ALI. Lipopolysaccharide (LPS)-stimulated human neutrophils, with or without inhibitor treatment, were analyzed for apoptosis. We found that the inhibitory effect of LPS on neutrophil apoptosis was blocked by treatment with the neutral sphingomyelinase (nSMase) inhibitor sphingolactone-24 (Sph-24), sphingosine kinase inhibitor II, and p38 mitogen-activated protein kinase (MAPK) inhibitor 4-[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-1H-imidazol-5-yl]pyridine (SB203580) but not by the acidic sphingomyelinase inhibitor chlorpromazine. LPS-activated phosphorylation of p38 MAPK also was attenuated by treatment with Sph-24 and sphingosine kinase inhibitor II. Furthermore, mice with LPS-induced lung injury were treated with the nSMase inhibitor Sph-24 to evaluate its impact on lung injury and survival. The severity of LPS-induced ALI was reduced, and the survival rate was increased in mice treated with Sph-24 compared with those given LPS alone. Intracellular levels of sphingolipids in alveolar neutrophils from patients with acute respiratory distress syndrome also were measured. We found that intracellular levels of ceramide and phospho-p38 MAPK were elevated in alveolar neutrophils from acute respiratory distress syndrome patients. Our results demonstrate that activation of the nSMase/sphingosine-1-phosphate pathway to induce p38 MAPK phosphorylation results in inhibition of neutrophil apoptosis, which may contribute to the development of ALI.

Introduction

Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), are characterized by overwhelming lung inflammation and increased microvascular permeability, causing diffuse lung edema and mechanical dysfunction (Wheeler and Bernard, 2007). ALI/ARDS contributes to a significant amount of mortality and morbidity in adult patients admitted to intensive care units. Mortality remains high (approximately 40%), although recent data have shown a reduction in mortality since the implementation of lung-protective ventilation strategies (Zambon and Vincent, 2008). So far, there is no specific pharmacological therapy that reduces mortality, and management is exclusively expectant and supportive, reflecting the lack of understanding of cellular and molecular mechanisms contributing to the pathogenesis of ALI. Nevertheless, accumulating evidence has indicated a consistent association between neutrophils and ALI in humans and animal models and the propensity of neutrophils and their products to cause tissue injury in experimental systems, leading to the conclusion that neutrophils have an important causative role in ALI (Zemans et al., 2009).
Neutrophil depletion is protective in many animal models of ALI, and blocking the major neutrophil chemoattractant interleukin (IL)-8 protects rabbits from ALI and death after severe acid aspiration (Folkesson et al., 1995). Studies showed that bronchoalveolar lavage (BAL) fluids from patients with early ARDS delay neutrophil apoptosis in vitro (Matute-Bello et al., 2000). The inhibitory effect of BAL fluids on neutrophil apoptosis is associated with various cytokines, such as granulocyte/macrophage colony-stimulating factor, granulocyte colony-stimulating factor, IL-8, and IL-2 (Aggarwal et al., 2000; Lesur et al., 2000). These factors cause the persistence of activated neutrophils in the lung and sustained lung inflammation and consequently increased organ damage and dysfunction. However, little is known about the intracellular signaling pathways regulating neutrophil activity in ALI. Efforts directed toward understanding key mechanisms to improve the detection and treatment of ALI are in urgent need.

Sphingolipids have been recognized as signaling molecules involved in a number of important cellular functions (Hannun and Obeid, 2008). Ceramide, generated from the hydrolysis of sphingomyelin by acid sphingomyelinase (aSMase) or neutral sphingomyelinase (nSMase) or through de novo synthesis, acts as a second messenger for apoptotic signaling triggered by various stresses, including oxidative stress, acid, and ionizing radiation, and extracellular stimuli, such as proinflammatory cytokines and lipopolysaccharide (LPS) (Mathias et al., 1998). A recent study showed that inhibition of aSMase improves pulmonary function by a decrease of ceramide generation in a neonatal piglet model of surfactant deficiency-induced ALI (von Bismarck et al., 2008). In contrast, a further metabolite of ceramide, sphingosine-1-phosphate (S1P), generated through ceramidase and sphingosine kinase, has been implicated in protection from apoptosis (Hannun and Obeid, 2008). A previous study showed that both spontaneous and anti-Fas-induced neutrophil apoptosis are antagonized by S1P via the modulation of the p38 mitogen-activated protein kinase (MAPK) pathway (Chihab et al., 2003). Moreover, a strategy to activate anti-apoptotic signaling of S1P targeting lung endothelial cells has been shown to prevent alveolar damage in a mouse emphysema model (Diab et al., 2010). In addition, S1P mediates diverse biological functions, including cell proliferation, angiogenesis, formation of adherence junctions, cell motility and migration, and lymphocyte trafficking, acting either intracellularly as a second messenger or extracellularly as a ligand for a family of five G protein-coupled receptors, termed S1P1-5 (Strub et al., 2010). Previous studies have indicated that S1P is capable of modulating vascular barrier integrity in ALI (McVerry and Garcia, 2005) and that inhibition of sphingosine kinase attenuates lung inflammation in an asthmatic mouse model (Nishiuma et al., 2008). However, the role of sphingolipids in regulating neutrophil apoptosis during ALI is unclear.

Previous reports have demonstrated that p38 MAPK is important in regulating neutrophil functions and is activated in response to many stimuli, such as LPS and tumor necrosis factor-α (Nick et al., 2002). In addition, p38 MAPK has been implicated in the regulation of apoptosis in different kinds of cells (Chen et al., 2008). In the present study, we found that after LPS challenge nSMase is induced to hydrolyze sphingomyelin into ceramide, which is metabolized further to S1P, leading to the inhibition of neutrophil apoptosis via phosphorylation of p38 MAPK. We also showed that treatment of mice with the nSMase inhibitor after LPS-induced lung injury improved mouse survival and decreased the severity of lung damage. The activation of the intracellular sphingolipid pathway and p38 MAPK in alveolar neutrophils from ARDS patients also was observed.

Materials and Methods

Patients. We enrolled three patients who were admitted to the intensive care unit because of respiratory failure caused by sepsis-induced ARDS, defined as the presence of the following standard criteria: 1) acute hypoxemic respiratory failure requiring mechanical ventilation; 2) diffuse bilateral alveolar infiltrates on the chest radiograph; 3) refractory hypoxemia with arterial partial pressure of oxygen/fraction of inspired oxygen <200, regardless of positive end-expiratory pressure level; 4) pulmonary artery occlusion pressure <18 mm H2O or no clinical evidence for left atrial hypertension; and 5) recognized sepsis syndrome for the development of ARDS. Sepsis was defined according to published consensus guidelines (Levy et al., 2003). Patients were excluded if they were <16 years of age, had refractory respiratory failure (partial pressure of oxygen <60 mm Hg with fraction of inspired oxygen 1.0), or had unstable hemodynamic status and lethal arrhythmia even under the use of a high-dose vasopressor and antiarrhythmia drug. We also included three patients with cardiogenic lung edema who were intubated and required mechanical ventilation in the absence of lung or systemic inflammation as the control subjects. The protocols and procedures were approved by the Institutional Review Board of the National Cheng Kung University Hospital. Written informed consent was obtained from all of the subjects or their relatives.

Fiberoptic Bronchoscopy Sampling. All of the enrolled patients underwent fiberoptic bronchoscopy (LF2 or P40; Olympus, Tokyo, Japan) for BAL fluids sampling within 24 h after intensive care unit admission. All of the patients were ventilated mechanically with fraction of inspired oxygen 100%, sedated with midazolam, and paralyzed with atracurium. No topical anesthetics were used before BAL. The fiberoptic bronchoscope was introduced without bronchial suctioning, except after BAL. Heart rate, blood pressure, and arterial oxygen saturation were monitored throughout the procedure. The bronchoscope was wedged into the right middle lobe or lingular division in patients with diffuse pulmonary infiltrates. Six aliquots (20 ml each) of sterile normal saline were instilled, and the fluid was aspirated immediately after each instillation. The first retrieved BAL fluids, reflecting a bronchial sample, were discarded, and the remaining BAL fluids were pooled in ice-cold tubes for study. BAL was filtered through 70-μm nylon cell-strainer filters (Falcon; BD Biosciences Discovery Labware, Bedford, MA) to remove mucus and then centrifuged at 500g for 15 min at 4°C to obtain a pellet corresponding to BAL cells, which were resuspended in 1 ml of phosphate-buffered saline (PBS). BAL cells were fixed with 1% paraformaldehyde in PBS and kept at 4°C for immunostaining.

Reagents and Antibodies. Ficol-Hypaque and dextran were obtained from Sigma-Aldrich (St. Louis, MO). aSMase inhibitor chlorpromazine (CHL), nSMase inhibitor sphingolactone-24 (Sph-24), and sphingosine kinase inhibitor II (SKI-II) also were purchased from Sigma-Aldrich, and the p38 MAPK inhibitor 4-[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-1H-imidazol-5-yl]pyridine (SB203580) was purchased from Toeric Bioscience (Ellisville, MO). Polyclonal antibodies against phospho-p38 MAPK, p38 MAPK, and caspase-3 were obtained from Cell Signaling Technology (Danvers, MA), anti-ceramide monoclonal antibody was obtained from Enzo Life Sciences, Inc. (Farmington, NY), and anti-S1P monoclonal antibody was obtained from Funakoshi (Tokyo, Japan). RPMI 1640, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Invitrogen (Carlsbad, CA).
Isolation of Human Neutrophils. Blood from healthy volunteers was fractionated on Ficoll-Hypaque and dextran gradients to separate neutrophils from lymphocytes, monocytes, and platelets. The neutrophil layer was transferred to a fresh tube and centrifuged for 10 min at 4°C. Hypotonic lysis with 0.2% NaCl was performed to remove the remaining erythrocytes. The neutrophils were ~95% pure as assessed by Liu's staining and ~98% viable by trypan blue exclusion assay. Isolated neutrophils were used for LPS stimulation and chemical inhibitor treatment studies.

Mice. C57Bl/6N breeder mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained on standard laboratory food and water ad libitum in our medical college laboratory animal center. Their 6- to 8-week-old progeny were used for the experiments. The protocols were approved by the Institutional Animal Care and Use Committee at National Cheng Kung University.

LPS-Induced Lung Injury and Treatment with Sph-24. Lung injury was induced by intranasal instillation of LPS (10 mg/ml) from Escherichia coli O111:B6 (Sigma-Aldrich). When mice lost voluntary movement after anesthesia, LPS suspended in 50 μl of sterilized PBS or PBS alone was instilled slowly into the lung via the nostrils. Neutrophils were recruited into the alveolar space within 24 h after LPS challenge. Each mouse was instilled with PBS or PBS alone or LPS followed by intraperitoneal injection of Sph-24 (1 mg/kg b.wt.) 3 and 9 h thereafter. The dose of Sph-24 was chosen based on our preliminary experiments. After 24 h, mice were killed by the procedures below. In survival experiments, mice were injected intraperitoneally with Sph-24 at 3 h after PBS or LPS instillation or with LPS instillation alone. This procedure was repeated after 24 h, and subsequently the 6-day survival rate was assessed.

Assays of Mouse Lung Injury. Mice were killed 24 h after PBS or LPS intranasal instillation. Mice were anesthetized, the trachea was cannulated, and BAL was performed twice with 1 ml of PBS (pH 7.4). The recovered fluid was centrifuged, and the supernatant was analyzed for protein concentration by Bio- rad assay (Bio-Rad Laboratories, Hercules, CA). Cell pellets were pooled and resuspended in PBS, and the number of viable cells was determined by trypan blue exclusion assay. Thereafter, the left lung was inflated and fixed with 4% paraformaldehyde for histologic evaluation.

Apoptosis and Caspase Analysis. Isolated human neutrophils were incubated in 10% FBS-RPMI, with or without pretreatment with Sph-24 (50 μM), SKI-II (5 and 10 μM), CHL (4 μM), and SB203580 (12.5 μM) at 30 min before the addition of LPS (500 ng/ml). The doses of the inhibitors were chosen based on previous studies and preliminary experiments (French et al., 2003; Jung et al., 2007; Chen et al., 2008). At 24 h, cells were fixed with 7% ethanol in PBS for propidium iodide (PI) (Sigma-Aldrich) staining and then were analyzed using flow cytometry (FACScan; BD Biosciences, San Jose, CA). A minimum of 10,000 events were counted per sample. Results are shown as the percentage of hypodiploid DNA (subG1; percentage apoptosis) corresponding to fragmented DNA characteristics for apoptotic cells. Apoptosis of BAL cells from mice untreated or treated with Sph-24 (1 mg/kg) at 3 and 9 h after LPS (10 mg/ml) instillation was measured using the Cell Death Detection ELISA kit, which detects cystolic histone-complexed DNA fragments, according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN). Cell apoptosis was determined by the optical density at 405 nm. Activation of caspase-3 was detected by Western blot analysis as described below.

Sphingomyelinase Assay. Sphingomyelinase activity was determined in cellular extracts (Amplex Red Sphingomyelinase assay kit; Invitrogen) according to the manufacturer's instructions. Isolated human neutrophils were incubated in 10% FBS-RPMI, with or without pretreatment with Sph-24 (50 μM), CHL (4 μM), and SKI-II (10 μM) at 30 min before the addition of LPS (500 ng/ml) for 24 h. Cells (1 x 10^6) were then placed in 100 μl of lysis buffer with an equal amount of kit reagent (200 μl per well in a 96-well plate). In brief, each reaction contained 50 μM Amplex Red reagent, 1 U/ml horseradish peroxidase, 0.1 U/ml choline oxidase, 4 U/ml alkaline phosphatase, and 0.25 mM sphingomyelin in the reaction buffer and was incubated at 37°C for 1 h. Fluorescence was measured using a microplate reader (Fluoroskan Ascent; Thermo Fisher Scientific, Waltham, MA) with excitation at 530 nm and emission at 590 nm.

Immunostaining and Flow Cytometric Analysis. For intracellular immunostaining, BAL cells from ARDS patients and patients with cardiogenic lung edema were fixed with 1% paraformaldehyde in PBS and permeabilized with 0.01% Triton X-100 in PBS. For measurement of S1P levels, isolated human neutrophils were incubated in 10% PBS-RPMI, with or without pretreatment with SKI-II (10 μM), and CHL (4 μM) at 30 min before the addition of LPS (500 ng/ml). At 24 h, cells were fixed with 1% paraformaldehyde in PBS and permeabilized with 0.01% Triton X-100 in PBS. A series of antibodies were used as indicated, followed by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG, anti-mouse IgG, or anti-mouse IgM (Calbiochem, San Diego, CA) staining. Mouse anti-ceramide and anti-S1P and rabbit anti-phospho-p38 MAPK, or S1P-positive neutrophils.

Histopathology. Mouse left lungs were infused with 4% buffered paraformaldehyde, embedded in paraffin, sectioned at 5-μm thickness, and stained with hematoxylin and eosin.

Western Blot Analysis. Isolated human neutrophils were placed in 10% PBS-RPMI, and then Sph-24 (50 μM), SKI-II (10 μM), and CHL (4 μM) were added at 30 min before LPS (500 ng/ml) treatment or LPS treatment alone. After 24 h, cells were washed in ice-cold PBS and then pelleted and lysed in a Triton X-100-based lysis buffer (1% Triton X-100, 150 mM NaCl, and 10 mM Tris (pH 7.5), 5 mM EDTA, 5 mM Na2O4, 10 mM NaF, and 10 mM sodium pyrophosphate) with a protease inhibitor mix and a Phosphatase Inhibitor Cocktail I (Sigma-Aldrich). The protein concentrations of the lysates were determined by Bio-Rad assay. Equal amounts of the proteins were loaded in each lane of a 10% SDS-PAGE minigel and separated by electrophoresis. Thereafter, the proteins were electrotransferred (100 V, 90 min) to polyvinylidene difluoride membranes in a buffer containing 48 mM Tris (pH 9.2), 39 mM glycine, 20% methanol, and 0.037% SDS. Non-specific protein binding was blocked by preincubation with 5% milk in PBS supplemented with 0.05% Tween 20 for 1 h. The membranes were incubated overnight at 4°C with antibodies specific for p38 MAPK, phospho-p38 MAPK, and caspase-3. Mouse antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA) was used as an internal control. After being washed in PBS supplemented with 0.05% Tween 20, the membranes were incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG (1: 5000) to detect caspase-3, phospho-p38, and total p38 MAPK and anti-mouse IgG (1:3000) for GAPDH. All of the immunoblots were washed thoroughly in PBS supplemented with 0.05% Tween 20 and detected using the enhanced chemiluminescence kit (Millipore Corporation, Billerica, MA) according to the manufacturer's instructions.

Statistical Analysis. Data were expressed as mean ± S.E.M. Statistical analysis was performed by Student's t test or nonparametric Mann-Whitney test if indicated. Data with three or more groups were analyzed by one-way analysis of variance, followed by Tukey's test as a method of post hoc analysis. All of the tests were two-tailed and P < 0.05 was considered significant. Survival curves were compared using the log-rank test. All of the data were analyzed using Prism 5.0 (GraphPad Software, Inc., San Diego, CA).
Results

Sphingolipids Are Involved in LPS-Induced Inhibition of Neutrophil Apoptosis. To determine the role of sphingolipids in modulating neutrophil apoptosis, we used LPS, which has been recognized as a principal pathogenic component in ALI, in inhibiting spontaneous apoptosis of human peripheral blood neutrophils (Fig. 1A and Supplemental Fig. 1A). LPS-induced suppression of neutrophil apoptosis also was confirmed by the blockade of caspase-3 activation (Supplemental Fig. 1B). On the basis of the fact that neutrophils are terminally differentiated and short-lived cells not amenable to in vitro genetic manipulation, we used chemical inhibitors to test our hypothesis. We found that Sph-24 (nSMase inhibitor) and SKI-II (sphingosine kinase inhibitor), but not CHL (aSMase inhibitor), antagonized the antiapoptotic effect of LPS (Fig. 1B). SKI-II, a potent and selective inhibitor of sphingosine kinase 1, has been shown to have a weak direct inhibitory effect on sphingosine kinase 2 (Ren et al., 2010). Sphingosine kinase 1 has been implicated in cell growth, survival, movement, and cytoskeletal rearrangement. In contrast, sphingosine kinase 2 has been shown to suppress cell growth and enhance apoptosis (Maceyka et al., 2005). In this study, we suggest that SKI-II mainly inhibits sphingosine kinase 1 to antagonize LPS-induced suppression of neutrophil apoptosis by decreasing S1P levels in LPS-treated neutrophils (Supplemental Fig. 2). We also demonstrated that LPS stimulated sphingomyelinase activity, which was diminished by pretreatment with Sph-24 but not CHL (Fig. 1C), indicating the involvement of nSMase but not aSMase activation. Likewise, SKI-II treatment did not cause any effect on nSMase activity (Fig. 1C). Together, these results suggest that S1P generation through the activation of nSMase, but not aSMase, mediates the antiapoptotic effect of LPS in neutrophils.

LPS-Induced Sphingolipid-Mediated Inhibition of Neutrophil Apoptosis Occurs via Phosphorylation of p38 MAPK. We next investigated whether p38 MAPK is regulated by sphingolipids, leading to the inhibition of neutrophil apoptosis. By Western blot analysis of neutrophil lysates, we found that LPS-induced p38 MAPK phosphorylation was inhibited by Sph-24 and SKI-II, but not CHL (Fig. 2, A and B). In addition, pretreatment with the p38 MAPK inhibitor SB203580 blocked the antiapoptotic effect of LPS (Fig. 2C). These results suggest that nSMase/S1P-mediated inhibition of neutrophil apoptosis occurs via p38 MAPK activation.

nSMase Inhibitor Sph-24 Ameliorates Mouse Lung Injury Induced by LPS and Improves Survival. To further explore whether the sphingolipid pathway is involved in delayed neutrophil apoptosis during ALI development, we administered mice the nSMase inhibitor Sph-24 (1 mg/kg) 3 and 9 h after LPS instillation. After 24 h, we measured total protein and cell counts in BAL fluids to evaluate lung injury severity. Mice treated with Sph-24 had significantly lower total protein and cell counts in BAL fluids compared with those in mice treated with LPS alone (Fig. 3, A and B). Likewise, mice treated with Sph-24 showed less distortion of alveolar architecture, fewer

Fig. 1. nSMase and sphingosine kinase, but not aSMase, regulate LPS-mediated inhibition of neutrophil apoptosis. A, spontaneous apoptosis of human neutrophils treated with various doses of LPS for 24 h was determined using PI staining and flow cytometry. M1 denotes a range that we defined to measure the percentage of apoptotic cells. B, human neutrophils pretreated with the nSMase inhibitor Sph-24 (50 μM), aSMase inhibitor CHL (4 μM) or sphingosine kinase inhibitor SKI-II (5 and 10 μM) were treated with LPS (500 ng/ml) for 24 h. Neutrophil apoptosis was measured using PI staining and flow cytometry. C, SMase activity was measured in LPS-treated (500 ng/ml) human neutrophils in the absence or presence of Sph-24 (50 μM), CHL (4 μM), or SKI-II (10 μM). The results shown are representative of three independent experiments performed in triplicate. Data are shown as the mean ± S.E.M. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
thickened and disrupted septa, and less intra-alveolar bleeding and neutrophil infiltration compared with those in mice treated with LPS alone (Fig. 3C). To further determine whether the decrease in lung inflammation is caused by alveolar neutrophil apoptosis, we determined apoptotic levels in BAL cells from mice with or without Sph-24 treatment. The results demonstrated that cell apoptosis was increased in BAL fluids from Sph-24-treated mice compared with those treated with LPS alone (Fig. 3C).

**Fig. 2.** LPS suppresses neutrophil apoptosis through the activation of p38 MAPK. A, human neutrophils were treated with LPS (500 ng/ml) for 24 h with or without pretreatment with inhibitors, including Sph-24 (50 μM), SKI-II (10 μM), and CHL (4 μM). Western blot analysis was used to determine phosphorylation of p38 MAPK. GAPDH was used as an internal control. B, p38 MAPK activation was measured as the ratio of phosphorylated p38 MAPK to GAPDH. Data represent the mean ± S.E.M. of four independent experiments. *, *P < 0.05. C, PI staining and flow cytometry were used to determine LPS-suppressed human neutrophil apoptosis in the presence or absence of the p38 MAPK inhibitor SB203580 (12.5 μM). The results shown are representative of three independent experiments performed in triplicate. Data are shown as the mean ± S.E.M. ***, *P < 0.001.

**Fig. 3.** Effect of inhibiting nSMase on LPS-induced mouse lung injury. The total protein level (A) and viable cell count (B) in BAL fluid were determined at 24 h after 50 μl of LPS (10 mg/ml) instillation in mice treated with or without Sph-24 (1 mg/kg) at 3 and 9 h after LPS instillation. C, histology of lung sections with hematoxylin and eosin staining from mice with or without Sph-24 treatment after LPS instillation for 24 h. Scale bars, 500 μm (top panel), 200 μm (bottom panel). D, apoptosis of BAL cells from mice treated with or without Sph-24 was measured with the Cell Death Detection ELISA kit. Each point represents an individual mouse. Mean values are indicated (n = 7, except for the LPS + Sph-24 group in A, where n = 6). The Mann-Whitney test was used to perform statistical analysis in D. E, mice were treated with or without Sph-24 (1 mg/kg) after LPS instillation. The survival rate was observed at 6 days (n = 9 in the LPS alone group, n = 8 in the Sph-24 group and LPS + Sph-24 group). *, *P < 0.05; **, *P < 0.01; ***, *P < 0.001.
mice compared with that in mice treated with LPS alone (Fig. 3D). Furthermore, LPS-treated mice given Sph-24 had improved survival compared with that in mice treated with LPS alone (Fig. 3E).

Increased Levels of Sphingolipids in Alveolar Neutrophils from ARDS Patients. We then sought to correlate the extent of sphingolipid signaling in alveolar neutrophils with the pathogenesis of ARDS. Patients with cardiogenic lung edema share similar clinical features with patients with ARDS. They all are characterized by increased alveolar fluids causing severe hypoxemia. However, the etiology of cardiogenic lung edema, mainly attributed to increased pulmonary microvascular hydrostatic pressure, is quite different from that of ARDS, in which increased permeability of the alveolar capillary barrier caused by lung inflammation is prominent. Therefore, we used alveolar neutrophils of patients with cardiogenic lung edema as a control to probe the intracellular signaling cascades in alveolar neutrophils of ARDS patients. Because ceramide up-regulation is a critical step in sphingolipid metabolism, we compared ceramide levels in alveolar neutrophils of ARDS patients with those of cardiogenic lung edema patients, in which the lung function impairment is not related to lung inflammation, as indicated by markedly lower alveolar neutrophil counts compared with those from ARDS patients (Fig. 4B). Ceramide content was measured by immunocytochemistry (Cogolludo et al., 2009). Immunostaining and flow cytometry results showed significantly higher ceramide levels in alveolar neutrophils from ARDS patients than those from cardiogenic lung edema patients (Fig. 4, A and B). A previous in vitro study showed that S1P may counteract neutrophil apoptosis via the p38 MAPK pathway (Chihab et al., 2003). In the present study, we show by immunostaining and flow cytometry that alveolar neutrophils from ARDS patients had higher levels of phospho-p38 MAPK compared with those from cardiogenic lung edema patients (Fig. 5, A and B).

Discussion

Sphingolipid signaling through aSMase-dependent ceramide production has been shown previously to induce apoptosis of lung endothelial cells and epithelial cells in ALI (Göggel et al., 2004). In contrast to the induction of apoptosis in endothelial and epithelial cells, apoptosis of neutrophils is decreased during sepsis (Gilroy et al., 2004). The current study demonstrates that suppression of pulmonary neutrophil apoptosis in ALI is mediated, at least in part, through the nSMase, ceramide, S1P, and p38 MAPK pathway (Fig. 6).

The important role of neutrophils in ALI has been demonstrated by previous studies in which the severity of lung injury is decreased when neutrophils are eliminated (Abraham et al., 2000). Neutrophil apoptosis is critical for the resolution of inflammation, which appears to be inhibited in ALI. Proinflammatory cytokines, such as IL-1β, tumor necrosis factor-α, interferon-γ, granulocyte/macrophage colony-stimulating factor, and granulocyte colony-stimulating factor, or bacterial products, such as LPS, inhibit neutrophil apoptosis, whereas IL-10 counteracts this inhibition (Keel et al., 1997; Aggarwal et al., 2000; Lesur et al., 2000). To unravel the molecular mechanisms responsible for decreased

![Fig. 4. Levels of sphingolipids in alveolar neutrophils of ARDS patients.](#)

A, the levels of ceramide (green) in alveolar neutrophils of patients with ARDS or cardiogenic lung edema were determined using immunostaining and costained with PI for nuclei (red), followed by flow cytometry. B, the averaged percentages of ceramide-positive neutrophils determined by flow cytometry (black bar) and neutrophil counts (white bar) in BAL fluid from patients with ARDS and cardiogenic lung edema are shown (n = 3 per group). Data are shown as the mean ± S.E.M. **, P < 0.01; ***, P < 0.001.

![Fig. 5. Increased phosphorylation of p38 MAPK in alveolar neutrophils of ARDS patients.](#)

A, the phosphorylation of p38 MAPK (green) in alveolar neutrophils of patients with ARDS or cardiogenic lung edema was determined using immunostaining and costained with PI for nuclei (red), followed by flow cytometry. B, the averaged percentages of phospho-p38 MAPK-positive neutrophils determined by flow cytometry in BAL fluid from patients with ARDS or cardiogenic lung edema are shown (n = 3 per group). Data are shown as the mean ± S.E.M. *, P < 0.05.
nuclear factor
pressed through a mechanism that involves activation of
neutrophils from patients with sepsis are profoundly sup-
neutrophil apoptosis, a previous study found that circulating
Subsequent phosphorylation of p38 MAPK leads to the inhibition of
nSMase is induced to hydrolyze sphingomyelin into ceramide. Ceramide
Schematic mechanism of sphingolipid-mediated neutrophil
Fig. 6.
nering apoptosis (Chihab et al., 2003; Hannun and Obeid,
2010). In contrast to ceramide, S1P prevents cells from un-
acting as an intracellular second messenger (Strub et al.,
2003). In a murine model of LPS-
lial barrier functions via different S1P receptor subtypes
of cell proliferation and survival, angiogenesis, and endothe-
dase and sphingosine kinase and is recognized as a regulator
Filosto, 2010). S1P is derived from ceramide through cerami-
and glial cells have shown that nSMase, but not aSMase, is
increase vascular permeability, leading to edema formation
and innate and acquired immunity, and to contribute to a
variety of lung diseases, such as pulmonary edema, emphy-
sema, cystic fibrosis, and pneumonia (Uhlig and Gulbins,
2008). In ALI, increased aSMase activity and ceramide con-
tent induce apoptosis of pulmonary endothelial cells and
increase vascular permeability, leading to edema formation
(Göggel et al., 2004; von Bismarck et al., 2008). However,
studied in human airway epithelial and cerebral endothelial
and glial cells have shown that nSMase, but not aSMase, is
activated to induce cell death by cigarette smoke and amyloid
β-peptide, respectively (Yang et al., 2004; Goldkorn and
Filosto, 2010). S1P is derived from ceramide through cerami-
dase and sphingosine kinase and is recognized as a regulator
of cell proliferation and survival, angiogenesis, and endothe-
lial barrier functions via different S1P receptor subtypes
(Spiegel and Milstien, 2003). In a murine model of LPS-
induced ALI, S1P significantly decreased pulmonary vascu-
lar leakage and inflammation (Peng et al., 2004). S1P also
can function inside the cell, independently of S1P receptors,
acting as an intracellular second messenger (Strub et al.,
2010). In contrast to ceramide, S1P prevents cells from un-
dergoing apoptosis (Chihab et al., 2003; Hannun and Obeid,
2008). In this study, we found that LPS increased nSMase
activity and that pretreatment with nSMase inhibitor Sph-24 and sphingosine kinase inhibitor SKI-II antagonized the
antiapoptotic effect of LPS on neutrophils. Of note, treatment
with Sph-24 appeared to be more effective in preventing
inhibition of neutrophil apoptosis by LPS compared with
SKI-II. Sph-24 is a selective and irreversible nSMase inhib-
itor (Delgado et al., 2006). Likewise, SKI-II acts as a potent
and selective inhibitor of sphingosine kinase 1, with no inhibi-
tion of protein kinase C-α, p42 MAPK/extracellular signal-
regulated kinase 2, or phosphatidylinositol 3-kinase (French
et al., 2003). However, a recent study has shown that SKI-II
has a weak direct inhibitory effect on sphingosine kinase 1
and 2 activities in vitro in addition to triggering lysosomal
degradation of sphingosine kinase 1 (Ren et al., 2010). As we
mentioned previously, sphingosine kinase 1 is related to cell
growth and survival, whereas sphingosine kinase 2 has been
shown to suppress cell growth and enhance apoptosis. On the
basis of the opposite effects of sphingosine kinase 1 and 2
on the regulation of cell apoptosis, we reasonably propose that
the efficacy of SKI-II in antagonizing LPS-induced suppress-
ion of neutrophil apoptosis may be compromised slightly. Of
interest, the aSMase inhibitor CHL had no effect on the
regulation of neutrophil apoptosis in response to LPS stimu-
lation. CHL has been used frequently as a selective aSMase
inhibitor. This compound induces proteolytic degradation of
aSMase by interfering with the binding of the enzyme to the
membranes of acidic vesicles and thereby rendering it sus-
ceptible to proteolytic cleavage by lysosomal proteases
(Verdurmen et al., 2010). Taken together, we suggest that de-
layed neutrophil apoptosis is mediated by S1P, which is
derived from ceramide generated through the activation of
nSMase, but not aSMase. Furthermore, in vivo data are in
line with these findings in an LPS-induced lung injury model,
because we observed decreased severity of ALI and increased
survival by treatment with the nSMase inhibitor. In this
study, we used the nSMase inhibitor Sph-24 for ALI treat-
ment based on our in vitro findings, in which nSMase
appeared to be more important in regulating neutrophil activ-
tion than aSMase; nevertheless, aSMase has been dem-
onstrated to be more crucial in maintaining the alveolar-
capillary barrier (Göggel et al., 2004) and, based on the
previous report that S1P has protective effects in LPS-in-
duced ALI through the enhancement of endothelial vascular
integrity (Peng et al., 2004), preventing us from using the
sphingosine kinase inhibitor SKI-II as an ALI treatment
because of its potential side effects on epithelial and endo-
thelial cells. Therefore, the nSMase inhibitor used for the
treatment of ALI may target primarily neutrophils with
fewer effects on epithelial and endothelial cells. In contrast,
ystemic or intratracheal administration of SKI-II may not
only act on neutrophils but also on lung epithelial and endo-
thelial cells, although a previous study has shown that SKI-II
attenuates shock-induced lung injury in a rat model of trau-
ma/hemorrhagic shock through the suppression of neutrophil
activation (Lee et al., 2004).
Moreover, S1P has been linked to the regulation of inflam-
matory responses, because S1P might suppress IL-2-induced
or T cell-related inflammatory responses. However, S1P also
has been implicated in the formation of adherence junctions,
proliferation, survival, angiogenesis, and the trafficking of
immune cells (Strub et al., 2010). In this study, we suggest
that S1P could be produced to prevent neutrophils from undergoing apoptosis in response to external stimuli, leading to sustained inflammation. Nevertheless, 2-amino-2-[4-octylphenoxy]propane-1,3-diol (FTY720), a synthetic S1P ligand, acts as an immunosuppressive agent in which the mechanism involves the action of phosphorylated FTY720 in lymphoid tissues not only in activating S1P receptors but also in down-regulating them. As a result, lymphocytes lose their responsiveness to S1P, blocking their egress from the thymus or lymph nodes (Sensken et al., 2009). FTY720 has been shown to protect against LPS-induced ALI in a murine model (Peng et al., 2004). However, the protective effect of FTY720 is produced by endothelial cell barrier enhancement or immunosuppression. So far, its role in the regulation of neutrophil activation and apoptosis remains unclear. In addition, the use of adrenocorticotropin hormone as an ARDS treatment (Steinberg et al., 2006), because of its systemic effect on immunosuppression and a possible increase in infection rate and mortality, may not be ideal.

Activation of p38 MAPK has been shown to be involved in spontaneous neutrophil apoptosis (Chihab et al., 2003). In contrast, other studies have indicated that p38 MAPK signaling contributes to the survival of mouse and human neutrophils (Alvarado-Kristensson et al., 2002). In animal studies, treatment with the p38 MAPK inhibitor before or during LPS-induced lung inflammation has been shown to result in significant decreases in the release of cytokines and chemokines and accumulation of neutrophils in the airspaces (Nick et al., 2002). However, another study showed that inhibition of p38 MAPK did not decrease neutrophil accumulation in the lung or development of ALI in the murine model of hemorrhage or endotoxemia (Arcaroli et al., 2001). In our study, we found increased expression of phospho-p38 MAPK in BAL neutrophils from patients with ARDS. The increased phospho-p38 MAPK levels in isolated human neutrophils treated with LPS were attenuated by pretreatment with nSMase and sphingosine kinase inhibitors, but not by the aSMase inhibitor. Furthermore, inhibiting p38 MAPK signaling abrogated the antiapoptotic effect in LPS-treated human neutrophils. Thus, the prevention of neutrophil apoptosis via p38 MAPK involves activation of nSMase and subsequent activation of sphingosine kinase. Our in vivo data also support this finding, because the severity of ALI and mouse mortality are mitigated by blocking this signaling cascade using the nSMase inhibitor Sph-24. This indicates that inhibiting the sphingomyelin hydrolytic pathway by targeting activation of nSMase may represent a promising therapeutic strategy for regulating neutrophil apoptosis and ameliorating development of ALI.

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