Inhibition of Neutrophil Apoptosis via Sphingolipid Signaling in Acute Lung Injury

Wei-Chieh Lin, Chiou-Feng Lin, Chia-Ling Chen, Chang-Wen Chen, and Yee-Shin Lin

Institute of Clinical Medicine (W.C.L., C.F.L.) and Department of Microbiology and Immunology (C.L.C., Y.S.L.), National Cheng Kung University Medical College; and Medical Intensive Care Unit, Department of Internal Medicine, National Cheng Kung University Hospital (C.W.C.), Tainan, Taiwan
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Address correspondence to: Dr. Yee-Shin Lin, Department of Microbiology and Immunology, National Cheng Kung University Medical College, Tainan 701, Taiwan.

E-mail: yslin1@mail.ncku.edu.tw

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ABBREVIATIONS: ALI, acute lung injury; LPS, lipopolysaccharide; nSMase, neutral sphingomyelinase; Sph-24, sphingolactone-24; SKI, sphingosine kinase inhibitor; MAPK, mitogen-activated protein kinase; ARDS, acute respiratory distress syndrome; S1P, sphingosine-1-phosphate; ICU, intensive care unit; IL, interleukin; BAL, bronchoalveolar lavage; GM-CSF, granulocyte/macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; aSMase, acidic
sphingomyelinase; TNF-α, tumor necrosis factor-α; PaO₂/FiO₂, arterial partial pressure of oxygen/fraction of inspired oxygen; PBS, phosphate-buffered saline; CHL, chlorpromazine; FBS, fetal bovine serum; PI, propidium iodide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

**Recommended section:** Inflammation, Immunopharmacology, and Asthma
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 which 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 neutral sphingomyelinase (nSMase) inhibitor sphingolactone-24 (Sph-24), sphingosine kinase inhibitor (SKI)-II, and p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580, but not by acidic sphingomyelinase inhibitor chlorpromazine. LPS-activated phosphorylation of p38 MAPK was also attenuated by treatment with Sph-24 and SKI-II. Furthermore, mice with LPS-induced lung injury were treated with 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 as compared with those given LPS alone. Intracellular levels of sphingolipids in alveolar neutrophils from patients with acute respiratory distress syndrome (ARDS) were also
measured. We found that intracellular levels of ceramide and phospho-p38MAPK were elevated in alveolar neutrophils from ARDS patients. Our results demonstrate that activation of the nSMase/S1P 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 (ICU). Mortality remains high (approximately 40%), although recent data have shown a reduction in mortality since the implementation of lung protective ventilatory 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 the 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 following severe acid aspiration (Folkesson et al., 1995). Studies showed that...
bronchoalveolar lavage (BAL) fluids from patients with early ARDS delay neutrophil apoptosis \textit{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 (GM-CSF), granulocyte colony-stimulating factor (G-CSF), IL-8, and IL-2 (Aggarwal et al., 2000; Lesur et al., 2000). These factors cause 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 or neutral sphingomyelinase (aSMase or nSMase) or through \textit{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 through decrease of ceramide generation in a neonatal piglet model of surfactant deficiency-induced ALI (von Bismarck et al.,
A further metabolite of ceramide, sphingosine-1-phosphate (S1P) generated through ceramidase and sphingosine kinase, has in contrast 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 modulation of the p38 mitogen-activated protein kinase (MAPK) pathway (Chihab et al., 2003). Moreover, a strategy to activate the antiapoptotic 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 the 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 (TNF-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 following LPS challenge, nSMase is induced to hydrolyze sphingomyelin into ceramide, which is further metabolized to S1P, leading to the inhibition of neutrophil apoptosis via the phosphorylation of p38 MAPK. We also showed that treatment of mice with nSMase inhibitor following LPS-induced lung injury improved mouse survival and decreased the severity of lung damage. The activation of intracellular sphingolipid pathway and p38 MAPK in alveolar neutrophils from ARDS patients was also observed.
Materials and Methods

Patients. We enrolled three patients who were admitted to ICU 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 (PaO₂/FiO₉) < 200, regardless of positive end-expiratory pressure level; 4) pulmonary artery occlusion pressure of < 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 the published consensus guidelines (Levy et al., 2003). Patients were excluded if they were < 16 years of age, had refractory respiratory failure (PaO₂ < 60 mmHg with FiO₂ = 1.0), or had unstable hemodynamic status and lethal arrhythmia even under the use of high-dose vasopressor and antiarrhythmia drug. We also included three patients with cardiogenic lung edema who were intubated requiring mechanical ventilation and 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 subjects or their relatives.

Fiberoptic Brochoscopy Sampling. All enrolled patients underwent fiberoptic
bronchoscopy (Olympus LF2 or P40; Olympus Optic, Tokyo, Japan) for BAL fluids sampling within 24 h after ICU admission. All the patients were mechanically ventilated with FiO₂ 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 the 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, Becton Dickinson, Franklin Lakes, NJ) to remove mucus and then centrifuged at 500 × g 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. Ficoll-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 (SKI)-II were
also purchased from Sigma-Aldrich and p3 MAPK inhibitor SB203580 was from TOCRIS (Ellisville, MO). Polyclonal antibodies against phospho-p38 MAPK, p38 MAPK and caspase-3 were obtained from Cell Signaling (Beverly, MA), anti-ceramide monoclonal antibody was from Alexis (San Diego, CA), and anti-S1P monoclonal antibody was from Funakoshi Co. (Tokyo, Japan). RPMI 1640, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Gibco BRL (Grand Island, NY).

**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 then 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. Isolated neutrophils were used for LPS stimulation and chemical inhibitor treatment studies.

**Mice.** C3H/HeN 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 6-8 wk-old progeny were used for 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 anesthetization, LPS suspended in 50 μl of sterilized PBS or PBS alone was slowly instilled 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 LPS alone, or LPS followed by intraperitoneal injection of Sph-24 (1 mg/kg of body weight) 3 h and 9 h thereafter. The dose of Sph-24 was chosen based on our preliminary experiments. After 24 h, mice were killed by following the procedures as below. In survival experiments, mice were injected intraperitoneally with Sph-24 3 h after PBS or LPS instillation, or with LPS instillation alone. This procedure was repeated after 24 h and subsequently the 6 days 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 PBS (pH 7.4). The recovered fluid was centrifuged and the supernatant was analyzed for protein concentration by Bio-Rad assay (Bio-Rad, Hercules, CA). Cell pellets were pooled, resuspended in PBS, and the number of viable cells was determined by trypan blue exclusion. 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) 30 min before addition of LPS (500 ng/ml). The doses of 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 70% 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 (sub-G1; 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) 3 and 9 h after LPS (10 mg/ml) instillation was measured using Cell Death Detection ELISA kit, which detects cytosolic histone-complexed DNA fragments, according to the manufacturer’s instructions (Roche, Indianapolis, IN). Cell apoptosis was determined by the optical density (O.D.) at 405 nm. Activation of caspase-3 was detected by Western blotting as described below.

**Sphingomyelinase Assay.** Sphingomyelinase activity was determined in cellular extracts (Amplex Red Sphingomyelinase assay kit; Invitrogen, Carlsbad, CA) 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) 30 min before addition of LPS (500 ng/ml) for 24 h. Cells (1 × 10^6) were then placed in 100 μl lysis buffer with an equal amount of kit reagent (200 μl per well in 96-well plate). Briefly, each reaction contained 50 μM of Amplex Red reagent, 1 U/ml of horseradish peroxidase, 0.1 U/ml of choline oxidase, 4 U/ml of alkaline phosphatase, and 0.25 mM of sphingomyelin in the reaction buffer, and incubated at 37°C for 1 h. Fluorescence was measured using a microplate reader (Fluoroskan Ascent; Thermo Electron Corporation, Milford, 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% FBS-RPMI, with or without pretreatment with SKI-II (10 μM), and CHL (4 μM) 30 min before 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 FITC-conjugated goat anti-rabbit IgG, anti-mouse IgG, or anti-mouse IgM (Calbiochem, La Jolla, CA) staining. Mouse anti-ceramide and anti-S1P, and rabbit anti-phospho-p38 MAPK were
used for confocal microscopy (Leica TCS SP2; Leica Microsystems, Heidelberg, Germany) and flow cytometric analysis. PI was used for nuclear staining. In the samples of BAL cells, neutrophils were assessed by forward and side scatter characteristics of FACScan. At least 10,000 events were counted per sample. Results are represented as the percentage of ceramide, 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% FBS-RPMI, and then Sph-24 (50 μM), SKI-II (10 μM), and CHL (4 μM) were added 30 min before LPS (500 ng/ml) treatment, or LPS treatment alone. After 24 h, cells were washed in cold PBS and then pelleted and lysed in a Triton X-100-based lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris [pH 7.5], 5 mM EDTA, 5 mM NaN₃, 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 proteins were loaded in each lane of a 10% SDS-PAGE mini-gel 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. Nonspecific protein binding was blocked by pre-incubation with 5% milk in PBS supplemented with 0.05% Tween-20 (designated PBST) 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 internal control. After washing in PBST, 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 immunoblots were thoroughly washed in PBST, and detected using the ECL kit (Millipore, Billerica, MA) according to the manufacturer’s instructions.

Statistical Analysis. Data were expressed as means ± 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 ANOVA, followed by Tukey test as a method of post hoc analysis. All tests were two-tailed and $P < 0.05$ was considered significant. Survival curves were compared using the Log Rank test. All data were analyzed using GraphPad Prism 5.0 (GraphPad Software, 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 was also confirmed by blockade of caspase-3 activation (Supplemental Fig. 1B). Based on 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 (sphigosine kinase inhibitor), but not CHL (aSMase inhibitor), antagonized the anti-apoptotic 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 kinases 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 SMase
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 anti-apoptotic 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 blotting of neutrophil lysates, we found that LPS-induced p38 MAPK phosphorylation was inhibited by Sph-24 and SKI-II, but not CHL (Fig. 2A and B). In addition, pretreatment with p38 MAPK inhibitor SB203580 blocked the anti-apoptotic 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 the delayed neutrophil apoptosis during ALI development, we administered mice with 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 mice treated with LPS alone (Fig. 3A and B). Similarly, mice treated with Sph-24 showed less distortion of alveolar architecture, fewer thickened and disrupted septa and less intraalveolar bleeding and neutrophil infiltration, as compared with 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 mice treated with LPS alone (Fig. 3D). Furthermore, LPS-treated mice given Sph-24 had an improved survival as compared with 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 ALI. 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 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 upregulation is a critical step in sphingolipid metabolism, we compared ceramide levels in alveolar neutrophils of ARDS patients with those of cardiogenic lung edema, 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). Using immunostaining and flow cytometry, results showed significantly higher ceramide levels in alveolar neutrophils from ARDS patients than those from cardiogenic lung edema patients (Fig. 4A and B).

A previous \textit{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, as compared with those from cardiogenic lung edema patients (Fig. 5A and B).
Discussion

Sphingolipid signaling through aSMase-dependent ceramide production has been previously shown to induce apoptosis of lung endothelial cells and epithelial cells in ALI (Goggel et al., 2004). In contrast to 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, at least in part, mediated 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, TNF-α, IFN-γ, GM-CSF, and G-CSF or bacterial products such as LPS inhibit neutrophil apoptosis, while IL-10 counteracts this inhibition (Keel et al., 1997; Aggarwal et al., 2000; Lesur et al., 2000). To unravel the molecular mechanisms responsible for the decreased neutrophil apoptosis, a previous study found that circulating neutrophils from patients with sepsis are profoundly suppressed through a mechanism that involves activation of NF-κB, with a concomitant reduction in the activity of caspase-9 and -3 and the maintenance of mitochondrial membrane
potential (Taneja et al., 2004). A recent report showed that impaired cytoplasmic accumulation of myeloid nuclear differentiation antigen contributes to delayed neutrophil apoptosis during sepsis (Fotouhi-Ardakani et al., 2010). In this study, we show that neutrophils in BAL fluids from patients with ARDS have increased levels of ceramide. Together with the in vitro studies using human neutrophils and in vivo mouse model, we suggest that the intracellular sphingolipids may play a role in regulating neutrophil apoptosis during the development of ALI.

Sphingolipids such as sphingomyelin, ceramide, and S1P are known to regulate various cell functions and physiological processes, including apoptosis, vascular permeability, innate and acquired immunity, and to contribute to a variety of lung diseases such as pulmonary edema, emphysema, cystic fibrosis, and pneumonia (Uhlig and Gulbins, 2008). In ALI, the increased aSMase activity and ceramide content induce apoptosis of pulmonary endothelial cells and increase vascular permeability, leading to edema formation (Goggel et al., 2004; von Bismarck et al., 2008). However, the studies 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 beta-peptide, respectively (Yang et al., 2004; Goldkorn and Filosto, 2010). S1P is derived from ceramide through ceramidase and sphingosine kinase, and is recognized as a regulator of cell proliferation and survival,
angiogenesis and endothelial barrier functions via different S1P receptor subtypes (Spiegel and Milstien, 2003). In a murine model of LPS-induced ALI, S1P significantly decreased pulmonary vascular leakage and inflammation (Peng et al., 2004). S1P can also 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 undergoing apoptosis (Chihab et al., 2003; Hannun and Obeid, 2008). In this study, we found that LPS increased nSMase activity, and pretreatment of nSMase inhibitor Sph-24 and sphingosine kinase inhibitor SKI-II antagonized the anti-apoptotic effect of LPS on neutrophils. Of note, treatment of Sph-24 appeared to be more effective in preventing the inhibition of neutrophil apoptosis by LPS as compared with SKI-II. Sph-24 is a selective and irreversible nSMase inhibitor (Delgado et al., 2006). Similarly, SKI-II acts as a potent and selective inhibitor of sphingosine kinase 1 with no inhibition of protein kinase C-α, p42-MAPK/ERK2, 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 a 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. Based on the opposite effects of
sphingosine kinase 1 and 2 on regulation of cell apoptosis, we reasonably propose that the efficacy of SKI-II on antagonizing LPS-induced suppression of neutrophil apoptosis may be slightly compromised. Of interest, aSMase inhibitor CHL had no effect on regulation of neutrophil apoptosis in response to LPS stimulation. CHL has been frequently used 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 susceptible to proteolytic cleavage by lysosomal proteases (Verdurmen et al., 2010). Taken together, we suggest that delayed 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 as we observed decreased severity of ALI and increased survival by treatment with nSMase inhibitor. In this study, we used nSMase inhibitor Sph-24 for ALI treatment based on our in vitro findings, in which nSMase appeared to be more important in regulating neutrophil activation than aSMase, nevertheless, aSMase has been demonstrated to be more crucial in maintaining the alveolar-capillary barrier (Goggel et al., 2004), and based on the previous report that S1P has protective effects in LPS-induced ALI through enhancement of endothelial vascular integrity (Peng et al., 2004), preventing us to use sphingosine kinase inhibitor SKI-II as an ALI treatment because of its
potential unwilling effects on epithelial and endothelial cells. Therefore, nSMase inhibitor used for treatment of ALI may primarily target on neutrophils with less effects on epithelial and endothelial cells. In contrast, systemic or intratracheal administration of SKI-II may not only act on neutrophils but also on lung epithelial and endothelial cells, although a previous study has shown that SKI-II attenuates shock-induced lung injury in a rat model of trauma/hemorrhagic shock through suppression of neutrophil activation (Lee et al., 2004).

Moreover, S1P has been linked to regulation of inflammatory responses as S1P might suppress IL-2-induced or T cell related inflammatory responses. However, S1P has also been implicated in 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 neutrophil from undergoing apoptosis in response to external stimuli, leading to sustained inflammation. Nevertheless, FTY720, a synthetic S1P ligand, acts as an immunosuppressive agent in which the mechanism involves the action of phosphorylated FTY720 in lymphoid tissues in not only activating S1P receptors but also down-regulating them. As a result, lymphocytes lose their responsiveness to S1P, blocking their egress from thymus or lymph nodes (Sensken et al., 2009). FTY720 has been shown to protect from LPS-induced ALI in a murine model (Peng et al., 2004). However, the protective effect of FTY720 is
produced by endothelial cell barrier enhancement through the ligation of S1P1 receptors, but not by immunosuppression. So far, its role in regulation of neutrophil activation and apoptosis remains unclear. In addition, the use of adrenocorticotropic 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., 2001). In animal studies, treatment with 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 the 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 aSMase inhibitor. Furthermore,
inhibiting p38 MAPK signaling abrogated the anti-apoptotic effect in LPS-treated human neutrophils. Thus, the prevention of neutrophil apoptosis via p38 MAPK involves the activation of nSMase and the subsequent activation of sphingosine kinase. Our in vivo data also support this finding as the severity of ALI and mouse mortality are mitigated by blocking this signaling cascade using nSMase inhibitor Sph-24. This indicates that inhibiting the sphingomyelin hydrolytic pathway by targeting the activation of nSMase may represent a promising therapeutic strategy for regulating neutrophil apoptosis and ameliorating the development of ALI.
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Authorship Contributions


*Conducted experiments:* W.C. Lin.


*Wrote or contributed to the writing of the manuscript:* W.C. Lin, C.W. Chen, and Y.S. Lin.
References


Footnotes

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Requests for reprints should be addressed to Dr. Yee-Shin Lin, Department of Microbiology and Immunology, National Cheng Kung University Medical College, Tainan 701, Taiwan. E-mail: ysLin1@mail.ncku.edu.tw
Legends for figures

**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 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 (500 ng/ml)-treated 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 means ± S.E.M. *, P < 0.05. **, P < 0.01. ***, P < 0.001.

**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 the 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 means ± 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 p38 MAPK inhibitor SB203580 (12.5 μM). The results shown are representative of three independent experiments performed in triplicate. Data are shown as means ± 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 the BAL fluid were determined 24 h after 50 μl 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 (upper panel), 200 μm (lower 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 (A) LPS + Sph-24 group n = 6). Mann-Whitney test was used to perform statistical analysis for Fig. D. E, mice were treated with or without Sph-24 (1 mg/kg) after LPS instillation. The survival rate was observed at six days (n = 9 in LPS alone group, n = 8 in Sph-24 group and LPS +
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 co-stained 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 from patients with ARDS and cardiogenic lung edema are shown (n = 3 per group). Data are shown as means ± 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 co-stained with PI for nuclei (red), followed by flow cytometry. B, the averaged percentages of phospho-p38 positive neutrophils determined by flow cytometry in BAL from patients with ARDS or cardiogenic lung edema are shown (n = 3 per group). Data are shown as means ± S.E.M. *, P < 0.05.

Fig. 6. Schematic mechanism of sphingolipid-mediated neutrophil apoptosis
inhibition during ALI development. Upon LPS challenge, nSMase is induced to
hydrolyze sphingomyelin into ceramide. Ceramide is further hydrolyzed to
sphingosine which is phosphorylated to S1P. Subsequent phosphorylation of p38
MAPK leads to the inhibition of neutrophil apoptosis.
Figure 1

A

B

C

Neutrophil apoptosis (%)

Time (h) 0 24 24 24 24 24 24
LPS (500 ng/ml) - - + + + + +
Sph-24 (50 μM) - - - + - - -
CHL (4 μM) - - - + - - -
SKI-II (5 μM) - - - - + - -
SKI-II (10 μM) - - - - - + +

SMase activity (Relative fluorescence)

Time (h) 0 24 24 24 24 24 24
LPS (500 ng/ml) - - - - - - -
Sph-24 (50 μM) - - - - - - -
CHL (4 μM) - - - - - - -
SKI-II (5 μM) - - - - - - -
SKI-II (10 μM) - - - - - - -
Figure 2

A

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B

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Neutrophil apoptosis (%)

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Figure 5

A

ARDS
Cardiogenic lung edema

Relative cell number
87.7%
40.5%

Fluorescence intensity, phospho-p38

B

Percentage of phospho-p38 positive neutrophils

ARDS
Cardiogenic lung edema

*