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 II 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 that in 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 sphingomyelinasen (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 nSMase improves pulmonary function through a decrease of ceramide generation in a neonatal piglet model of surfactant deficiency-induced ALI (von Bischmark et al., 2008). In contrast, a further metabolite of ceramide, sphingosine-1-phosphate (SIP), 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 SIP 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 SIP targeting lung endothelial cells has been shown to prevent alveolar damage in a mouse model (Diab et al., 2010). In addition, SIP 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 SIP1-5 (Strub et al., 2010). Previous studies have indicated that SIP 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 SIP, 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. 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 II (SKI-II) also were purchased from Sigma-Aldrich, and the p38 MAPK inhibitor 4-(4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-1H-imidazol-5-yl)pyridine (SB203580) was purchased from Toeris Bioscience (Ellisville, MO). Polyclonal antibodies against phospho-p38 MAPK, p38 MAPK, and caspase-3 were obtained from Cell Signaling Technology (Danvers, MA), anti-keratin monoclonal antibody was obtained from Enzo Life Sciences, Inc. (Farmington, NY), and anti-SIP 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. C57/B6J or C57BL/6J 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:B4 (Sigma-Aldrich). When mice lost voluntary movement after anesthetization, LPS suspended in 50 μl of sterilized PBS or PBS alone was instilled slowly into the lung via the nares. 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.w) at 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 PBS 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% PBS-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 70% ethanol in PBS for propidium iodide (PI) staining (Sigma-Aldrich) and then analyzed by 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; percent 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% PBS-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 × 10^6) then were 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.

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. 2A 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.](https://jpet.aspetjournals.org/doi/10.1124/jpet.117.242931)
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 from mice treated with LPS alone. 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 < 0.01; ***, 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 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 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 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...
nuclear factor pressed through a mechanism that involves activation of neutrophil apoptosis, a previous study found that circulating neutrophil apoptosis. Subsequent phosphorylation of p38 MAPK leads to the inhibition of apoptosis inhibition during ALI development. Upon LPS challenge, schematic mechanism of sphingolipid-mediated neutrophil undergoing apoptosis (Chihab et al., 2003; Hannun and Obeid, 2003). 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 inhibitor (Delgado et al., 2006). Likewise, SKI-II acts as a potent and selective inhibitor of sphingosine kinase 1, with no inhibition 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 suppression 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 stimulation. 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 susceptible to proteolytic cleavage by lysosomal proteases (Verbelen et al., 2009). 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, 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 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 (Göggel et al., 2004) and, based on the previous report that S1P has protective effects in LPS-induced 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 endothelial 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, 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 the suppression of neutrophil activation (Lee et al., 2004). Moreover, S1P has been linked to the regulation of inflammatory 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

**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 hydrolyzed further to sphingosine, which is phosphorylated to S1P. Subsequent phosphorylation of p38 MAPK leads to the inhibition of neutrophil apoptosis.
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-octylphenyl(ethyl)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 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 also 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 hemorrhoage or endotoxemia (Arcaroli et al., 2001). In our study, 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 aSMA 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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Authorship Contributions

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

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Supplemental Fig. 1. LPS prevents neutrophils from spontaneous apoptosis via inhibition of caspase-3 activation. A, human neutrophils were treated with various doses of LPS for the indicated times and then stained with PI for apoptosis analysis by flow cytometry. Mean values ± S.E.M. are shown (n = 3). B, Western blot analysis was used to determine the expression of pro-caspase-3 and cleaved caspase-3 in LPS (500 ng/ml)-treated neutrophils for 24 h. GAPDH was used as an internal control.
Supplemental Fig. 2. SKI-II decreases S1P levels in neutrophils treated with LPS. Human neutrophils were treated with LPS (500 ng/ml) for 24 h without or with CHL (4 μM) or SKI-II (10 μM). The S1P levels were determined by flow cytometry and shown as percentage of S1P positive neutrophils. Upper panel shows the means ± S.E.M. of triplicate experiments. ***: $P < 0.001$. Lower panel shows a set of representative experiment.