The Effect of Mechanical Ventilation on Lung Epithelial Cell Fate

by

Joyce Lee

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of the Institute of Medical Science
University of Toronto

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Abstract

Premature infants subjected to mechanical ventilation (MV) are prone to lung injury that may result in bronchopulmonary dysplasia (BPD). MV induces pulmonary apoptosis and halts alveolar development. However, it is unknown whether ventilation-induced apoptosis is associated with autophagy. I propose that lung epithelial cells subjected to injurious mechanical stretch exhibit cell death by an interplay between autophagy and apoptosis. The aims of this thesis are to determine the role of autophagy in stretch-induced lung epithelial cell death, and to determine the significance of ceramide signaling. I demonstrate that stretch of fetal rat lung epithelial (FRLE) cells induces apoptosis via autophagy. Ceramide levels were elevated in response to stretch. Sphingomyelin phosphodiesterase 1 (SMPD1) inhibition prevented cell death by autophagy and apoptosis. These results demonstrate that stretch-induced SMPD1 activity leads to ceramide accumulation, resulting in lung epithelial apoptosis via autophagy, and identifies SMPD1 as a potential therapeutic target for limiting ventilation-induced BPD.
Contributions

The author performed all of the experiments, results, and analysis presented in this thesis, with the exception of the mentioned contributions.

Dr. Leonardo Ermini and members of the Analytical Facility for Bioactive Molecules (AFBM) at The Hospital for Sick Children performed all liquid chromatography-mass spectrometry measurements.

Dr. Cameron Ackerley processed and imaged all transmission electron microscopy experiments.

Dr. Behzad Yeganeh processed and imaged all immunohistochemistry experiments.

Dr. Andreas Kroon performed all in-vivo rat ventilation experiments.
Acknowledgments

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<tbody>
<tr>
<td>3-MA</td>
<td>3-methyladenine</td>
</tr>
<tr>
<td>ACTB</td>
<td>β- actin</td>
</tr>
<tr>
<td>ALI</td>
<td>Acute lung injury</td>
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<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptotic protease-activating factor-1</td>
</tr>
<tr>
<td>ARDS</td>
<td>Acute respiratory distress syndrome</td>
</tr>
<tr>
<td>ATG</td>
<td>Autophagy Related Genes</td>
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<tr>
<td>Baf A1</td>
<td>Bafilomycin A1</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BECN1</td>
<td>BECN1, autophagy related</td>
</tr>
<tr>
<td>BPD</td>
<td>Bronchopulmonary dysplasia</td>
</tr>
<tr>
<td>Caspase</td>
<td>Cysteine aspartate-specific proteases</td>
</tr>
<tr>
<td>CASP3</td>
<td>Caspase-3</td>
</tr>
<tr>
<td>CER</td>
<td>Ceramide synthase</td>
</tr>
<tr>
<td>CERS5</td>
<td>Ceramide synthase 5</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>CMA</td>
<td>Chaperone-mediated autophagy</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole, dihydrochloride</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DISC</td>
<td>Death-inducing-signaling complex</td>
</tr>
<tr>
<td>ENPP7</td>
<td>Alkaline sphingomyelinase</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated protein with death domain</td>
</tr>
<tr>
<td>FB₁</td>
<td>Fumonisin B₁</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FEV₁</td>
<td>Forced expiratory volume in 1 second</td>
</tr>
<tr>
<td>FRLE</td>
<td>Fetal (E19) rat lung epithelial</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s buffered salt solution</td>
</tr>
<tr>
<td>HIER</td>
<td>Heat-induced epitope retrieval</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>JNK₁</td>
<td>c-Jun NH2-terminal kinase 1</td>
</tr>
<tr>
<td>LASS5</td>
<td>Longevity assurance homolog 5</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography-tandem mass spectrometry</td>
</tr>
<tr>
<td>LC3B</td>
<td>Microtubule-associated protein 1 light chain 3 b</td>
</tr>
<tr>
<td>LPC</td>
<td>Lysophosphatidylcholine</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCRM</td>
<td>Mitochondrial ceramide-rich macrodomains</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
</tr>
<tr>
<td>MLE</td>
<td>Murine lung epithelial</td>
</tr>
<tr>
<td>MOMP</td>
<td>Mitochondrial outer membrane permeabilization</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reaction monitoring</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Mammalian target of rapamycin complex 1</td>
</tr>
<tr>
<td>MV</td>
<td>Mechanical ventilation</td>
</tr>
<tr>
<td>mVT</td>
<td>Moderate tidal volume</td>
</tr>
<tr>
<td>NF</td>
<td>Nuclear factor</td>
</tr>
<tr>
<td>PARP</td>
<td>poly ADP-ribose polymerase</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PCD</td>
<td>Programmed cell death</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>Class III phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PI3P</td>
<td>Phosphatidylinositol-3-phosphate</td>
</tr>
<tr>
<td>RIP</td>
<td>Receptor interacting protein</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>S1P</td>
<td>Sphingosine-1-phosphate</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small-interfering ribonucleic acid</td>
</tr>
<tr>
<td>SM</td>
<td>Sphingomyelin</td>
</tr>
<tr>
<td>SMPD1</td>
<td>Acid sphingomyelinase</td>
</tr>
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<td>SMPD2</td>
<td>Neutral sphingomyelinase</td>
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<tr>
<td>SPH</td>
<td>Sphingosine</td>
</tr>
<tr>
<td>Sph-24</td>
<td>Sphingolactone-24</td>
</tr>
<tr>
<td>SphK</td>
<td>Sphingosine kinase</td>
</tr>
<tr>
<td>SPT</td>
<td>Serine palmitoyltransferase</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>ULK1</td>
<td>UNC-51-like kinase</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VILI</td>
<td>Ventilator-induced lung injury</td>
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</table>
Chapter 1
Introduction

1 Introduction

1.1 Bronchopulmonary Dysplasia

Bronchopulmonary dysplasia (BPD) was originally described in 1967 by Northway and colleagues in preterm infants who were subjected to aggressive mechanical ventilation and high oxygen concentrations (Northway et al., 1967). These infants suffered from significant respiratory symptoms and functional abnormalities as a result of diffuse airway damage, mucosal inflammation, fibrosis, and airway collapse (Baraldi and Filippone, 2007). Advancements in neonatal care and the development of preventative treatments including antenatal corticosteroids, surfactant replacement, and gentler ventilation strategies brought a decrease in the ‘old’ form of BPD (Baraldi and Filippone, 2007). However, with improved survival rates of infants born at earlier gestational ages, BPD remains one of the most common chronic lung diseases of neonates.

1.1.1 Pathogenesis and epidemiology

Very premature infants (23-26 weeks gestation) subject to intubation and positive pressure ventilation suffer from ventilator-induced lung injury (VILI) and many develop the ‘new’ form of BPD. The ‘new’ BPD is interpreted as a lung developmental disorder and is characterized by a halt in lung development, with fewer and larger alveoli, vascular abnormalities, and an overall simplification of normal lung complexity (A J Jobe, 1999; Carvalho et al., 2013). The infants that are at greatest risk of developing BPD are born during the late canalicular/early saccular stage of lung development, a period in which respiratory structures are premature and alveolar structures have not yet formed (Fig. 1) (Alan H Jobe, 2003). Therefore, mechanical ventilation and oxygen supplementation due to respiratory dependence at this stage of lung development remain primary causes of BPD which develops in 52% and 7% of infants born weighing between 500 and 1500 g, respectively (Ehrenkranz et al., 2005; Baraldi and Filippone, 2007).
Figure 1. Stages of lung development, damaging factors, and types of lung injury.

The severity of lung injury may range from structural damage (old bronchopulmonary dysplasia) to early developmental arrest (new bronchopulmonary dysplasia) depending on timing and extent of damaging factors. Premature infants born between 23 to 30 weeks of gestational age (late canalicular to early saccular stages of lung development) are at greatest risk for bronchopulmonary dysplasia. Reproduced and modified with permission from Baraldi and Filippone; The New England Journal of Medicine, 2007 (Baraldi and Filippone, 2007).
1.1.2 Symptoms and pulmonary function

Affected infants suffer from lifelong consequences including increased susceptibility to respiratory infections, pulmonary hypertension, and frequent hospital visits (Morley et al., 2008; Carvalho et al., 2013). There have been several studies that report increased coughing, wheezing, and asthma-like symptoms in children that were born prematurely, particularly those that required mechanical ventilation (Rona et al., 1993; Greenough et al., 1996). These factors culminate in resource consumption and present a significant financial burden on healthcare systems (Donn, 2005; Stuart et al., 2013). Therefore, it is critical to better understand the pathogenesis of BPD to develop therapeutic strategies targeted at preventing VILI.

A study reported significantly lower forced expiratory volume in 1 second (FEV\textsubscript{1}) in survivors of BPD compared to their respective healthy controls (Mitchell and Teague, 1998). Currently, it is difficult to gather information on long-term lung function in survivors with the ‘new’ form of BPD, as much of the data available reflects those patients who were diagnosed before the era of surfactant treatment and antenatal corticosteroids. However, it is important to understand the significance of lung injury, and the long-term consequences of chronic lung diseases, such as BPD, need to be further investigated.

1.1.3 Treatment

The use of corticosteroids has brought about rapid improvement of lung mechanics in premature infants with BPD (Jacob et al., 1997). However, the routine use of corticosteroids remains controversial due to the risk of neurodevelopmental impairment in premature newborns (Jefferyes, 2012). Inhaled bronchodilators, including β2-agonists and anticholinergic agents provide short-term improvement to lung function, however do not seem to confer long-term effects (Pantalitschka and Poets, 2006). The use of other treatment options such as diuretics has shown great potential in improving lung mechanics, however the effects of long-term treatment are not well understood (Kinsella et al., 2006). It is evident that treatment options are still empirical and a better understanding of the course of BPD will reveal more specific therapeutic targets to prevent and treat the disease.
1.2 Mechanical Ventilation

1.2.1 Ventilator-induced lung injury (VILI)

MV is frequently used to provide respiratory assistance to very premature infants. Despite the movement towards gentler ventilation strategies, the incidence of BPD has not reduced and there is strong evidence that MV leads to both short- and long-term injurious consequences, including arrested alveolar development (Alan H Jobe, 2001; Kornecki et al., 2005; Alan H Jobe, 2015). The pathogenesis of VILI is multi-factorial, complex and may be caused by high airway pressures (barotrauma), over-distension of the lung due to excessive tidal volume (volutrauma), repeated opening and closing of lung units (atelectrauma), or as a result of molecule mediators released by lung tissues (biotrauma) (Slutsky, 1999). Baro-, volu-, and atelec-trauma are types of injury that are largely believed to cause harm within the lung as a result of physical forces. Contrastingly, there has been increasing evidence that mechanical forces can lead to lung injury as a result of abnormal molecular signaling at the intracellular level (Slutsky, 1999) ADD more recent citations.

1.2.2 Mechanotransduction in the lung

The process by which a physical stimulus, such as mechanical stretch, is converted into biochemical signals within the cell is referred to as mechanotransduction (French, 1992).Mechanosensors at the cell membrane sense physical forces and trigger a cascade of signaling molecules called second messengers. Second messengers then act on and alter the activity of downstream components of the signaling pathway to ultimately result in the amplification or reduction of a cellular response by evoking a transcriptional or post-translational modification (M Liu et al., 1999). Four known mechanosensing mechanisms control mechanotransduction in the lung. First, stretch receptors that detect forces exerted by extrapulmonary airway smooth muscle and thoracic movement, and transmit information to respiratory neurons (Sant'Ambrogio, 1987). Second, stretch-activated ion channels allow the exchange of cations or anions between the extra- and intra-cellular spaces in response to mechanical stimuli (Sackin, 1995). Third, integrins are transmembrane adhesion receptors that act as a bridge between components of the extracellular matrix and the actin cytoskeleton to regulate complex physiological processes including cell migration and tissue organization (Giancotti and Ruoslahti, 1999). Finally, there is
also strong evidence that G-proteins have critical involvement in mechanotransduction (Labrador et al., 2003; Torday and Rehan, 2003; Gudi et al., 2003), especially within the lung (Correa-Meyer et al., 2002).

1.2.3 Biotrauma hypothesis

The ‘Biotrauma Hypothesis’ holds that upregulation of mediators triggered by mechanical ventilation can cause or worsen lung injury (Slutsky and Tremblay, 1998). The biotrauma hypothesis also holds that these molecular mediators have systemic biological actions systemically (Slutsky and Tremblay, 1998) which can lead to multi-organ failure.

Several classes of such mediators have been proposed; for example, proinflammatory cytokines including tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) (Tremblay et al., 1997; Ranieri et al., 1999), and angiotensin II (Jerng et al., 2007) have been correlated with lung injury. More recently, a study implicated lipid-soluble protein-bound molecules not only as biomarkers in VILI, but also as pathogenic mediators circulated during high tidal volume ventilation worsening lung injury (Jaecklin et al., 2011). Amongst suggested liposoluble compounds, sphingolipids were identified as potential pathogenic mediators in VILI. As addressed in this thesis, I was particularly interested in sphingolipids and their potential role in the pathogenesis of BPD.

1.2.4 Effect of ventilation on lung development

Currently, the molecular mechanisms of VILI are not completely understood, and how MV arrests lung development remains unknown. Apoptosis has been suggested as a potentially important mechanism in the reduction of alveolar number and the surface area for gas exchange in the lung. In fact, a previous study demonstrated that prolonged MV of developing lungs with room air induces apoptosis and causes failure of alveolar septation and angiogenesis in lungs of newborn mice (Mokres et al., 2009). Complementary to these findings, Kroon and colleagues previously reported increased apoptosis via the extrinsic death pathway in alveolar type II cells with MV of newborn rats (Kroon et al., 2013). Moreover, a role in lung development has been proposed for the Fas ligand (FasL)/Fas signaling pathway has been localized to the respiratory epithelia corresponding with maximal airway distention and increasing apoptosis (De Paepe et
al., 2000; Wajant, 2002), suggesting a potential role of FasL/Fas signaling in ventilation-induced apoptosis. Kroon and colleagues showed that prolonged ventilation significantly increased the expression of FasL during MV, and universal caspase inhibition and neutralization of FasL abrogated stretch-induced apoptosis (Kroon et al., 2013). However, the mechanism leading to apoptotic cell death has yet to be studied. In contrast to apoptosis, autophagy is a catabolic process that serves to maintain homeostasis and cell survival in response to cellular stress (Mizushima, 2007; Klionsky et al., 2012), and is often activated upstream to apoptosis. The focus of this thesis is to better understand the molecular mechanistic signaling and activation of cell fate pathways that occurs during VILI.
1.3 Sphingolipids

Sphingolipids are a class of lipids that were named after the mythological Sphinx because of their enigmatic nature. They were initially thought to serve strictly as structural components of the membrane bilayer, but have now been implicated in various cell-signaling pathways including cell proliferation, differentiation, and programmed cell death (PCD) (Spiegel et al., 1996). These molecules display amphipathic properties in which the hydrophobic end is comprised of a sphingoid base that is linked to a fatty acid, while the hydrophilic end varies in structures consisting of hydroxyl groups, phosphates and sugar residues. Different lengths, saturations, and hydroxylations of fatty acids, as well as different head groups result in an immense diversity of sphingolipid species (Yang Yang & Uhlig, 2011). All sphingolipids can be derived from ceramide. For example, ceramide can be deacylated by ceramidase to sphingosine that then can be phosphorylated by a sphingosine kinase isoenzyme (SphK₁ or SphK₂) to form sphingosine-1-phosphate (S1P) (Hong Liu et al., 2002; Spiegel and Milstien, 2003). Alternatively, sphingosine can be acylated by ceramide synthase (CER) to give rise to ceramide. Both ceramide and sphingosine can act as second messengers to promote apoptosis, cellular senescence, and growth arrest (Kolesnick and Krönke, 1998). Sphingomyelin synthase converts ceramide to sphingomyelin, a structural lipid mainly localized to the outer membrane leaflet, while sphingomyelin can give rise to ceramide by the action of sphingomyelinase (SMase) isoenzymes (Teichgräber et al., 2008). Ceramide and S1P have received attention as they appear to play opposing roles in a dynamic relationship known as the “sphingolipid rheostat” (Spiegel et al., 1996; Huwiler et al., 2000; Young et al., 2013). At one end of the scale, ceramide is typically recognized to initiate apoptosis and growth arrest, whereas S1P, at the other end, promotes cell proliferation, survival, mobility, and cell-to-cell adhesion (Olivera and Spiegel, 1993; Hannun and Obeid, 1995; Susan Pyne and Nigel J Pyne, 2000). These two sphingolipids play a role in lung homeostasis and cell fate processes such as apoptosis, and more recently, autophagy.
Figure 2. Possible roles for ceramide and sphingosine-1-phosphate in lung diseases.

1.3.1 Sphingolipids in the lung

Sphingolipids have significant roles in various organ systems (Cervia et al., 2013; Tibboel, Joza, et al., 2013; Tibboel et al., 2014; Pullmannová et al., 2014; Awojoodu et al., 2014), but their role in the regulation of lung development has been of recent interest. Ceramide, S1P and their dynamic relationship have been of particular interest. For instance, the de novo pathway of ceramide synthesis has been linked to apoptotic endothelial cell death and decreased pulmonary barrier function (Petrache et al., 2005; Medler et al., 2008). Medler and colleagues (Medler et al., 2008), it was found that de novo ceramide synthesis resulted in apoptotic cell death in lung endothelial cells by both paracellular and TNF-α-stimulated intracellular ceramide signaling. Increased ceramide synthesis has also been linked to the development of emphysema-like disease in the murine lung, suggesting that the balance between a pro-apoptotic molecule and a pro-survival factor, such as S1P, is essential in maintaining homeostasis in the vasculature of the lung (Petrache et al., 2011). In-depth investigation into the levels of sphingolipids in a developing rat lung revealed that maintenance of sphingolipid levels and their metabolism are highly regulated. In the embryonic rat lung, sphingomyelin and sphingosine levels increase during development and plateau at birth. Interestingly, the activity profiles of serine palmitoyltransferase (SPT), acid sphingomyelinase (SMPD1) and neutral sphingomyelinase (SMPD2) correlate accordingly (Longo et al., 1997). By contrast to the rat lung, sphingomyelin levels in the fetal lung of the monkey and lamb decrease during development (Adams et al., 1971; Perelman et al., 1982). Changes in sphingolipid levels may affect proper lung development and function, as Tibboel and colleagues demonstrated in newborn mice that hyperoxic conditions increased multiple sphingolipids, including ceramide (Tibboel, Joza, et al., 2013). This caused abnormal alveolar morphology and obstructive lung function that were only partially recovered in room air (Tibboel, Joza, et al., 2013). D-sphingosine reduced ceramide levels and partially halt in alveolarization, demonstrating that altered sphingolipid levels may be a factor in hyperoxia-induced lung injury or BPD (Tibboel, Joza, et al., 2013). These findings suggest that regulation of sphingolipid metabolism is important in proper lung development and that imbalances in sphingolipid regulation can lead to improper lung structure or disease states (Fig. 2).
1.3.2 Sphingolipids, apoptosis, and autophagy in pulmonary disease

This section explores sphingolipids and their important role in pathological processes within the lung and disease states. The vast number of sphingolipids is reflected in the spectrum of signaling mechanisms they are involved in. This section highlights the key roles of sphingolipids and the interconnected relationship between apoptosis and autophagy, and the significance of the interrelationship within the lungs and its diseases such as acute lung injury (ALI)/acute respiratory distress syndrome (ARDS), BPD, asthma, chronic obstructive pulmonary disease (COPD), emphysema, and cystic fibrosis (CF).

1.3.2.1 Bronchopulmonary dysplasia

As mentioned previously, very premature infants that are subjected to mechanical ventilation and oxygen supplementation due to respiratory failure are prone to lung injury that may result in chronic lung disease, such as BPD, with lifelong consequences (Baraldi and Filippone, 2007). The lungs of present day BPD patients are characterized by a halt in lung development, with simplification of normal lung complexity as seen with fewer and larger alveoli and vascular abnormalities (Alan H Jobe, 2001). Newborn mice exposed to hyperoxic conditions displayed an increase in multiple sphingolipids, including ceramide (Tibboel, Joza, et al., 2013). This caused abnormal alveolar morphology and obstructive lung function that were only partially recovered in room air (Tibboel, Joza, et al., 2013). The use of D-sphingosine reduced ceramide levels and partially minimized halt in alveolarization, demonstrating that altered sphingolipid levels may be a factor in hyperoxia-induced lung injury or BPD (Tibboel, Joza, et al., 2013). Levels of vascular endothelial growth factor (VEGF), a critical factor in pulmonary vascular development, were decreased in a rodent animal model of BPD (Hosford and Olson, 2003), thereby most likely reducing alveolarization (Jakkula et al., 2000; Le Cras et al., 2002). Increased ceramide production also decreases VEGF by suppression of hypoxia-inducible factor-1α (Yasuo et al., 2013). These findings suggest that sphingolipids play a role in abnormal lung vascular development in BPD through VEGF signaling. In another study, apoptosis was detected in alveolar epithelial cells in the lungs of preterm infants that were subjected to ventilation and oxygen treatment (May, Strobel, et al., 2004). Similar results have been reported in mouse models of BPD (Dieperink et al., 2006). Kroon and his colleagues observed that mechanical
ventilation of newborn rat resulted in increased numbers of apoptotic alveolar type II cells (Kroon et al., 2013). Prolonged maximal cyclic stretch was also associated with increased expression of cleaved caspase-3, -7, and -8, as well as the apoptotic mediator Fas ligand (FasL), suggesting that the extrinsic death pathway via the FasL/Fas system is involved in ventilation-induced apoptosis of alveolar type II cells (Kroon et al., 2013). Given these findings, it is worthwhile to investigate the role of ventilation-induced ceramide production in epithelial cell apoptosis as a mechanism responsible for pulmonary apoptosis and inhibition of alveolar development in preterm infants with BPD. The possibility that altered levels of various sphingolipids can change cell fate during MV opens potential areas of therapeutic interventions for BPD to reduce lung damage in premature infants that require respiratory assistance.

1.3.2.2 Acute respiratory distress syndrome

ARDS is another important respiratory disease that leads to mortality in preterm infants and is characterized by lung inflammation, decreased surfactant, increased microvascular permeability, and edema (Rubenfeld, 2007; Tibboel et al., 2014). The contributing role of sphingolipids in attenuating injury to the lung during ALI and ARDS has been recently explored and is now well established that there are a number of mechanisms including the SMPD2, ceramide, S1P, and the p38 MAPK pathway, that appear to be involved in the pathological process (Lin et al., 2011).

The influence of sphingolipids on neutrophil cell fate in ALI/ARDS is of particular interest as it is well established that the longevity of neutrophils correlates with disease severity (Zemans et al., 2009; Uhlig and Yang Yang, 2013). Under homeostatic conditions, neutrophils will undergo unprompted apoptosis (Sarah Fox et al., 2010). However, neutrophils that are recruited to occupy the alveolar space during lung injury contribute to the pathogenesis of ALI and ARDS by production and secretion of proteases and ROS for the duration of their survival (Grommes and Soehnlein, 2011). Thus, neutrophil apoptosis is essential for resolution of inflammation, especially in the context of lung injury.

Apoptosis of neutrophils from patients with sepsis was suppressed via a mechanism involving nuclear factor (NF)-κB, reduced caspase-9 and caspase-3 activity, and maintenance of mitochondrial membrane potential (Taneja et al., 2004). Another study found that
sphingolactone-24 (Sph-24) (SMPD2 inhibitor) and SKI-II (sphingosine kinase inhibitor) prevented the anti-apoptotic effect of lipopolysaccharides (LPS) on neutrophils and that LPS stimulated SMase activity, suggesting that sphingolipids are involved in inhibition of neutrophil apoptosis (Lin et al., 2011). When Sph-24 was administered to LPS-challenged mouse lungs, leukocyte counts decreased after 24 hrs suggesting that SMPD2 accelerates the progression of lung injury by deceleration of neutrophil apoptosis (Lin et al., 2011). LPS challenge of neutrophils that have been recruited to the lung also display an increase in S1P levels. This study also revealed that the increased levels of anti-apoptotic S1P are likely the result of activation of the SMPD2-S1P pathway (Lin et al., 2011). S1P is generated by SphKs that are stimulated by factors such as histamines and cytokines, which could explain the increase of SphK activity in the injured lung (Mastrandrea et al., 2005; Lin et al., 2011). Thus, both SMPD2 and S1P play key roles in the pathogenic process of ALI/ARDS, particularly in their influence of preventing neutrophil apoptosis. In ALI, increased SMPD2 activity augments ceramide and subsequent formation of sphingosine, that then is phosphorylated to S1P by activated sphingosine kinase, leading to phosphorylation of p38 mitogen-activated protein kinase (MAPK), resulting in inhibition of neutrophil apoptosis (Chihab et al., 2003; Lin et al., 2011). Greater understanding of the pathogenesis of ALI/ARDS and of the role sphingolipids play in cell fate will improve detection and treatment of this critical condition.

1.3.2.3 Other respiratory disorders

Asthma is a complex, chronic inflammatory lung disease that is characterized by airway wall remodeling, airway smooth muscle contraction and hyperreactivity, increased mucus production, and inflammatory cell gathering (Worgall et al., 2013). It has become apparent that sphingolipid metabolites play a regulatory role in the pathogenesis of asthma (Maceyka & Spiegel, 2014). S1P is thought to act as a pro-survival signal in cells (Susan Pyne and Nigel J Pyne, 2000), and increased levels in the asthmatic airway appear to play a role in perpetuating the asthmatic response (Ammit et al., 2001). The role of S1P in the pathogenesis of asthma is well illustrated by the fact that S1P appears to act as a mitogenic factor and a stimulator of proliferation of the airway smooth muscle cells. S1P increases G₁/S progression in the cell cycle which results in augmented thrombin-induced deoxyribonucleic acid (DNA) proliferation (Ediger and Toews,
2000; Ammit et al., 2001). SphK plays important roles in other inflammatory and hyperproliferative diseases, and has received attention with the prospect of developing specific SphK inhibitors for therapeutic purposes (Plano et al., 2014).

COPD encompasses a spectrum of pathological conditions, including emphysema, that are characterized by difficulties breathing due to obstructed airflow (Dr David M Mannino MD and A Sonia Buist MD, 2007). Emphysema is found almost exclusively in adults, often associated with inhalation of cigarette smoke, but can also result from exposure to environmental pollutants, typically worsening over time (Yoshida and Tuder, 2007). In particular, patients with emphysema have a loss of alveolar surface area available for gas exchange due to destruction of alveolar septa (Yoshida and Tuder, 2007). The pathogenesis of COPD includes inflammatory responses in the airway, loss of barrier function, oxidative, and endoplasmic reticulum (ER) stress responses. In addition, both apoptosis and autophagy of airway cells in the airways are generally accepted to be important events in the pathogenesis of COPD and pulmonary emphysema (Ryter et al., 2009). It is now recognized that sphingolipids play important roles in the development of COPD, including ceramide appears to be a crucial mediator in the apoptosis of alveolar cells (Petrache et al., 2005; Tibboel, Reiss, et al., 2013). For instance, in rat and mice emphysema models, inhibition of de novo synthesis of ceramide by VEGF blockade prevented alveolar cell apoptosis and oxidative stress (Petrache et al., 2005). Moreover, lung ceramide levels are markedly higher in individuals with emphysema from chronic cigarette smoking compared to individuals without emphysema (Petrache et al., 2005). Tibboel and colleagues recently revealed that the addition of a SPT inhibitor to a rodent model of elastase-induced emphysema diminished the increase in ceramides and improved lung function (Tibboel, Reiss, et al., 2013). The authors suggested that ceramide upregulation may be a critical factor in the development of alveolar destruction in emphysema, thus suggesting a potential therapeutic target that warrants further investigation. The functional significance of autophagy in disease states such as COPD has yet to be established since relatively few studies have been done on the lung. Few in vivo and in vitro specimens of COPD lung exhibit increased levels of autophagy markers when exposed to cigarette smoke or cigarette smoke extract, possibly as a response to a source of stress on the lung (Zhi-Hua Chen et al., 2008; Kim, Xue Wang, Chen, Lee, Huang, Yong Wang, Ryter, and Choi, 2008a; Hwang et al., 2010). There have been reports suggesting that the
autophagy plays an important role in the pathogenesis of COPD, perhaps via promoting epithelial cell death (Zhi-Hua Chen et al., 2008; Ryter et al., 2009; Zhi-Hua Chen, Lam, Jin, Kim, Cao, Seon-Jin Lee, Ifedigbo, Parameswaran, Ryter, and Choi, 2010a). As mentioned earlier, increased expression of ceramide has been detected in emphysema and may play a critical role in impaired autophagy in the context of COPD. However, the role of ceramide in smoke-induced COPD is not yet well studied. It may be worthwhile to investigate whether the increase of ceramide content in lungs when exposed to cigarette smoke also has an impact on autophagy, and if the pathway plays a pathogenic role in COPD.

CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that codes for a chloride channel that mediates proper movement of chloride and other ions across the apical surface of epithelial cells (Rommens et al., 1989; Welsh and Smith, 1993). In Canada, the disease has a median predicted age of survival of 49.7 years (Stephenson et al., 2014). It is characterized by reduced mucociliary clearance, accumulation of mucus in the bronchi ultimately leading to recurrent and/or chronic infections with bacteria including S. aureus and P. aeruginosa (Uhlig and Gulbins, 2008). Ceramide has been found at high concentrations in CF airways and appears to be an important factor in the hallmark features of CF and cellular fate in the affected lungs (Teichgräber et al., 2008). There is evidence that CFTR dysfunction leads to an imbalance in sphingolipid homeostasis (Teichgräber et al., 2008) and that its localization in the lipid raft influences membrane ceramide composition (Hamai et al., 2009). Ceramide appears to be a critical regulator of P. aeruginosa infection in CFTR-deficient mice (Teichgräber et al., 2008). Accumulation of ceramide in the cells led to apoptosis and DNA deposition in the upper airways that facilitated P. aeruginosa infection. Treatment of CFTR-deficient mice with amitriptyline (SMPD1 inhibitor) normalized ceramide levels and prevented pulmonary infection with P. aeruginosa (Teichgräber et al., 2008). It has yet to be fully understood if these findings can be transferred into a human system, but results thus far have suggested that disruption of ceramide formation may serve as a prospective treatment for patients with CF.
Table 1. Sphingolipids in pulmonary diseases

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<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Sphingolipid</th>
<th>Cell Fate</th>
<th>Pathophysiological Effect</th>
<th>Disease State</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sepsis, inhalation of harmful substances</td>
<td>↑S1P</td>
<td>↓ neutrophil apoptosis</td>
<td>expedites inflammation, increase in proteases and ROS resulting in damage to lung tissues</td>
<td>ALI/ARDS</td>
<td>(Lin et al., 2011)</td>
</tr>
<tr>
<td>Mechanical ventilation, high oxygen</td>
<td>↑ ceramide</td>
<td>↑ alveolar epithelial cell apoptosis</td>
<td>halt in alveolarization, fewer &amp; larger alveoli</td>
<td>BPD</td>
<td>(Tibboel, Joza, et al., 2013; Kroon et al., 2013)</td>
</tr>
<tr>
<td>Inhalation of cigarette smoke/ pollutants</td>
<td>↑ ceramide</td>
<td>↑ alveolar epithelial cell apoptosis</td>
<td>loss of alveolar surface area available for gas exchange</td>
<td>COPD/Emphysema</td>
<td>(Petrach e et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>↑ ceramide</td>
<td>↑ impaired autophagy</td>
<td>Accumulation of impaired autophagy marker, p62 in autophagosomes</td>
<td>COPD/Emphysema</td>
<td>(Ryter et al., 2010)</td>
</tr>
<tr>
<td>Infection in CFTR-deficient mice</td>
<td>↑ ceramide</td>
<td>↑ bronchial cell apoptosis</td>
<td>bronchial cell apoptosis and DNA deposition in the upper airways</td>
<td>CF</td>
<td>(Teichgräber et al., 2008)</td>
</tr>
</tbody>
</table>
1.4 Ceramide

Ceramide is the simplest of all sphingolipids and is a well-known critical mediator of various cell death pathways, including apoptosis and necrosis (Engedal and Saatcioglu, 2001; Todaro et al., 2002). Increased ceramide levels have been associated with apoptosis in both homeostatic systems as well as pathological settings as a result of cellular insults including oxidative stress, chemotherapeutic agents, ischemia and radiation (Verheij et al., 1996; Hannun, 1996; Kolesnick and Krönke, 1998; Hannun and Luberto, 2000; Petrache et al., 2005). Ceramide can act on both the intrinsic (mitochondrial) and extrinsic pathways of apoptosis in a context-dependent manner (Siskind et al., 2002; Kroon et al., 2013). Moreover, ceramide can induce apoptosis by recruitment of death receptors to lipid rafts and assembly of channels in the outer membrane of the mitochondria promoting the release of cytochrome c, only to mention two amongst several investigated pathways (Siskind and Colombini, 2000; Siskind et al., 2002; Schenck et al., 2007). It has more recently been shown that ceramide is associated with the induction of autophagy, influencing cellular fate under stress conditions such as amino acid deprivation, mitochondrial damage, and ER stress (Daido et al., 2004; Guenther et al., 2008; Spassieva et al., 2009; Ponnusamy et al., 2010; Sentelle et al., 2012; Taniguchi et al., 2012).

1.4.1 Ceramide metabolic pathways

Ceramide can be generated through three known pathways. De novo synthesis of ceramide is characterized by the rate-limiting step catalyzed by SPT, in which serine and palmitoyl-CoA undergo condensation in the cytoplasmic leaflet of the ER (Dawkins et al., 2001). Generation of ceramide from sphingomyelin can be catalyzed by several sphingomyelinase isoenzymes with different optimal pH levels: SMPD1 is found in the lysosome and mediates sphingolipid turnover and degradation; Mg\(^{2+}\) dependent SMPD2 is localized to the ER, Golgi apparatus, and the plasma membrane; and finally, alkaline sphingomyelinase (ENPP7) (Levade and Jaffrézou, 1999). Lastly, release of ceramide through a recycling loop from sphingosine involves hydrolysis of glycosphingolipids and can also occur by the reverse activity of ceramidase (Okino et al., 2003) (Fig. 3).
Figure 3. Overview of sphingolipid metabolic pathways and their major functions.

Condensation of serine and palmitoyl-CoA by the enzyme serine palmitoyltransferase (SPT) is the rate-limiting step of *de novo* synthesis of ceramide. Generation of ceramide from sphingomyelin can be catalyzed by several sphingomyelinase isoenzymes with various optimal pH levels. Ceramide generated through a recycling loop from sphingosine involves hydrolysis of glycosphingolipids and can also occur by the reverse activity of ceramidase. Reproduced and modified with permission from Lee and colleagues; Apoptosis, 2015 (Joyce Lee *et al*., 2015).
1.4.2 Ceramides in the lungs

It is important to emphasize that a regulated level of ceramide may be necessary for maintenance of homeostasis in the lung. It is evident now that the preservation of the sphingolipid rheostat is important in the formation of lung structures at all stages of lung development and physiology (Tibboel et al., 2014). Pigs exposed to fumonisin B1 (FB₁) (an inhibitor of CER) resulted in elevated levels of sphingolipids, including sphinganine and sphingosine, which resulted in alveolar endothelial cell damage and fatal pulmonary edema (Gumprecht et al., 1998). Chronic exposure to FB₁ in rats not only induced renal tumors, but also resulted in metastatic invasion of the lungs (Hard et al., 2001). These findings suggest that ceramide synthesis is required for an underlying level of apoptosis in the lungs as an anti-tumorigenic factor (Hard et al., 2001). Xu and colleagues demonstrated that in murine lung epithelia the de novo ceramide synthesis pathway is the major contributing pathway for ceramide production (Zhiwei Xu et al., 2005). Longevity assurance homolog 5 (LASS5) was found to be the predominant CER in murine primary type II epithelial cells and SV40-transformed murine lung epithelial (MLE) cells. Indeed, inhibition of CER activity by FB₁ was shown to inhibit the production of ceramide (Zhiwei Xu et al., 2005). Furthermore, this study also showed that overexpression of LASS5 reduced phosphatidylcholine (PC) production, suggesting that ceramide may regulate PC metabolism in pulmonary cells (Zhiwei Xu et al., 2005).
1.5 Apoptosis

1.5.1 Programmed cell death

PCD is an essential physiological process involved in development, aging, and tissue homeostasis which maintains normal cellular fate in different organisms (Ameisen, 2002). Apoptosis is a widely recognized mode of PCD in which complex molecular signaling systems trigger an orderly, energy-dependent enzymatic breakdown of DNA, lipids, and other macromolecules (Fuchs and Steller, 2011). Cells undergoing apoptosis show typical, well-defined morphological changes characterized as rounding of the cell, shrinkage of pseudopods, decreased cellular volume, chromatin condensation (pyknosis), nuclear fragmentation (karyorrhexis) along with little or no ultrastructural reformations of organelles in the cytoplasm followed by plasma membrane blebbing, and ingestion by phagocytes (Kerr et al., 1972; Fuchs and Steller, 2011).

1.5.2 Apoptotic signaling pathway

Apoptosis does not induce inflammation, since apoptotic cells do not release their cellular contents into the surrounding interstitial tissue; rather they are quickly engulfed by macrophages or adjacent normal cells (Savill and Fadok, 2000; Elmore, 2007). The mechanism of apoptosis is highly synchronized and is coordinated by an extensively studied group of cysteine proteases known as cysteine aspartate-specific proteases (caspases). Caspases are widely expressed in an inactive proenzyme form (procaspases), localized in the nucleus, cytoplasm, ER, and the mitochondrial intermediate space, and can be translocated to the plasma membrane (Fuchs and Steller, 2011). Caspases have proteolytic activity and are able to cleave proteins at an internal aspartic acid site, although different caspases have specificities involving recognition of neighboring amino acids (Stennicke and Salvesen, 1998; Creagh et al., 2003). According to their order of activation, caspases are classified into two groups: 1) the initiator caspases (i.e. caspase-2, -8, -9, and -10), 2) and effector caspases (i.e. caspase-3, -6, and -7) (Fischer et al., 2003; Yi et al., 2007). Once activated, caspases can often activate other procaspases, allowing initiation of a protease cascade that leads to an irreversible commitment towards cell death.
There are at least two major pathways in mammals that are involved in the initiation of apoptosis, namely the extrinsic and intrinsic pathways (Fig. 4). Initiator caspases are activated by extrinsic or intrinsic stimuli that lead to the activation of executioner caspases (Fischer et al., 2003; Yi et al., 2007). Extrinsic and intrinsic pathways differ in their induction and regulation, although there is now evidence that the two pathways are linked and intersect at different stages where molecules in one pathway can influence the other (Igney and Krammer, 2002).

1.5.3 Extrinsic pathway

The extrinsic pathway, also commonly referred to as the death receptor pathway, is initiated through the ligation of death receptors (Fas, TNF-R1) by their specific ligands (e.g., FasL, TNF) (Ghavami et al., 2009). Interaction of a death ligand with its corresponding receptor leads to activation of initiator caspase-8. Active caspase-8 can affect the mitochondria via truncated BID and causes mitochondrial initiator caspase (caspase-9) activation (Lakhani et al., 2006; Fuchs and Steller, 2011). All of these events culminate in effector caspase activation (caspase-3, -7, -6) (Ghavami et al., 2009), resulting in cleavage of different substrates like cytokeratins, poly ADP-ribose polymerase (PARP), plasma membrane cytoskeletal proteins (alpha fodrin), and subsequently provokes morphological and biochemical aspects of apoptosis.

1.5.4 Intrinsic pathway

The intrinsic (or mitochondrial) pathway can be initiated following ER stress, exposure to stresses such as cytotoxic drugs, ultraviolet radiation, and free radicals which cause DNA damage (Soengas et al., 1999; Runyan et al., 2006; Kulik et al., 2008; Ahn et al., 2011). DNA damage and ER stress activates pro-apoptotic members of the Bcl-2 family (Bax/Bak) and induces mitochondrial outer membrane permeabilization (MOMP), ultimately leading to caspase-dependent or independent apoptosis (Lorenzo et al., 1999; Ghavami et al., 2009). Anti-apoptotic Bcl-2 proteins (Bcl-2 and Bcl-xL) counteracts pro-apoptotic proteins and can delay or inhibit apoptosis (Newmeyer et al., 2000). Following MOMP, release of various polypeptides such as cytochrome c from the mitochondrial intermembrane space promotes caspase activation and apoptosis. Cytochrome c in the cytosol binds with apaf-1 (apoptotic protease-activating factor-1), inducing its oligomerization and thereby forming a structure termed the apoptosome.
Formation of the death-inducing signaling complex (DISC) and apoptosome results in activation of initiator caspases 8 and 9, respectively (Assuncao Guimaraes and Linden, 2004; Young et al., 2013). Each then activates effector caspase-3, which ultimately results in hallmark apoptotic morphological signatures such as loss of cytoplasm, blebbing of the plasma membrane, and fragmentation of DNA in the nucleus (Nagata, 2000; Assuncao Guimaraes and Linden, 2004).

1.5.5 Ceramides & other sphingolipids in apoptosis

Sphingolipids have direct effects on regulators of the extrinsic pathway of apoptosis. For instance, ceramide activates protein kinase C, which regulates the activation of c-Jun NH2-terminal kinase 1 (JNK1) and inhibition of Akt to induce apoptosis (Y M Wang et al., 1999; Bourbon et al., 2000; Bourbon et al., 2002; Todd E Fox et al., 2007). Ceramides can also directly bind and activate the lysosomal protease cathepsin D, a direct effector of apoptosis (Heinrich et al., 2004; Dumitru et al., 2009). Upon stimulation by TNF-α, increased levels of ceramide stimulate cathepsin D-mediated cleavage of BID to activate the apoptotic pathway (Heinrich et al., 2004; Dumitru et al., 2009). Sphingosine acts also as a pro-apoptotic signaling lipid via suppression of the MAPK/ERK signaling pathway (Jarvis et al., 1997). By contrast, S1P is a suppressor of ceramide-mediated activation of JNK1 by activating pro-survival Akt/mammalian target of rapamycin (mTOR) complex 1 (mTORC1), MAPK/ERK, and NF-κB signaling pathways (Cuvillier et al., 1996). Thus, regulators of the extrinsic pathway of apoptosis are affected in response to various sphingolipids (Fig. 4).

Several studies have also highlighted the regulatory roles of sphingolipids on the intrinsic pathway of apoptosis (Fig. 4). Ceramides promote the intrinsic pathway by formation of channels in the outer membrane of the mitochondria to promote the release of cytochrome c resulting in caspase-9 activation (Siskind and Colombini, 2000; Siskind et al., 2002). On the other hand, dihydroceramide inhibits ceramide channel formation (Stiban et al., 2006). In addition, the intrinsic pathway can also be stimulated by the inhibitory action of ceramide on mitochondrial complex III to generate reactive oxygen species (ROS) (García-Ruiz et al., 1997; Gudz et al., 1997). The apoptotic action of ceramide can also be mediated by the recruitment and activation of pro-apoptotic Bax at the mitochondria through the PP2A-dependent dephosphorylation of Bax and formation of mitochondrial ceramide-rich macrodomains (MCRMs) (Haefen et al., 2002;
Intriguingly, ceramides synthesized in the ER are transported to the mitochondria, where they transiently permeabilize its outer membrane and stimulate the release of cytochrome c (Stiban et al., 2008). Such exchange may limit the need for ceramide synthesis in the mitochondria to reach ceramide levels required for initiation of the intrinsic apoptotic pathway (Stiban et al., 2008). SMPD1-released ceramide is able to bind directly to lysosomal protease cathepsin D, leading to cathepsin D activation. The activated cathepsin D subsequently cleaves BH3-only protein Bid and promotes the mitochondrial apoptotic pathway (Heinrich et al., 2004). Furthermore, while ceramide-mediated activation of pro-apoptotic protein, BAD, promotes apoptosis, S1P suppresses apoptosis via BAD inactivation (Basu et al., 1998; Stoica et al., 2003; Betito and Cuvillier, 2006). Sphingosine downregulates expression of anti-apoptotic proteins Bcl-2 and Bcl-xL to enhance apoptosis (Sakakura et al., 1996; Shirahama et al., 1997).
Figure 4. Schematic representation of the apoptotic pathway and regulation by sphingolipids.

There are two main apoptotic pathways: A) extrinsic pathway and the B) intrinsic pathway. Sphingolipids have been shown to modulate apoptosis at multiple steps of the process. Reproduced and modified with permission from Lee and colleagues; Apoptosis, 2015 (Joyce Lee et al., 2015).
1.6 Autophagy

1.6.1 Programmed “self-eating”

Autophagy is a tightly regulated catabolic process that supplies energy during development and in response to nutrient stress by carrying out lysosomal degradation of cell contents (Mizushima, 2007). Despite its major role as a survival mechanism, autophagy has previously been classified as a mechanism of PCD based on morphological grounds, termed “autophagic cell death” to describe a form of caspase-independent necrosis-like cell death associated with the accumulation of autophagosomes in cells (Shimizu et al., 2004). The existence of autophagic cell death as a bona fide death process is still controversial, and the casual relationship between autophagy and cell death remains unproven (Kroemer and Levine, 2008; Shen and Codogno, 2011). Nevertheless, many studies have pointed to intimate relationships between autophagy and cellular death programs, which are not yet fully understood (Jain et al., 2013).

1.6.2 Autophagy pathway and mediators

The autophagy pathway is evolutionarily conserved from early eukaryotes to mammals with as many as 38 Autophagy Related Genes (ATG) identified in yeasts and their human orthologs (Araki et al., 2013). Autophagy is divided into three distinct forms: chaperone-mediated autophagy (CMA), microautophagy and macroautophagy (Klionsky et al., 2012). A variety of stress stimuli including long term starvation, exposure to cytotoxic compounds, or oxidative stress can lead to CMA activation which selectively degrades cytosolic proteins in lysosomes (Kaushik and Cuervo, 2012). The exact molecular mechanism that triggers microautophagy remains unknown. However, GTP hydrolysis and calcium ions are considered as major initiators of this event in yeast (Uittenweiler and Mayer, 2008). Macroautophagy (referred to here as autophagy) degrades the bulk of damaged cytoplasmic organelles and proteins. Autophagy includes mitophagy (mitochondrial autophagy), ribophagy (ribosomal autophagy), pexophagy (peroxisome autophagy), ER-phagy (endoplasmic reticulum autophagy), aggrephagy (protein aggregate autophagy) and lipophagy (fat autophagy) (Klionsky et al., 2012).

Autophagosomes are the major particles that are formed and processed during the autophagy pathway. An autophagosome includes a double-membrane vesicle destined for degradation of
proteins and organelles (cargo) which finally fuse with lysosomes to form autophagolysosomes (Hsin-Yi Chen and White, 2011). Autophagosome formation requires the expression of ATG genes which control levels of Atg proteins (Xie and Klionsky, 2007). Formation of the structures occurs via three main steps: 1) initiation (induction), 2) elongation, and 3) maturation (closure), with subsequent fusion with lysosomes to form the autolysosome or amphisome (Klionsky et al., 2012) (Fig. 5).

The mTOR constitutes a central checkpoint that suppresses autophagy via restraining the kinase activity of UNC-51-like kinase (ULK1) (Codogno and Meijer, 2005; Glick et al., 2010). mTORC1 contains the mTOR catalytic subunit (raptor/GβL/PRAS40/deptor) and phosphorylates ULK1 in the absence of amino acid and growth factor signals (Mehrpour et al., 2010). ULK1 Ser/Thr protein kinase, Atg13, and FIP200 (FIP200 is the mammalian homolog of the yeast Atg17) form the ULK1 complex (Hosokawa et al., 2009; Jung et al., 2009; Ganley et al., 2009) and regulates autophagy by phosphorylation of Atg13 and FIP200 (Edmond Y W Chan et al., 2007). Under stresses such as nutrient starvation, mTORC1 is inhibited by AMP-activated protein kinase (AMPK) resulting in the disassociation of ULK1 and Atg 13. The ULK1 complex is then phosphorylated by AMPK and then initiates vesicle nucleation (Ganley et al., 2009).

Beclin-1 (BECN1) is a platform protein and its complex with class III phosphatidylinositol 3-kinase (PI3K) has a key regulatory role in nucleation and assembly of the initial phagophore membrane (Zhong, Wang, Li, et al., 2009; Zhong, Wang, and Yue, 2009). BECN1 associates with Vps15, Vps34, and Ambra1 to form a complex that regulates class III PI3K, which forms phosphatidylinositol-3-phosphate (PI3P). Furthermore, BECN1 complex can also trigger autophagy via JNK1, and death-associated protein kinase (DAPK) (Pattingre et al., 2008). PI3P is needed for recruitment of other Atg proteins as well as formation of the Ω-shape of initial vesicle nucleation which can be sourced from the outer mitochondrial membrane, ER and/or the plasma membrane (Zhifen Yang and Klionsky, 2010; Kroemer et al., 2010). Elongation of the vesicle requires two ubiquitin-like conjugation systems. The first involves the formation of the Atg12-Atg5 conjugate, which appears to act as the E3 ligase of microtubule-associated protein 1 light chain 3 (LC3). The second system initiates autophagosome formation and involves the conjugation of LC3 to the polar head of phosphatidylethanolamine (PE) by release of Atg8 from
Atg3 (Klionsky et al., 2012). This conjugation leads to the conversion of the soluble LC3 (LC3-I) to its lipidated form, LC3-II, which is incorporated into the autophagosome membrane (Takao Hanada et al., 2007; Kroemer et al., 2010). Atg9 has also been shown to be recruited by the Atg1-Atg13 signaling complex and has an important role in expansion of the autophagosome precursor (Orsi et al., 2012). The conversion of LC3 from LC3-I to LC3-II is regarded as a critical step in autophagosome formation (Kabeya et al., 2000) and also represents a hallmark for detecting autophagy (Kabeya et al., 2000; Klionsky et al., 2012). Following elongation, expansion, and closure, the autophagosome with cytoplasmic material finally fuses with the lysosome forming an autophagolysosome, and its contents are subsequently digested by lysosomal enzymes (Klionsky et al., 2012).

When the autophagosome proceeds to fuse with a lysosome, its vesicular contents are degraded into macromolecules and are recycled back to the cytosol (Mizushima, 2007; Zhifen Yang and Klionsky, 2010; Young et al., 2013). This process is conserved in all eukaryotic cells and serves to maintain homeostasis under normal conditions to prevent accumulation of excess/damaged organelles and proteins. However, under conditions of stress such as nutrient starvation, oxidative stress, pathogen infection or hypoxia, autophagy serves as an adaptive cell survival response that could also lead to cell death in situations of defective or excessive autophagy (Codogno and Meijer, 2005; Zhifen Yang and Klionsky, 2010).

1.6.3 Ceramides in autophagy

Ceramide has been demonstrated to induce autophagy by various mechanisms. Because of the paradoxical roles of autophagy, ceramide-dependent autophagy could either promote cell death (by inducing autophagy cell death, or “switching” the cell from autophagy to apoptosis) (Daido et al., 2004; Pattingre et al., 2009; Lépine et al., 2011; Young et al., 2012) or under certain conditions may induce cytoprotective autophagy (Park et al., 2008; Guenther et al., 2008). Recently, it has been demonstrated that stimulation of de novo ceramide synthesis results in dissociation of the complex formed between BECN1 and Bcl-2 (anti-apoptotic protein) through stimulating the phosphorylation of Bcl-2 by JNK1 leading to autophagy activation (Pattingre et al., 2009). Likewise, inhibition of ceramide synthesis resulted in suppression of autophagy (Pattingre et al., 2009). Ceramide suppresses the Akt signaling pathway, resulting in autophagy
activation via negative regulation of mTOR signaling (Scarlatti et al., 2004), suggesting an upstream regulatory target of the autophagy pathway. Furthermore, ceramide-induced autophagy, either by targeting the mitochondria or by upregulation of BECN1, has been reported to be linked to autophagic cell death (Scarlati et al., 2004; Daido et al., 2004; Li et al., 2009). The role of ceramide-induced autophagy in the context of the ‘sphingolipid rheostat’ has yet to be fully understood and further investigation is required to determine the mechanisms involved in determination of cell fate when autophagy is activated.
Figure 5. A schematic overview of autophagy machinery and its regulation by sphingolipids.

Autophagy induction, membrane nucleation, phagophore formation and closure, and autophagosome maturation hallmark critical steps of the autophagy pathway. Sphingolipids have direct effects on various stages of autophagy. Reproduced and modified with permission from Lee and colleagues; Apoptosis, 2015 (Joyce Lee et al., 2015).
1.7 Rationale, Hypothesis, and Research Aims

In the preceding text, I have outlined two cellular mechanisms, apoptosis and autophagy, with particular emphasis upon their respective signaling pathways. It is evident that although the two pathways are highly interconnected, they are functionally distinct and play different roles in both homeostatic and pathophysiological systems within the lung. Apoptosis is an essential physiological process that is involved in development, aging, and tissue homeostasis, while the process can also serve as the crux of several mentioned disease states. Autophagy is a tightly regulated process that supplies energy during development and in response to nutrient stress, but can also lead to cell death upon failed cell survival.

I have also highlighted one of the most common chronic lung diseases that affect neonates: BPD. BPD is characterized by arrested lung development, with larger and fewer alveoli and abnormal vasculature, ultimately leading to lifelong reduction in respiratory potential. Treatment options are empirical, and the disorder presents a considerable demand on healthcare systems. It remains critical to better understand the molecular mechanisms involved in the effects of VILI to identify potential therapeutic targets.

Recent investigation into sphingolipid signaling and the activation of cell fate pathways (Tibboel, Joza, et al., 2013; Kroon et al., 2013) have identified potential candidates for the development of therapies that can treat or prevent BPD. Moreover, the potential for sphingolipids to act as a regulator of autophagy and apoptosis in response to mechanical ventilation remains an unexplored territory.
Hypothesis

I hypothesize that injurious mechanical ventilation (stretch) triggers stretch-induced lung epithelial cell death by autophagy and apoptosis via an increase in ceramide production, thereby affecting lung development in ventilation-induced lung injury.

Research Aims

To test this hypothesis, I have defined 2 related objectives that will be further addressed:

1. To determine whether mechanical ventilation (stretch) alters lung epithelial cell fate by inducing autophagy and/or apoptosis.

2. To determine the role of ceramides in mediating ventilation (stretch)-induced lung epithelial cell death.
Chapter 2
Materials and Methods

2 Materials and Methods

2.1 Materials

Female timed-pregnant Wistar rats were obtained from Charles River (St. Constant, QC) and were housed in Lab Animal Services at the Hospital for Sick Children. The following chemicals and reagents were used in preparation of this thesis: Phosphate Buffered Saline (PBS; 14190), Hank’s Buffered Salt Solution (HBSS; 14175), Dulbecco’s Modified Eagle Medium (DMEM; 11095), gentamycin reagent solution (Gentamycin; 15750), and 4', 6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI; D1306) were from Invitrogen (Waltham, MA). TrypLE Express Enzyme phenol red (TrypLE; 12605) and Fetal Bovine Serum (FBS; Life Technologies, 12483) were purchased from Life Technologies (Carlsbad, CA). Deoxyribonucleic I (DNase; LS002006) and collagenase type I (Collagenase; LS004196) was from Worthington (Lakewood, NJ). Complete protease inhibitor cocktail tablets (Pl; 04 693 159001) were from Roche (Basel, Switzerland). Bovine Serum Albumin (BSA; A8806) and goat serum (G9023) were from Sigma-Aldrich (St. Louis, MO). Fluorescence mounting medium (Mounting medium; S3023) was from Dako (Santa Clara, CA). A list of chemical inhibitors used, concentrations, and suppliers are provided in Table 2. A list of antibodies used, applications, respective concentrations, and suppliers are provided in Table 3.
Table 2. Chemical inhibitors used to study autophagy, apoptosis, and ceramide metabolism

<table>
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<th>Supplier</th>
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WB: Western blot, IF: Immunofluorescent staining, IHC: Immunohistochemistry
2.2 Primary Cell Culture: Isolation and separation of fetal lung epithelial cells

Time pregnant Wistar rats were euthanized by an excess of carbon dioxide on day 19 of gestation (term=22 days). Fetal rat lungs from three litters were used for each distal epithelial cell isolation procedure. The lungs were dissected and excess adherent tissues were removed. The lungs were rinsed twice in cold Hanks’ balanced salt solution (HBSS) and transferred to a glass petridish. The lungs were minced into very fine pieces, washed twice with HBSS, and centrifuged at 100 xg for one minute. The lung tissue was trypsinized with 0.125% Trypsin (GIBCO), 0.002% DNAse (Worthington) in HBSS, in a trypsinizing flask under constant stirring for 20 minutes in a 37°C water bath. After trypsinization, the cells were neutralized in Minimum Essential Medium (MEM) + 5% (v/v) fetal bovine serum (FBS) (with 50 µg/mL gentamycin) and then centrifuged in a 50 mL centrifuge tube at 450 xg for 5 minutes. The cell pellet was resuspended in 30 mL of MEM + 0.1% (w/v) collagenase and incubated for 15 minutes in a 37°C water bath. An equal amount of MEM + 5% FBS was added to the cell suspension and centrifuged at 450 xg for 5 minutes. The cell pellet was resuspended in MEM + 5% FBS and seeded into 75-cm² tissue culture flasks (15 mL per flask) and incubated for one hr in 5% CO₂ incubator at 37°C to allow fibroblast cells to adhere. After this incubation period, the floating cells from the flasks were transferred to a 50 mL tube and the flasks were washed with MEM to collect all floating cells. The cells in the 75-cm² tissue culture flasks were discarded. The collected cells were centrifuged at 450 xg for 5 minutes, and then reseeded into a second set of 75-cm² tissue culture flasks (15 mL per flask) and incubated for one hr in 5% CO₂ incubator at 37°C to allow the remaining fibroblast cells to adhere. The floating cells were collected as before and centrifuged at 450 xg for 5 minutes. The cells in the 75-cm² tissue culture flasks were discarded. The collected cells were centrifuged in 35 mL MEM and centrifuged 4 times in 200 xg for 3 minutes. After the final centrifugation, the cell pellet was resuspended in MEM + 5% FBS and seeded into 75-cm² tissue culture flasks (approximately 10 mL per flask) and placed in the 5% CO₂ incubator at 37°C to allow the epithelial cells to adhere overnight. After 24 hrs of isolation, the FRLE cells were trypsinized and seeded onto 6-well collagen type-I coated Bioflex plate (~7.5x10⁵ cells•well⁻¹) in 2 mL of MEM + 5% (v/v) FBS. The cells were incubated overnight, and then changed to fresh MEM + 5% (v/v) FBS for another 24 hrs. The cells were then changed to MEM
+ 0.5% (v/v) FBS for 1-2 hrs and then changed again to fresh MEM + 0.5% (v/v) FBS right before they were subjected to mechanical stretch or static culture treatment.

2.3 Mechanical stretch of FRLE cells

The Flexercell Strain Unit, FX-4000 (Flexercell Int., NC) was used to apply mechanical strain to the primary rat lung epithelial cells. The computer controlled unit mimics mechanical strain by applying equibiaxial strain across a flexible, matrix-coated membrane on which cells are cultured. A vacuum pressure is applied, which pulls the flexible membrane over a stationary post subjecting the cells to a specific elongation. The cells were stretched at various intervals using:

- Continuous cyclic high stretch – 30 cycles•minute⁻¹; 20% elongation

Continuous cyclic high stretch elongations of 20% were applied at intervals of 30 cycles per minute. Control cells were also grown on collagen-I coated Bioflex 6-well plates and were treated in the same manner, but remained static. After a time interval, medium from each well was collected and stored in an eppendorf tube at -80°C for future ceramide analyses. Cell lysates were also collected for RNA, protein, or ceramide analyses.

2.4 Mechanical ventilation of newborn rat lung

Dr. Andreas Kroon ventilated 7-day old rat pups (FlexiVent; Scireq, Quebec) with room air and moderate tidal volume [inspired tidal volume (VT): 7–9 ml/kg; respiratory rate: 150 min⁻¹; positive end-expiratory pressure: 2 cmH₂O] for 12 and 24 hrs as described in his published work (Kroon et al., 2010; Kroon et al., 2011; Kroon et al., 2013). Lung tissue was processed for histology or was fresh frozen, and was used for this study.

2.5 Protein extraction and Western blotting

The cells were washed on ice with ice cold HBSS, scraped, and centrifuged at 200 xg for 5 minutes at 4°C. The cell pellet was resuspended in RIPA lysis buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton-X-100 (v/v), 1% sodium deoxycholate (w/v) and 0.1% SDS (w/v)] containing protease inhibitor (1mM PMSF, 2 µg•mL⁻¹ leupeptin, 100 mM sodium orthovanadate, 1 µg•mL⁻¹ pepstatin A and 2 µg•mL⁻¹ aprotinin), and homogenized by a dounce homogenizer. After homogenization, the cell suspension was incubated for 30 minutes on ice followed by
centrifugation at 15,000 xg for 15 minutes at 4°C. The supernatant was collected and a Bradford assay was conducted to determine protein concentration (Bradford, 1976).

After determination of protein concentration for each sample, 30 µg of total protein was diluted in NuPAGE LDS sample buffer (4X) (Life Technologies, Carlsbad, CA) and boiled at 70°C for 10 minutes. The proteins were then subjected to 15% SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA), and blocked with 5% (w/v) non-fat milk (Bio-Rad, Hercules, CA) in Tris-buffered saline with 0.1% (v/v) Tween 20 (TBST) for 1 hr at room temperature. The antibodies used were diluted in blocking solution, added to the membrane, and incubated overnight at 4°C under gentle agitation. Primary antibodies and respective dilutions used are outlined in Table 3. After overnight incubation the membrane was washed three times for 15 minutes each wash with TBST and then incubated for 1 hr at room temperature (gentle agitation) with horseradish peroxidase conjugated goat anti-rabbit or -mouse IgG diluted 1:10,000 in blocking solution. Chemiluminescence solution (Amersham International, England) was applied to the membranes and protein bands were detected and visualized on Clinicselect Blue x-ray film (Carestream, Rochester, NY). Films were digitally scanned and bands were analyzed for optical density using ImageJ (NIH, USA). Densities of protein bands were measured and expressed as a ratio of protein of interest over the loading control (β-actin).

2.6 Exogenous ceramide treatment

After isolation, the FRLE cells were trypsinized and seeded onto plastic 6-well plates (~7.5x10⁵ cells•well⁻¹) in 2 mL of MEM + 5% (v/v) FBS. The cells were incubated overnight, and then changed to fresh MEM + 5% (v/v) FBS for another 24 hrs. The cells were then rinsed twice with MEM + 0.1% (v/v) and changed again to fresh MEM + 0.1% (v/v) FBS right before they were treated with exogenous ceramide 16:0 (C16 Ceramide d18:1/16:0) N-palmitoyl-D-erythro-sphingosine (Avanti Polar Lipids, Alabaster)). Ceramide 16:0 was diluted in MEM + 0.1% (v/v) FBS at 25 µM. The vehicle was 100% ethanol diluted in MEM + 0.1% (v/v) FBS at 25 µM. The primary lung epithelial cells were exposed to ceramide 16:0 for 4, 8, or 12 hrs and cell lysates were collected for protein analyses. The vehicle control was collected at 12 hrs of incubation.
2.7 Primary epithelial cell transfection

Freshly isolated day-19 fetal rat lung epithelial cells were rinsed twice with warm PBS (37°C) and dissociated using TrypLE Express (Invitrogen, Carlsbad) for 20 min. Floating cells were neutralized with MEM + 5% (v/v) FBS. The cells were transferred to a 15 mL conical tube and centrifuged at 450 xg for 5 minutes at room temperature. Cells were washed with warm PBS twice by centrifugation at 450 xg for 5 minutes at room temperature. The PBS was aspirated and the cell pellet was resuspended in the provided Resuspension Buffer R at a density of 1.0x10^7 cells/mL. A Neon™ Tube was prepared with 3 mL of provided Electrolytic Buffer and placed into the Neon™ Pipette Station. In a sterile, 1.5 mL microcentrifuge tube, 2 µL of one of three unique 27mer siRNA duplexes corresponding to rat Atg7 or a trilencer-27 universal scrambled negative control siRNA duplex from OriGene Technologies Inc., (Rockville, Maryland), along with 5x10^5 cells were mixed gently. The Neon™ Tip and Neon™ Pipette were used to collect the mixture and apply the electroporation protocol (3 pulses, 1200 V, 20 msec) using the Neon™ Transfection System (Invitrogen). Transfected cells were plated in a well on a 6-well plate and incubated in MEM + 5% (v/v) FBS for 24 hrs before further experimentation.
2.8 Pharmacologic inhibitors

2.8.1 PI3K and autophagic flux inhibition

FRLE cells were gently washed twice with 37°C HBSS and treated with MEM + 0.5% (v/v) containing either DMSO (vehicle control), an autophagic flux inhibitor: 10 nM Bafilomycin A1 (Baf A1; Sigma), or a phosphatidylinositol 3-kinase inhibitor: 5-10 mM 3-Methyladenine (3MA; Sigma), 5-10 µM KU55933 (Tocris Biosciences) or 5-10 µM Gö6976 (Tocris Biosciences). After a 1 hr pre-treatment, the medium was removed and fresh media containing the respective components was added (1.5 mL/well), and the cells were subject to further experimentation.

2.8.2 Pan-caspase inhibition

FRLE cells were rinsed twice with 37°C HBSS and pretreated (1 hr) with MEM + 0.5% (v/v) containing either DMSO (vehicle control) or 20 µM pan caspase inhibitor benzylcarbonyl-Val-Ala-Asp-fluromethylketone (z-VAD-FMK; R&D Systems Minneapolis, MN). After pre-treatment, the media was removed and replaced with fresh media containing the respective components, and cells were subjected to further experimentation.

2.8.3 Ceramide metabolic pathway inhibition

FRLE cells were washed twice with 37°C HBSS and cells were pretreated (2 hr) with MEM + 0.5% (v/v) containing either DMSO (vehicle control) or an inhibitor of ceramide generation: 5-20 µM myriocin (Sigma-Aldrich, M1177), 25-50 µM imipramine hydrochloride (imipramine; Sigma-Aldrich, 17379), 1-10 µM fluoxetine hydrochloride (fluoxetine; Sigma-Aldrich, F132), or GW 4869 (Cayman Chemical, 13127). The media was replaced with a fresh solution, and cells were subject to further experimentation.
2.9 Immunofluorescence microscopy

After various stretch intervals, the cells on collagen-I coated Bioflex membranes were rinsed twice with ice cold sterile PBS and then incubated with 4% paraformaldehyde (PFA) on ice for 10 minutes. The cells were then washed twice with ice cold sterile PBS and the membrane was removed from the well using a sterile blade. The membrane was cut into smaller pieces and stored in sterile PBS at 4°C. A piece of the membrane was then blocked with 5% normal goat serum and 1% BSA in PBS at room temperature for 1 hr and then rinsed three times with PBS. Samples were then incubated overnight with anti-LC3B (Cell Signaling Technology, Danvers, MA) and anti-E-cadherin (BDBiosciences, San Jose, CA) antibodies at 4°C in a dark humidified chamber. The membranes were then washed three times for 5 minutes each with PBS and incubated for 1 hr at room temperature with goat anti-rabbit IgG (H+L) secondary antibody Alexa Fluor 546 conjugate (Life Technologies, A11035) or goat anti-mouse IgG (H+L) secondary antibody Alexa Fluor 488 conjugate (Life Technologies, A11034), respectively, for 1 hr at room temperature. The membrane was washed three times for 5 minutes each with PBS and incubated with DAPI (Invitrogen, Carlsbad, CA) for 10 minutes. The membranes were then mounted using fluorescence mounting medium (Dako, Markham), onto slides and allowed to dry overnight. Once dried, the perimeter of the membrane was sealed with clear nail varnish to prevent any degradation of the antibodies. The proteins and nuclei were visualized with a Leica CTRMIC 6000 confocal microscope and Hamamatsu C910013 spinning disc camera (Leica Microsystems Inc.), and analyzed with Volocity software (PerkinElmer).

2.10 Transmission electron microscopy

Following stretch intervals, FRLE cells on collagen-I coated Bioflex membranes were rinsed twice with ice cold sterile PBS and the membrane was removed from the well using a sterile blade. The membrane was then fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and stored at 4°C. The membrane was post-fixed in osmium tetroxide, dehydrated in an ascending series of acetone, infiltrated, embedded in Epon Araldite, and subject to polymerization at 60°C overnight. Ultrathin sections were prepared and mounted on grids,
stained with uranyl acetate and lead citrate, then imaged using a JEOL JSM 6700F transmission electron microscope (JEOL, Peabody, Massachusetts) and a CCD camera (AMT corp.).

2.11 Mass spectral analysis of ceramides

**Extraction procedure:** Cells were washed on ice with HBSS, scraped, and centrifuged at 200 xg for 5 minutes at 4°C. The cell pellet was resuspended in ice cold HBSS and kept at -80°C until extraction. Cells were transferred to siliconized glass tubes and homogenized in 2 mL of methanol/water (1:1). A mixture of internal standards (C17 (d18:1/17:0) ceramide, 17:0 (d18:1/17:0) sphingomyelin; 10 ng of each) and 2 mL of chloroform were added to the samples. The samples were vortexed for 1 minute, kept still on ice for 10 minutes, and centrifuged at 1000 xg for 5 minutes. The chloroform layer was extracted and the sample was dried under a stream of nitrogen. Samples were resuspended in 1 ml of ethanol.

**Mass spectrometry:** Liquid chromatography tandem mass spectrometry was performed by an Agilent 1200 Series binary pump (Agilent Technologies Inc., Santa Clara, CA, USA) coupled to an API4000 triple-quadrupole mass spectrometer (SCIEX, Concord, ON, Canada). Reverse phase high performance liquid chromatography (HPLC) was performed using a Kinetex C18 column (Phenomenex, Torrance, CA, USA). Multiple Reaction Monitoring (MRM) mass transition parameters were optimized using a combination of pure standards (5 µL/min of 1 µg/mL), and the sample injection volume was 1-5 µL. The mobile phase consisted of (A) water/acetonitrile/methanol (2/1/1, v/v/v) and (B) tetrahydrofuran/acetonitrile/methanol (2/1/1, v/v/v). Both components contained 0.05% formic acid. The HPLC gradient flow was 400 µL/min and was as follows: a 60:40 (A:B) initial condition was held for 4.5 min before injection, held for 2 min after injection, and shifted to 15:85 (A:B) over a 13 min period. Conditions were held for 15 min and returned to initial conditions for 17 min. MS analysis was performed in the positive electrospray ionization mode. Nitrogen was used as the Collision Induced Dissociation gas, the ion spray voltage was set at 5,000 V, and the source temperature was maintained at 400°C. MRM Mass Transitions and Chromatographic Retention Times parameters follow those that have been published previously (Tibboel, Joza, et al., 2013). A separate standard curve was generated for each analyte measured using MRM area ratios for quantitative analysis (Analyte Peak Area/IS Peak Area) and the results were then calculated by plotting the sample area ratios
against their respective analyte specific standard curve (Bielawski et al., 2006; Yoo et al., 2006). Data integration and quantitation was performed using ABSciex Analyst 1.6 software.

2.12 Flow cytometry

FRLE cells subjected to mechanical stretch or static culture were washed twice with 4°C PBS and dissociated using 0.5 mL/well TrypLE express (Invitrogen) for 15 minutes at 37°C. Floating cells were pelleted by centrifugation at 450 xg for 5 minutes, and washed twice with 4°C PBS. The cells were resuspended in 1X Binding Buffer, provided in the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, San Jose, CA) at a concentration of 1x10^6 cells/mL and 100 µL aliquots were allocated to 5 mL culture tubes. To each tube, 5 µL of FITC Annexin V and/or Propidium Iodide (PI) were added, gently mixed, and incubated for 15 minutes at room temperature (25°C) in the dark. Following incubation, each tube was washed twice with 3 mL of 1X Binding Buffer, centrifuged at 450 xg for 5 minutes. The final cell pellet was resuspended in 500 µL of 1X Binding Buffer and cell data was acquired by the Beckman Coulter Gallios 10/3 flow cytometer and analyzed using the Kaluza flow cytometry software (Beckman Coulter, Brea, CA). The FL-1 channel, 525 nm emission max, measured Annexin V positive events. The FL-4 channel, 670 nm emission max, measured PI positive events.

The following controls were used to set up compensation and quadrants:

1. Unstained cells
2. Cells stained with FITC Annexin V (no PI)
3. Cells stained with PI (no FITC Annexin V)
4. Cells treated with staurosporine (1 mM, Sigma-Aldrich) served as a positive control
2.13 Acid sphingomyelinase activity assay

SMPD1 activity was measured *in vitro* using an acid sphingomyelinase assay kit (Echelon Biosciences In., Salt Lake City, UT) following manufacturer’s recommendations. FRLE cells subjected to stretch were washed twice with HBSS and collected by a scraper on ice. Cellular debris was removed by centrifugation at 15,000 xg for 15 minutes and resuspended in 50 uL of 1 mM PMSO H₂O, and stored at -80°C until used. Protein quantification was determined by a Bradford assay, and 25 µg of protein was used for each sample in duplicate. The substrate buffer (150 uL) was added to the SMPD1 standard to make the 10 U/mL standard that was used to generate a standard curve of SMPD1 activity (Table 4). Diluted standards or samples (20 uL) were loaded into the wells of the provided 96-well plate. Then, 30 uL of Substrate Buffer was added to each well and the plate was mixed for 5 minutes. The SMPD1 Substrate was thawed and diluted (150 uL SMPD1 Substrate: 6 mL Substrate Buffer). Then 50 uL of diluted substrate was added to each well, and the plate was incubated for 3 hrs with shaking at 37°C in the dark. Following incubation, 50 uL Stop Buffer was added to each well and incubated for an additional 10 minutes at room temperature with shaking. The plate was read at 360 nm excitation and 460 nm emission using a fluorescence microplate reader (POLARstar Omega, BMG Labtech, Offenburg, Germany).
Table 4. Standard dilution series for SMPD1 activity assay

<table>
<thead>
<tr>
<th>SMPD1 Standard Points</th>
<th>uL of SMPD1 Standard or uL of previous dilution</th>
<th>uL Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Units/mL</td>
<td>ρmol/hr</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>200</td>
<td>SMPD1 Standard</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>50 uL of previous dilution solution</td>
</tr>
<tr>
<td>2.5</td>
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</tr>
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<td>1.25</td>
<td>25</td>
<td>50 uL of previous dilution solution</td>
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</tr>
<tr>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>
2.14 Data presentation and statistical analysis

All values are expressed as the mean ± standard error of the mean (SEM) of at least three independent culture experiments. Statistical significance was defined as $P < 0.05$, and was determined using GraphPad Prism Version 6.0 software (GraphPad Software Inc.). A Mann-Whitney test was used to compare two groups. The Kruskal-Wallis test was performed to compare three or more groups, followed in case of significance by multiple pair-wise comparisons using the post-hoc Dunn’s test.
3  Results

3.1  Aim 1: Determine if mechanical ventilation (stretch) alters lung epithelial cell fate by inducing autophagy and/or apoptosis

3.1.1  Mechanical ventilation of newborn rat lung induces apoptosis

It has previously been shown that mechanical ventilation induces apoptotic cell death in alveolar type II cells of newborn rats via the Fas/FasL system (Kroon et al., 2013). I sought to further investigate the mechanism of mechanical ventilation-induced cell death in the newborn rat lung. First, I confirmed that prolonged (24 hr) mechanical ventilation with moderate tidal volume (mVT) significantly increased amounts of cleaved caspase-3 (CASP3) and cleaved PARP protein in lungs of 7-day old rat pups (n=3) vs. non-ventilated 8-day rat pups (n=3) (Fig. 6 A-C). No significant differences in cleaved PARP and cleaved CASP3 protein were noted after 12 hrs of mechanical ventilation (Fig. 7 A-C).
Figure 6. Prolonged mechanical ventilation of newborn rat lung induces pulmonary apoptosis.

(A) Representative immunoblots of cleaved-CASP3 and cleaved-PARP proteins in tissue lysates of day-7 newborn rat lung subjected to 24 hrs mechanical ventilation (MV) with moderate tidal volume (mVT) of 7-9ml/kg (ventilated; n=3 separate newborn pups) compared with non-ventilated day-8 newborn rat lung control (control; n=3 separate newborn pups). Densitometric analysis of (B) cleaved-CASP3 and (C) cleaved-PARP protein in ventilated vs. control rat lung. Protein levels were normalized to β-actin. *P<0.05
Figure 7. Mechanical ventilation-induced cell death does not occur after 12 hrs.

(A) Representative immunoblot of day 7 newborn rat lungs subject to prolonged ventilation (moderate V_T of 7-9 ml/kg) for 12 hrs. (B) Cleaved CASP3 and (C) cleaved PARP protein content in ventilated (n=4) vs. control rat lung (n=4). Protein levels were normalized to β-actin. *P<0.05
3.1.2 Mechanical ventilation of newborn rat lung induces autophagy

Emerging evidence suggests an overlap between the autophagy and apoptotic cell death pathways (Codogno and Meijer, 2005; Kim, Xue Wang, Chen, Lee, Huang, Yong Wang, Ryter, and Choi, 2008b; Zhifen Yang and Klionsky, 2010; Cervia et al., 2013). To investigate whether autophagy is involved in the response to mechanical stretch in newborn rat lung, I measured the expression of LC3B-II and ATG5-12 complex proteins. Immunoblotting revealed a significant increase in the expression of ATG5-12 and LC3B-II proteins in lungs of D7 newborn rats ventilated for 12 hrs compared to non-ventilated controls (Fig. 8A-C). Immunohistochemistry (IHC) analysis using an anti-LC3B antibody corroborated the immunoblot findings (Fig. 8D-E). Interestingly, immunoblotting revealed no significant differences in LC3B-II levels after 24 hrs of mVT ventilation (Fig. 9 A, B), suggesting that MV-induced autophagy precedes apoptotic cell death.
Figure 8. Mechanical ventilation of newborn rat induces autophagy before apoptosis.

(A) Representative immunoblots for ATG5-12 and LC3B-II proteins in tissue lysates of day-7 newborn rat lung subjected to 12 hrs MV with mVT of 7-9 ml/kg (ventilated; n=4 separate newborn pups) compared with non-ventilated day-7.5 newborn rat lung control (control; n=4 separate newborn pups). Densitometric analysis of (B) ATG5-12 and (C) LC3B-II proteins in ventilated vs. control rat lung. Protein levels were normalized to β-actin. (D) Representative histological images of day-7 newborn rat lung stained for LC3B protein (brown colour, depicted by arrows) after 12 hrs of ventilation compared to non-ventilated day 7.5 rat lung control. (E) Quantitative analysis of LC3B staining signal in 12 hr ventilated rat lung (n=3 separate newborn pups) vs. control lung (n=3 separate newborn pups). Bar represents: 25 µm. *P< 0.05
Figure 9. Mechanical ventilation-induced autophagy precedes apoptotic cell death.

(A) Representative immunoblot of day 7 newborn rat lungs subject to prolonged ventilation (mVT of 7-9 ml/kg) for 24 hrs. (B) Immunoblot analysis of LC3B-II protein in ventilated (n=3) vs. control rat lung (n=3). Protein levels were normalized to β-actin. *P<0.05
3.1.3 Cyclic stretch of FRLE cells results in autophagy and apoptotic cell death

Using FRLE cells as an in vitro preterm lung model, Kroon and colleagues previously demonstrated that apoptotic cell death occurs in lung epithelial cells when subjected to mechanical stretch (Kroon et al., 2013). Day 19 of gestation in the embryonic rat lung represents the late canalicular/early saccular stage of lung development that is hallmarked by the formation of canalicular branches from the terminal bronchi, as well as the invasion of capillaries into the surrounding mesenchyme. This stage of lung development correlates with the human lung at 23-27 weeks gestation at which preterm infants are at greatest risk of developing ventilation-induced lung injury (A J Jobe, 1999). To further investigate the underlying mechanism that resulted in ventilation (stretch)-induced apoptotic cell death, I subjected FRLE cells to continuous 20% cyclic stretch (mimics high VT ventilation) for up to 12 hrs. I observed increased protein levels of cleaved CASP3 and cleaved PARP after 8 hrs of cyclic stretch (Fig. 10 A-C). In line with these findings, flow cytometry consistently showed a greater proportion of FRLE cells staining positive for Annexin V (with or without PI staining) after 8 hrs of stretch compared to static control cells (Fig. 10 D). Immunofluorescent analysis with E-cadherin positive FRLE cells confirmed loss of integrity as a result of mechanical stretch (results not shown). In order to analyze whether in vivo ventilation-induced autophagy could be recapitulated in an in vitro setting, I measured LC3B-II levels in cell lysate from FRLE cells exposed to various times (0, 4, 8, 12 hrs) of continuous 20% cyclic stretch. No changes in LC3B-II protein levels were noted when cells were analyzed at 4-hr intervals (Fig. 11 A,B). I then speculated that autophagy may be activated as an earlier response to mechanical stretch and, therefore, subjected FRLE cells to stretch and measured autophagy proteins (LC3B-II, ATG5-12 complex, and ATG7) at 15 minutes intervals (Fig. 12 A). A significant increase in LC3B-II, ATG5-12, and ATG7 proteins were observed within the first hr of stretch, consistently reaching greatest levels after 45 minutes (Fig. 12 B-D), suggesting stretch-induced autophagy is rapid. In agreement with these findings, immunofluorescent analysis showed an increase in LC3B puncta (red-orange) in the E-cadherin positive FRLE cells after 60 minutes exposure to cyclic stretch (Fig. 12 E). Transmission electron microscopy (TEM), the current golden standard for autophagy detection, confirmed the presence of autophagosomes in 45-min stretched FRLE cells compared to static controls (Fig. 12 F). No changes in LC3B-II and cleaved CASP3 protein levels were observed when FRLE cells
were subject to 10- and 15% cyclic stretch to suggest activation of autophagy and apoptosis is amplitude-dependent (Fig. 13).
Figure 10. Cyclic stretch induces apoptosis in FRLE cells.

(A) Representative immunoblots for cleaved-CASP3 and cleaved-PARP proteins in lysates from FRLE cells subjected to 20% cyclic stretch for 0, 4, 8 and 12 hrs (n=3 independent cell culture isolations). Densitometric analysis of (B) cleaved-CASP3 and (C) cleaved-PARP protein in FRLE cells during 4-hr intervals of stretch (n=3 independent cell culture isolations). Protein levels were normalized to β-actin. (D) Representative flow cytometry of stretched (8 hr) FRLE cells stained for Annexin V and propidium iodide (PI) (similar results were obtained with cells of 3 separate isolations). *P< 0.05
Figure 11. Stretch-induced autophagy is rapid and transient.

(A) Representative immunoblot of FRLE cells stretched for 12 hrs, observed at 4-hr intervals for LC3B protein. (B) Immunoblot analysis for LC3B-II protein in stretched FRLE cells (n=3). Protein levels were normalized to β-actin. *P<0.05
E-Cadherin, LC3B, DAPI

F  Static control  45 min Stretch
Figure 12. Stretch induces rapid autophagy in FRLE cells.

(A) Representative immunoblots for ATG7, ATG5-12 and LC3B-II proteins in lysates of FRLE cells subject to 20% cyclic stretch for 0, 15, 30, 45 and 60 minutes (n=3 independent cell culture isolations). Densitometric analysis of (B) ATG7, (C) ATG5-12, and (D) LC3B-II protein in FRLE cells after 15-minute intervals of stretch (n=3 independent cell culture isolations). Protein levels were normalized to β-actin. (E) Representative immunofluorescent images of FRLE cells stained for LC3 puncta (red-orange), E-cadherin (epithelial marker, green), and DAPI (nuclear marker, blue), when stretched for 60 minutes. Bar represents 20 µm. (F) Representative TEM images of a stretched (45 minutes) FRLE cell (right) with autophagosomes (arrow), compared to a static control cell (left). White bar represents 500 nm. *P<0.05
Figure 13. Activation of stretch-induced autophagy and apoptosis is amplitude dependent.

Immunoblots for (A) LC3B-II and (B) Cl. CASP 3 protein in lysates of FRLE cells subjected to 45 min and 8 hr stretch intervals at varying amplitudes of stretch (10%, 15%, 20%), and static culture. Protein levels were normalized to ACTB.
3.1.4 Mechanical stretch-induced autophagy mediates apoptosis

Previous studies have demonstrated that autophagy may lead to cell death in situations of defective or excessive autophagy (Codogno and Meijer, 2005; Zhifen Yang and Klionsky, 2010). Therefore, I explored the significance of autophagy in ventilation (stretch)-induced apoptosis. To inhibit autophagosome formation, FRLE cells were pretreated for 60 min with PI3K inhibitors, 3-methyladenine (3MA), KU5593, or Gö6976 (Sheng et al., 2014; Yanyang Wu et al., 2014; Bago et al., 2014), prior to stretching for 45 min (Fig. 14). Interestingly, when cells were treated with any of the aforementioned inhibitors, I observed a significant decrease in cleaved CASP3 protein levels when cells were stretched for 8 hrs compared to untreated stretched cells (Fig. 15A and B).

I also transfected FRLE cells with siRNAs targeting the rat Atg7 gene. Three siRNA duplexes were tested and all exhibited effective inhibitory effects (Fig. 16). The siRNA-C was used in subsequent knockdown assays. FRLE cells were transfected with Atg7 or scrambled siRNA and subjected to cyclic stretch for 45 min or 8 hrs (Fig. 15C). As anticipated, knockdown of Atg7 significantly decreased the amount of LC3B-II protein when FRLE cells were stretched for 45 minutes compared to scrambled siRNA controls (Fig. 15D). In accordance with the PI3K inhibition results, immunoblot analysis also revealed a significant decrease in the amount of cleaved CASP3 protein when Atg7 siRNA transfected cells were stretched for 8 hrs compared to stretched cells transfected with scramble siRNA (Fig. 15E).

To further explore the link between stretch-induced autophagy and apoptosis, I treated FRLE cells with Baf A1, an inhibitor of the vacuolar (V)-type ATPase that blocks autophagosome-lysosome fusion, and subjected the cells to stretch for 45 min or 8 hrs. Cell lysates were collected and immunoblotted for LC3B-II and cleaved CASP3 protein (Fig. 15F). As anticipated from autophagosome-lysosome blockage, the amount of LC3B-II was increased when stretched FRLE cells were treated with Baf A1 compared to stretched vehicle (DMSO) controls (Fig. 15G). Interestingly, increased LC3B-II accumulation correlated with significantly greater levels of cleaved CASP3 protein (Fig. 15H).
For affirmation that autophagy preceded apoptosis in the stretch-induced cell response, I analyzed the effect of Z-VAD-FMK treatment on stretched FRLE cells. The pan caspase inhibitor significantly decreased cl. CASP 3 levels in FRLE cells stretched for 8-12 hrs compared to non-treated stretched cells (Figs.5I, J). However, Z-VAD-FMK did not affect LC3B-II formation across a range of stretch intervals (Fig. 15K). Collectively, the data suggest that stretch-induced autophagy precedes apoptosis and that autophagosome formation may act as a link between the two cell fate pathways.
Figure 14. Inhibition of autophagy by PI3K inhibitors in FRLE cells.

A representative immunoblot for cleaved CASP3 and LC3B-II proteins when FRLE cells were stretched in the presence of early-autophagy inhibitors (phosphoinositide 3-kinase inhibitors: 3-methyladenine (3MA), KU5593 (KU), and Gö6976 (Gö)). Protein levels were normalized to β-actin.
Figure 15. Stretch-induced apoptosis is regulated by autophagic mediators.

(A) Representative immunoblots for LC3B-II and cleaved-CASP3 proteins in lysates of FRLE cells treated with vehicle (control) or class III PI3K inhibitors 3-methyladenine (3MA), KU5593, or Gö6976 (n=3 independent cell culture isolations). (B) Densitometric analysis of cleaved-CASP3 protein in stretched cells treated with vehicle (control), 3MA, KU5593, or Gö6976 (n=3 independent cell culture isolations). (C) Representative immunoblots for LC3B-II and cleaved-CASP3 proteins in lysates of FRLE cells transfected with scramble or Atg7 siRNA and stretched for 45 minutes or 8 hrs (n=3 independent cell culture isolations). (D) Densitometric analysis of LC3B-II protein after 45 minutes of stretch when FRLE cells were transfected with Atg7 siRNA compared to scramble siRNA controls. (E) Densitometric analysis of cleaved-CASP3 protein after 8 hrs of stretch when cells were transfected with Atg7 siRNA compared to scramble siRNA controls. (F) Representative immunoblots for LC3B-II and cleaved-CASP3 proteins in lysates of FRLE cells treated with Baf A1, an inhibitor of autophagosome-lysosome fusion, and stretched for 45-60 minutes, and 8-12 hrs (n=3 independent cell culture isolations). Densitometric analysis of (G) LC3B-II protein after increasing durations of stretch (n=3 independent cell culture isolations) and (H) cleaved-CASP3 protein in transfected FRLE cells stretched for 8-12 hrs compared to scramble siRNA controls (n=3 independent cell culture isolations). (I) Representative immunoblots for LC3B-II and cleaved-CASP3 in lysates of FRLE cells treated with Z-VAD-FMK, a pan-caspase inhibitor (n=3 independent cell culture isolations). Densitometric analysis of (J) cleaved-CASP3 and (K) LC3B-II protein after 12 hrs of stretch (n=3 independent cell culture isolations). Protein levels were normalized to β-actin. *P< 0.05
Figure 16. *Atg7* siRNA knockdown in FRLE cells.

Transfection of FRLE cells with *Atg7* siRNA A, B, or C, represented by immunoblot for ATG7 protein. Protein levels were normalized to ACTB.
3.2 Aim 2: To determine the role of ceramides in mediating ventilation (stretch)-induced lung epithelial cell death

3.2.1 Mechanical ventilation of newborn rats increases ceramide levels

Since studies have demonstrated that sphingolipids, particularly ceramides, are increased in lungs as a result of insult or disease (Ammit et al., 2001; Petrache et al., 2005; Lin et al., 2011; Tibboel, Joza, et al., 2013), we measured ceramide levels in bronchoalveolar lavage (BAL) fluid after mechanical ventilation of the newborn rat lung. Sphingolipids in BAL fluid measured by tandem mass spectrometry (LC-MS/MS) were assumed to be mainly from lung epithelial cells lining the airways. I found that mVT ventilation of 7-day old rat pups for 12 and 24 hrs significantly increased multiple ceramide species in BAL fluid compared to control 8-day rat pups (Fig. 17A, C and Table 4, 5). In particular, long chain C16 ceramide was greatly elevated (Fig. 17B, D and Table 4, 5). This ventilation-induced increase in ceramides was specific as no changes were noted in BAL content of any LPC and PC species (Table 6).
Figure 17. Increased ceramide during mechanical ventilation.

BAL fluid was collected and ceramide was measured by LC-MS/MS. Measurement of (A, C) total ceramide and (B, D) C16 ceramide content in BAL fluid of day-7 newborn pups after 12 hrs (n=8 separate pups) and 24 hrs (n=6 separate pups) of ventilation compared to day-7.5 (n=8 separate pups) and day-8 (n=6 separate pups) non-ventilated controls, respectively. *P<0.05
Table 4. Ceramide measurement in BAL fluid of control and 12 hr ventilated rat lungs

<table>
<thead>
<tr>
<th>Lipid species</th>
<th>Day 7.5 Control lungs (ng/mL BALF)</th>
<th>12 hr Ventilated lungs (ng/mL BALF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cer C14:0</td>
<td>1.87 ± 0.97</td>
<td>3.09 ± 0.93</td>
</tr>
<tr>
<td>Cer C16:0</td>
<td>48.89 ± 11.09</td>
<td>120.33 ± 40.13*</td>
</tr>
<tr>
<td>Cer C18:0</td>
<td>7.84 ± 1.87</td>
<td>16.47 ± 6.50</td>
</tr>
<tr>
<td>Cer C18:1</td>
<td>0.28 ± 0.10</td>
<td>0.70 ± 0.33</td>
</tr>
<tr>
<td>Cer C20:0</td>
<td>4.09 ± 1.06</td>
<td>9.40 ± 3.35</td>
</tr>
<tr>
<td>Cer C22:0</td>
<td>17.00 ± 5.66</td>
<td>29.50 ± 12.37</td>
</tr>
<tr>
<td>Cer C24:0</td>
<td>43.73 ± 12.29</td>
<td>75.93 ± 24.93</td>
</tr>
</tbody>
</table>

Data is represented as the mean ± SD (n=6 separate BAL fluids per group)

*P<0.05
Table 5. Ceramide measurement in BAL fluid of control and 24 hr ventilated rat lungs

<table>
<thead>
<tr>
<th>Lipid species</th>
<th>Control lungs (ng/mL BALF)</th>
<th>24 hr Ventilated lungs (ng/mL BALF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cer C14:0</td>
<td>0.25 ± 0.13</td>
<td>0.33 ± 0.06</td>
</tr>
<tr>
<td>Cer C16:0</td>
<td>59.03 ± 26.87</td>
<td>124.67 ± 19.66*</td>
</tr>
<tr>
<td>Cer C18:0</td>
<td>6.62 ± 2.12</td>
<td>12.32 ± 2.74</td>
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<tr>
<td>Cer C18:1</td>
<td>0.40 ± 0.16</td>
<td>0.76 ± 0.15</td>
</tr>
<tr>
<td>Cer C20:0</td>
<td>3.54 ± 0.97</td>
<td>7.00 ± 2.05</td>
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<tr>
<td>Cer C22:0</td>
<td>16.35 ± 4.20</td>
<td>25.11 ± 5.12</td>
</tr>
<tr>
<td>Cer C24:0</td>
<td>24.21 ± 7.35</td>
<td>44.40 ± 10.49*</td>
</tr>
</tbody>
</table>

Data is represented as the mean ± SD (n=6 separate BAL fluids per group)

*P<0.05
Table 3. Lysophosphatidylcholine (LPC) and phosphatidylcholine (PC) measurement in BAL fluid of control and 24 hr ventilated rat lungs (n=6)

<table>
<thead>
<tr>
<th>Lipid species</th>
<th>Control lungs (ng/mL BALF)</th>
<th>24 hr Ventilated lungs (ng/mL BALF)</th>
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</thead>
<tbody>
<tr>
<td>LPC 16:0</td>
<td>1.74 ± 0.79</td>
<td>1.46 ± 0.41</td>
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<tr>
<td>LPC 16:1</td>
<td>0.14 ± 0.09</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td>LPC 18:0</td>
<td>0.42 ± 0.15</td>
<td>0.53 ± 0.18</td>
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<tr>
<td>LPC 18:1</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.00</td>
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<tr>
<td>LPC 20:0</td>
<td>0.16 ± 0.09</td>
<td>0.08 ± 0.01</td>
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<tr>
<td>LPC 20:3</td>
<td>0.15 ± 0.06</td>
<td>0.19 ± 0.06</td>
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<tr>
<td>LPC 20:5</td>
<td>0.02 ± 0.01</td>
<td>0.01 ± 0.00</td>
</tr>
<tr>
<td>LPC 22:5</td>
<td>0.02 ± 0.01</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>LPC 22:6</td>
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<td>PC 30:0</td>
<td>59.33 ± 5.33</td>
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<td>PC 32:0</td>
<td>160.58 ± 24.68</td>
<td>144.42 ± 33.31</td>
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<td>63.50 ± 6.64</td>
<td>45.55 ± 9.96</td>
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<td>PC 32:2</td>
<td>3.21 ± 0.86</td>
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<td>PC 32:3</td>
<td>0.49 ± 0.17</td>
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<td>PC 34:0</td>
<td>2.54 ± 0.44</td>
<td>2.85 ± 0.72</td>
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<tr>
<td>PC 34:1</td>
<td>22.48 ± 3.17</td>
<td>26.12 ± 6.22</td>
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<td>16.14 ± 3.38</td>
<td>10.83 ± 3.18</td>
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<tr>
<td>PC 34:3</td>
<td>1.83 ± 0.44</td>
<td>1.66 ± 0.35</td>
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<tr>
<td>PC 34:4</td>
<td>0.50 ± 0.15</td>
<td>0.37 ± 0.07</td>
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<tr>
<td>PC 36:0</td>
<td>0.06 ± 0.01</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>PC 36:1</td>
<td>0.13 ± 0.03</td>
<td>0.22 ± 0.04</td>
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<tr>
<td>PC 36:2</td>
<td>1.17 ± 0.31</td>
<td>1.42 ± 0.36</td>
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<tr>
<td>PC 36:3</td>
<td>3.85 ± 0.63</td>
<td>3.50 ± 1.07</td>
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<tr>
<td>PC 36:4</td>
<td>26.69 ± 2.46</td>
<td>25.55 ± 8.55</td>
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<tr>
<td>PC 38:3</td>
<td>0.42 ± 0.09</td>
<td>0.51 ± 0.13</td>
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<tr>
<td>PC 38:2</td>
<td>0.02 ± 0.01</td>
<td>0.03 ± 0.01</td>
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<tr>
<td>PC 38:4</td>
<td>2.10 ± 0.44</td>
<td>2.59 ± 0.72</td>
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<tr>
<td>PC 38:5</td>
<td>3.60 ± 0.64</td>
<td>3.45 ± 1.02</td>
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<tr>
<td>PC 38:6</td>
<td>1.61 ± 0.59</td>
<td>2.03 ± 0.35</td>
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<tr>
<td>PC 40:6</td>
<td>0.10 ± 0.02</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>PC 40:7</td>
<td>0.09 ± 0.02</td>
<td>0.13 ± 0.02</td>
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*Data is represented as the mean ± SD (n=6 separate BAL fluids per group)*
3.2.2  *In vitro* mechanical stretch of FRLE cells results in increased ceramide

Sphingolipid measurement by LC-MS/MS revealed an increase in C16 ceramide in FRLE cells within 30 minutes of exposure to cyclic stretch (Fig. 18A), just prior to the peak of LC3B-II protein expression that occurred after 45 min of stretch. Recent studies have demonstrated that ceramides can act as an activation signal for autophagy (Scarlatti *et al.*, 2004; Daido *et al.*, 2004; Pattingre *et al.*, 2009; Li *et al.*, 2009). Therefore, I investigated the potential of C16 ceramide to induce autophagy in FRLE cells. When FRLE cells were treated with 25 µM C16 ceramide, I observed increases in LC3B-II protein within 45 minutes of incubation (Fig. 18B,C). This pattern of LC3B-II protein expression was strikingly similar to that observed in stretch-induced autophagy (Fig. 12A-D). C16 ceramide also increased cleaved CASP3 levels (Fig. 18D,E) with a time pattern that was similar to stretch-induced apoptosis (Fig. 10A-C). These results collectively suggested that the stretch-induced increase in ceramide-16:0 might play a role in activating the cell fate pathways. To further investigate the potential role of ceramide-16:0 in stretch-induced autophagy, I transfected FRLE cells with Atg7 siRNA prior to exogenous C16 ceramide exposure and observed a significant decrease in ceramide-induced LC3B-II expression compared to scrambled siRNA-treated controls (Fig. 19A, B). When FRLE cells transfected with Atg7 siRNA were treated with C16 ceramide for 8 hrs, there was also a significant decrease in cleaved-CASP3 levels compared to scrambled siRNA-treated controls (Fig. 19A, C). These results suggest that the activation of autophagy and apoptosis during stretch may be a result of a stretch-induced increase in C16 ceramide levels.
Figure 18. Ceramide induces autophagy and apoptosis in lung epithelial cells.

(A) FRLE cell lysate was collected and ceramide was measured by LC-MS/MS after 1 hr of stretch or static culture. Change in C16 ceramide levels are presented as Δ%=(stretch-static)/static x100 (n=3 independent cell culture isolations in triplicate). FRLE cells were treated with exogenous C16 ceramide to investigate potential autophagy- and apoptosis-inducing effects. (B) Representative immunoblot and (C) densitometric analysis for LC3B-II protein in cell lysates of FRLE cells treated with C16 ceramide for 60 min (n=3 independent cell culture isolations). (D) Representative immunoblot and (E) densitometric analysis for cleaved-CASP3 protein in lysates of FRLE cells exposed to C16 ceramide for 12 hrs (n=3 independent cell culture isolations). Protein levels were normalized to ACTB. *P<0.05
Figure 19. Role of ceramides in mechanical stretch-induced autophagy and apoptosis.

FRLE cells were transfected with scrambled or Atg7 siRNA and then treated with C16 ceramide for 45 minutes or 8 hrs. (A) Representative immunoblots for LC3B-II and cleaved-CASP3 in cell lysates of FRLE cells. Densitometric analysis of (B) LC3B-II protein after 45 minutes of C16 ceramide treatment and (C) cleaved-CASP3 protein after 8 hrs of C16 ceramide treatment (n=3 separate cell culture isolations). Protein levels were normalized to ACTB. *P< 0.05
3.2.3 Ceramide production in stretched FRLE cells triggers autophagy

To investigate whether stretch-induced ceramide generation was responsible for the induction of autophagy and apoptosis, I treated FRLE cells with various inhibitors of ceramide metabolic pathways and assayed for changes in stretch-induced LC3B-II protein levels. Pretreatment with myriocin that blocks the ceramide de novo synthesis pathway via SPT (Miyake et al., 1995; K Hanada et al., 2000; Kentaro Hanada, 2003; Wadsworth et al., 2013) had no significant effect on LC3B-II protein expression in stretched FRLE cells (Fig. 20). RT-PCR analysis also revealed no significant changes in expression of ceramide synthase 5 (CERS5), an enzyme involved in the de novo synthesis pathway of C16 ceramide (Kroesen et al., 2001; Mizutani et al., 2005) in lungs of ventilated rat pups (not shown). Thus, the increase in ceramide observed in ventilated rat lung and stretched FRLE cells are likely not a result of increased de novo synthesis. Therefore, I investigated the role of sphingomyelin breakdown by sphingomyelinases in stretch-induced generation of ceramide. I treated FRLE cells with inhibitors of SMPD1, fluoxetine (Kornhuber et al., 2008; Becker, Riethmüller, et al., 2010; Gulbins et al., 2013) and imipramine (Brenner et al., 1998; Göggel et al., 2004; Kornhuber et al., 2008; Bismarck et al., 2008), and subjected them to mechanical stretch. I observed a significant decrease in LC3B-II levels when FRLE cells were stretched in the presence of imipramine or fluoxetine compared to vehicle-treated and stretched controls (Fig. 21 A, B). Treatment with SMPD2 inhibitor, GW4869 (Marchesini et al., 2003; Yanyang Wu et al., 2014), had no significant effects on LC3B-II protein levels (results not shown). I also observed a decrease in cleaved CASP3 levels when FRLE cells were stretched for 8 hrs in the presence of imipramine or fluoxetine compared to vehicle-treated and stretched controls (Fig. 21 C,D). These results suggest that breakdown of sphingomyelin by SMPD1 is responsible for stretch-induced increase in ceramide leading to autophagy and apoptosis activation. Indeed, SMPD1 activity was increased in rat lungs ventilated for 12 hrs (Fig. 21E) as well as FRLE cells stretched for 45 minutes (Fig. 21F).
Figure 20. Inhibition of *de novo* ceramide synthesis does not affect stretch-induced autophagy.

Representative immunoblot for LC3B protein in lysates of FRLE cells treated with various concentrations (5-20 µM) of myriocin and subjected to continuous 20% cyclic stretch for 45 minutes. Protein levels were normalized to β-actin.
Figure 21. Inhibition of acid sphingomyelinase activity attenuates ventilation (stretch)-induced autophagy.

FRLE cells were subjected to stretch in the presence and absence of SMPD1 inhibitors, fluoxetine and imipramine. (A-D) Representative immunoblots and densitometric analysis for (A, B) LC3B-II protein in lysates of FRLE cells stretched for 45 minutes (n=3 separate cell culture isolations), and for (C, D) cleaved CASP3 protein after 8 hrs of stretch (n=3 separate cell culture isolations). Protein levels were normalized to β-actin. (E, F) Measurement of SMPD1 activity in (E) tissue lysates of day-7 newborn rat lung subjected to 12 hrs of mechanical ventilation (n=4 separate newborn pups), and in (F) FRLE cell lysates stretched for 45 minutes (n=3 separate cell culture isolations). SMPD1 activities were compared to non-ventilated day-7.5 newborn rat lungs and static cultured FRLE cells, respectively. *P< 0.05
Chapter 4
Discussion

4 Discussion

In this study I support my hypothesis and demonstrate that mechanical stretch of lung epithelia causes apoptotic cell death by a mechanism that depends on bioactive ceramide signaling and activation of the autophagy pathway. My findings showed that autophagy was activated upstream of apoptosis in the cellular response to mechanical stretch. Moreover, blocking ceramide generation by inhibiting SMPD1 activity using imipramine or fluoxetine during mechanical stretch prevented autophagy and subsequent apoptotic cell death.

4.1 Apoptosis due to lung injury

Apoptosis is critical for both normal lung development, proper function, and also in responses following lung injury (Kresch et al., 1998). Several studies have shown activation of the apoptotic cell death pathway in the lung in response to harmful stimuli including mechanical ventilation, microbial lung infection, and cigarette smoke (Petrache et al., 2005; Teichgräber et al., 2008; Kroon et al., 2011; Lin et al., 2011; Tibboel, Joza, et al., 2013). Stretch-induced apoptosis was first demonstrated in vivo in fetal rabbits after tracheal occlusion (De Paepe et al., 1999). Moreover, various reports have implicated cell death in causation of the adverse effects of mechanical ventilation on alveolar development (Lukkarinen et al., 2003; Mokres et al., 2009; Allison et al., 2010). Kroon and colleagues previously demonstrated cell cycle arrest of epithelial lung cells in newborn rats after prolonged mechanical ventilation (Kroon et al., 2011), suggesting altered cell fate as a critical factor in alveolar simplification. In a more recent study, the same group of investigators showed that prolonged mechanical ventilation induced apoptosis predominately by the extrinsic death pathway via the FasL/Fas system in newborn rat lung (Kroon et al., 2013). In contrast, the same group demonstrated that the intrinsic pathway (via caspase-9 activation) did not appear to contribute to stretch-induced apoptosis (Kroon et al., 2013). In this thesis, I confirm these findings and reaffirm that in vivo mechanical ventilation of newborn rats and in vitro cyclic stretch of FRLE cells induces apoptotic cell death. Studies investigating the effect of MV on apoptosis in infants with BPD have been limited. Elevated
apoptotic signaling was detected in lung bronchiolar and epithelial cells (but not in fibroblasts) (Hargitai et al., 2001). Greater numbers of apoptotic epithelial cells are also detected in lung sections from ventilated preterm infants (May, Marx, et al., 2004). However, interpretation of the results should be cautioned because the infants from these studies were also treated with oxygen. Therefore, further investigation into the effect of MV alone is required to determine how treatment affects lung development. The activation of apoptotic mechanisms in response to mechanical ventilation may vary in an organ-specific manner. By contrast to the previous finding of MV-induced activation of the extrinsic apoptotic pathway, a study has reported activation of the intrinsic apoptotic pathway via the Fos/FoxO1/Stat3-Bim pathway in the diaphragm in response to mechanical ventilation (Tang et al., 2011).

4.2 Autophagy during lung injury

Autophagy often develops upstream as a primary response to stress stimuli in homeostatic and pathological settings that may be followed by a mode of PCD, such as apoptosis, upon failed cell-survival (Cui et al., 2007; Hussain et al., 2010; Franzetti et al., 2012; Maes et al., 2014; Gao et al., 2015). The role of autophagy and autophagy mediators in MV-induced injury has not previously been investigated and until recently, there has been a limited understanding of the potential role of autophagy in the initial response to lung injury and disease. Recent studies have reported activation of the autophagy pathway as a contributing factor to the pathology of lung pathologies. A study by Yang Sun and colleagues demonstrated that H5N1-infected lungs from human and mice contained accumulation of autophagosomes, and autophagic cell death in alveolar epithelial cells via the Akt and mTOR pathway (Yang Sun et al., 2012). In the present study, I found a temporal activation of both autophagy and apoptosis pathways following mechanical ventilation. In vitro, stretch induced an increase of autophagic markers including LC3B-II in FRLE cells within 45 min while in vivo, mVt ventilation upregulated LC3B-II protein in newborn rat lung after 12 hrs. Autophagy occurred prior to apparent induction of apoptosis after 8 hrs of stretch in vitro and 24 hrs of ventilation in vivo. Similar findings have been reported by Kim and colleagues (Kim, Xue Wang, Chen, Lee, Huang, Yong Wang, Ryter, and Choi, 2008b) in a study that treated human bronchial epithelial cells with cigarette smoke extract. Smoke extract increased LC3B-II expression within 1 hr of exposure, culminating in
DISC formation, activation of caspases (-8, -9, -3), and apoptotic cell death after a 12 hr period. The group showed that LC3B⁻/⁻ mice had significantly decreased levels of apoptosis in lungs exposed to cigarette smoke compared to their wildtype littermates. It was also found that LC3B interacted with Fas in DISC, and was required for enhanced caspase activation during exposure to cigarette smoke (Zhi-Hua Chen, Lam, Jin, Kim, Cao, Seon-Jin Lee, Ifedigbo, Parameswaran, Ryter, and Choi, 2010a). Interestingly, Tanaka and colleagues demonstrated that exposure to hyperoxia also induced a rapid conversion of LC3B-I to LC3B-II in mouse lung in vivo and in Beas-2B human bronchial epithelial cells in vitro (Tanaka et al., 2012). Contrastingly however, the group showed that LC3B siRNA knockdown promoted hyperoxia-induced epithelial cell death, whereas overexpression of LC3B conferred cytoprotective effects during hyperoxia via physical interaction with Fas and components of DISC (Tanaka et al., 2012). Both studies demonstrated rapid activation of autophagy signaling in response to insults to the lung, however LC3B protein appeared to act as a pro-survival signal during hyperoxia whereas in response to cigarette smoke, LC3B played a role in activating apoptotic epithelial cell death (Zhi-Hua Chen, Lam, Jin, Kim, Cao, Seon-Jin Lee, Ifedigbo, Parameswaran, Ryter, and Choi, 2010a; Tanaka et al., 2012). Hence, activation of autophagy and apoptosis in response to a lung injury insult appears to occur in a sequential pattern, though the relationship appears context-specific and further investigation is required to further understand the role of autophagy in stretch-induced apoptosis.

4.3 Crosstalk between autophagy and apoptosis

The dynamic relationship between autophagy and apoptosis is complex in the sense that determination of cell fate is specific to cell-type, cellular settings, and stimuli (Yu et al., 2006; Espert et al., 2006). Autophagy and apoptosis are highly regulated processes that are functionally distinct, yet are positively and negatively interconnected at different levels of regulation (Lavieu et al., 2006). Perturbations in the apoptotic machinery, such as inhibition of caspases, may lead to induction of autophagy and subsequent caspase-independent cell death (Yue Xu et al., 2006; Madden et al., 2007). Conversely, inhibition of autophagy may lead to apoptotic cell death as a result of failure of the cell to adapt to a particular stress stimuli (González-Polo et al., 2005; Yue Xu et al., 2006), possibly due to a bioenergetic shortage (Scott et al., 2004), or accumulation of
unwarranted organelles or misfolded proteins (Qin et al., 2003; Bjørkøy et al., 2005). However, autophagy activation as a primary cell-survival response to adverse stress may also mediate a “switch” to apoptosis (Espert et al., 2006) or necrosis (Yu et al., 2006). Here, I demonstrate a requirement for autophagy in mechanical-ventilation (stretch) induced apoptosis as inhibition of autophagy using class III PI3K inhibitors or Atg7 siRNA knockdown reduced CASP3 cleavage. The PI3K inhibitor 3-MA is one of the most widely used early-stage autophagy inhibitors; however, its use should be cautioned as it also inhibits cell survival promoting class I phosphoinositide 3-kinase (You-Tong Wu et al., 2010; Farkas et al., 2011). Therefore, I used more specific class III PI3K inhibitors, KU55933 and Gö6976 (Farkas et al., 2011), to confirm the 3-MA inhibition of the stretch-induced autophagy response. Our results also showed that preventing stretch-induced apoptosis through inhibition of caspase activities with Z-VAD-FMK had no effect on autophagy activation, in agreement with autophagy being activated upstream of apoptosis. These findings suggest an interdependence of an early prosurvival autophagic response to stretch for later stretch-induced apoptogenic signaling. Recent investigations have identified a few autophagic mediators that may act as potential molecular switches to adjust the cell from autophagy to apoptosis upon failed cell survival. Autophagic proteins such as ATG1 (Scott et al., 2007) and BECN1 (Zan-hong Wang et al., 2007; Wirawan et al., 2010) have been shown to act central in mounting an autophagy response and the capacity to coordinate induction of cell death. Increasing evidence suggests that accumulation of unprocessed autophagic protein LC3B-II plays a role in activation of PCD pathways such as apoptosis (Kanzawa et al., 2004; Zhi-Hua Chen, Lam, Jin, Kim, Cao, Seon-Jin Lee, Ifedigbo, Parameswaran, Ryter, and Choi, 2010b). Our in vitro findings with Baf A1 support a pathogenic role for LCB3-II in MV-induced lung injury by acting as a pro-apoptotic agent. It has been reported that Baf A1 can directly induce apoptosis (Kinoshita et al., 1996; Ohta et al., 1998). My observation that static FRLE cells treated with Baf A1 for 12 hrs did not increase cleaved CASP3 levels (results not shown), however, argues against Baf A1 inducing apoptosis in FRLE cells.
4.4 Ceramides in respiratory disorders

Sphingolipids may act as important mediators in lung injury and the pathogenesis of various respiratory disorders (Uhlig and Gulbins, 2008). Petrache and colleagues revealed that an excess of ceramide-induced oxidative stress triggered apoptotic cell death which led to alveolar enlargement (Petrache et al., 2008). Superoxide dismutase has been shown to play a protective role against apoptosis and alveolar enlargement induced by disproportionate ceramide, suggesting that there are protective mechanisms that can be activated to protect against or prevent unwarranted cell death in the healthy lung (Petrache et al., 2008). Here, I observed an increase in ceramides, specifically C16 ceramide, in both 12-24 hrs ventilated newborn rat lung. Interestingly, absolute ceramide amounts remain relatively unchanged in both control and ventilated lung after 12-24 hrs, suggesting that the peak of ceramide generation occurred before 12 hrs of ventilation with no further upregulation. The “many ceramides” paradigm hypothesizes that ceramide molecules are generated individually within specific pathways and intracellular compartments to exert unique functions (Young et al., 2013). Individual ceramide species may differentially regulate pro-survival and pro-apoptotic signaling pathways (Pettus et al., 2002). For example, very long chain ceramides (C24:0, C24:1) that are generated by the activity of ceramide synthase 2 have been shown to promote cell proliferation in cancer cell progression (Hartmann et al., 2012). In contrast, C16 ceramide has been identified as a pro-apoptotic mediator (Thomas et al., 1999; Yamada et al., 2001) and is frequently used to mimic endogenous ceramide signaling (Rénert et al., 2009). I found that exogenous C16 ceramide activated both autophagy and apoptosis in FRLE cells in similar temporal patterns as seen in the stretch-activated response. When I performed knockdown of Atg7 prior to C16 ceramide exposure, autophagy was inhibited while apoptosis was partially, but significantly, reduced. Furthermore, a loss in ceramide production that appears to occur after 30 minutes of mechanical stretch further suggests that the rapid and transient increase of C16 ceramide may serve as a signal for autophagy activation without direct activation of downstream apoptosis. Thus, C16 ceramide likely induces apoptotic cell death in immature lung epithelial cells partially via the autophagy pathway.
4.5 Pharmacological inhibition of ceramide synthesis

Ceramide can be generated through three known pathways. De novo synthesis of ceramide is hallmarked by the rate-limiting step of condensation between serine and palmitoyl-CoA catalyzed by SPT (Dawkins et al., 2001). Another important step in the de novo pathway, namely acetylation of sphinganine to dihydroceramide, is catalyzed by CER. Ceramide can also be generated by breakdown of sphingomyelin catalyzed any of the sphingomyelinase isoenzymes SMPD1, SMPD2, and ENPP7 (Levade and Jaffrézou, 1999). Recycling of sphingosine (SPH) and glycosphingolipids by reverse activity of ceramidase can also produce ceramide (Okino et al., 2003). Targeting the pathways of ceramide generation by pharmacological intervention has been used as means to prevent or minimize the pathophysiological effects of ceramide. Alveolar lung cell death and oxidative stress were prevented by inhibition of SPT (and thus the de novo synthesis pathway) by myriocin in an emphysema model (Petrache et al., 2008). A recent study has shown that oxidative stress and elevated de novo synthesis of ceramide in preeclampsia results in increased trophoblast cell autophagy (Melland-Smith et al., 2015). However, in the present stretch model, inhibition of the de novo synthesis pathway by myriocin did not have significant effects on stretch-induced autophagy. Also, I did not observe any differences in CERS5 expression in our ventilated newborn lung model. CERS5 is the predominant CERS in the lung involved in the formation of C16 ceramide (Zhiwei Xu et al., 2005). Thus, the de novo ceramide pathway is not responsible for the stretch-induced upregulation of C16 ceramide leading to autophagy activation. Airway ceramide levels have been reported to be elevated in animal models of CF and an improvement in lung function was observed after treatment with amitriptyline, an SMPD1 inhibitor (Becker, Grassme, et al., 2010; Becker, Riethmüller, et al., 2010). SMPD1-dependent production of ceramide has also been implicated in pulmonary edema formation in ALI (Göggel et al., 2004; Y Yang et al., 2010). In line with these studies, I found increased SMPD1 activity in in vivo ventilated rat lung and in vitro stretched FRLE cells compared to their respective controls. Inhibition of SMPD1 activity by imipramine or fluoxetine ameliorated stretch-induced autophagy and apoptosis, whereas inhibition of SMPD2 by GW4869 did not have any effect. It may be worthwhile to measure SMPD1 mRNA expression in response to MV and mechanical stretch. However, the rapid increase in ceramide during mechanical stretch suggests that this observation is due to an increase of enzymatic activity as opposed to
altered gene expression. The mechanism by which ceramide mediates stretch-induced autophagy remains unclear; however, it is plausible that ceramide targets an upstream regulator of the autophagy pathway. A previous study has shown that stimulation of *de novo* ceramide synthesis leads to phosphorylation of Bcl-2 and its dissociation from BECN1 resulting in autophagy activation (Pattingre et al., 2009). Ceramide has also been reported to suppress Akt signaling resulting in negative regulation of mTOR and autophagy activation (Scarlatti et al., 2004). Future studies need to investigate the effects of ceramide generation via SMPD1 inhibition in ventilation-induced newborn lung injury as a potential treatment strategy to prevent BPD.
4.6 Limitations

4.6.1 Other cell death pathways

There has been limited investigation into the mechanism of ventilation induced-lung injury in preterm infants with BPD. Increased apoptotic activity in epithelial cells of preterm infants has been observed, however, the role of autophagy in epithelial cell death is unknown (Hargitai et al., 2001). Furthermore, the role of other forms of PCD such as necroptosis has yet to be investigated. Necroptosis, a programmed form of necrosis, is a receptor interacting protein (RIP)-mediated form of cell death dependent on the TNF receptor, Fas, and TNF-related apoptosis-inducing ligand (TRAIL) receptor activation, and is morphologically distinct from apoptosis (Kawahara et al., 1998; Francis Ka-Ming Chan et al., 2003). Although fundamentally distinct, significant cross-talk between necroptosis, autophagy, and apoptosis has been described (Feng et al., 2007; Declercq et al., 2009). It is likely that autophagy and apoptosis are not exclusive, and a network of PCD mechanisms are activated in response to injurious ventilation, and further study must be conducted to better understand the molecular basis of lung injury.

4.6.2 Animal model studies

Animal studies have allowed for better understanding of the molecular mechanisms that are involved in lung development, delineation of factors critical to the pathogenesis of neonatal lung disease, and the study of potential novel strategies to be tested in a clinical setting. However, interpretation of data from animal model studies is limited because of their controlled environment and disparities between laboratory settings and the natural state; therefore, their limitations need to be considered. Postnatal undernutrition coinciding with the alveolar stages of rat lung development has been shown to result in larger and fewer alveoli, thicker septa, and altered proportions of club (Clara) and ciliated cells in the bronchiolar epithelium following neonatal exposure to hyperoxic gas (Das, 1984). This is particularly disadvantageous when investigating the independent effects of injury on lung development (O'Reilly and Thébaud, 2014). Although not carefully examined in this study, it is important that body weights of newborn rats are documented throughout the experiment, to ensure that postnatal growth restriction is not a confounding factor.
4.6.3  *In vitro* studies

In this study, I utilized FRLE cells and subjected them to mechanical stretch to investigate the molecular mechanisms involved in lung injury. Although *in vitro* studies allow for manipulation of the experimental model and the study of specific factors in pathophysiological conditions, the exclusion of lung structures can lead to the generation of results that do not accurately represent what occurs in the *in vivo* setting. For example in this study, I use a primary lung epithelial cell population that are stretched on membranes coated with collagen I, in contrast to collagen IV which is a major component of the complex matrix composition in basement membranes of the lung (West and Mathieu-Costello, 1999). Moreover, there is evidence that mechanical stress induces lung fibrosis, and epithelia-mesenchymal transition plays an important role in ventilator-induced lung fibrosis and dysregulated repair (Cabrera-Benítez et al., 2012). Likewise, components of the extracellular matrix, specifically glycosaminoglycan hyaluronan, have been implicated in the inflammatory response during VILI (Heise et al., 2011). Mechanical ventilation also leads to increased epithelial expression of inflammatory mediators TNF-α and IL-6 (Heise et al., 2011). Although epithelial injury is a primary event in the pathogenesis of lung injury and disease (Martin et al., 2005), it is evident that interactions between lung epithelia and surrounding tissues contribute to lung pathology. Therefore, the results from my *in vitro* studies should be used to provide reason for further investigation into the potential therapeutic applications of ceramide pathway inhibitors in the context of the lung during VILI.

4.6.4  Side effects of SMPD1 inhibitors

Based on the results in this study, I suggest the use of SMPD1 inhibitors as potential therapeutic agents in MV-induced lung injury. However, the inhibitors used in this study, imipramine and fluoxetine, also known as Tofranil and Prozac respectively, are antidepressants administered for the treatment of major depressive disorder. Despite their wide use as treatment for mental illness, there has been little investigation into their side effects in the lung. Studies have reported increased pulmonary hypertension in rat (Fornaro et al., 2007) and cat (Hong Liu et al., 2002) models, suggesting dose-dependent harmful effects of the inhibitors. Therefore, further investigation into the use of SMPD1 inhibitors as potential therapeutic agents should be carefully considered.
4.6.5 Bronchopulmonary dysplasia is multifactorial

Finally, BPD is caused by a combination of factors including hyperoxia (Hosford and Olson, 2003; Makena et al., 2011; Tibboel, Joza, et al., 2013), mechanical stretch (May, Strobel, et al., 2004; Kroon et al., 2011; Kroon et al., 2013), inflammation (Copland et al., 2004; Kroon et al., 2010), and possibly genetic predisposition (Hallman and Gluck, 1980; Bhandari et al., 2006; Hilgendorff et al., 2007). Although this thesis explores the pathogenesis of BPD due to mechanical stretch, the knowledge obtained from this study should be used to provide a more complete understanding of the other mechanisms involved so to be translated into novel strategies to treat neonatal lung disease.
Chapter 5
Conclusion

5 Conclusions

5.1 Summary of findings

Survival of very premature infants has improved considerably because of major advancements made in neonatal care, including use of postnatal surfactant replacement, antenatal glucocorticoid therapy, and enhanced nutritional and respiratory support (Bland et al., 2007). However, in chronic lung disease such as BPD, the need for prolonged mechanical ventilation can lead to a halt in lung development (Baraldi and Filippone, 2007). Since the first description of BPD in 1967 (Northway et al., 1967), substantial evidence has left little doubt that mechanical ventilation can alter normal lung development. However, identification of the mediators and mechanisms responsible for the resulting pathophysiological lung state has yet to be fully understood. The purpose of my experiments described in this thesis was to investigate the roles of two functionally distinct yet highly interconnected cell fate pathways, apoptosis and autophagy, in a mechanism through which a halt in lung development occurs as a result of mechanical ventilation.

Kroon and colleagues previously showed that prolonged mechanical ventilation induces apoptosis in alveolar type II cells in newborn rats by a mechanism that involves FasL/Fas signaling of the extrinsic death pathway (Kroon et al., 2013). I confirmed these findings and demonstrated that 24 hrs of MV induces apoptosis in newborn rat lung. I also reported autophagy activation after 12 hrs of MV. To further investigate, I subjected FRLE cells to continuous 20% cyclic stretch and demonstrated autophagy activation that preceded apoptotic cell death; moreover, autophagy is required in MV-induced apoptosis. Through these data I accomplished the first of two objectives of this thesis, suggesting an interconnected relationship between autophagy and apoptosis in the MV-induced cellular response.

In a previous study, Tibboel and colleagues showed increased ceramide levels in oxygen-induced BPD injury in newborn rats. The addition of D-sphingosine during recovery ameliorated
hyperoxia-induced alveolar arrest, suggesting a role for bioactive ceramides in neonatal lung damage (Tibboel, Joza, et al., 2013). These results gave me reason to speculate a role for ceramide signaling in MV-induced lung injury. In fact, I reported increased ceramide in both in vivo and in vitro models of MV that preceded autophagy activation. I demonstrated that C16 ceramide is capable of activating both autophagy and apoptosis in FRLE cells, and that apoptosis activation in response to C16 ceramide is partially due to autophagy mediators. My data also suggested that MV induces breakdown of sphingomyelin by SMPD1 to generate ceramide and induce apoptotic cell death via autophagy activation. By these findings, I accomplished the second objective of this thesis, demonstrating a role of ceramide signaling in MV-induced lung epithelial cell injury.
5.2 Final conclusion

The hypothesis driving the studies in this thesis stated that: injurious mechanical ventilation (stretch) triggers stretch-induced lung epithelial cell death by autophagy and apoptosis via an increase in ceramide production, thereby affecting lung development in ventilation-induced lung injury. My results demonstrate an interconnected relationship between autophagy and apoptosis in stretch-induced cell death. Inhibition of SMPD1 protected against stretch-induced cell death by down regulating autophagy and subsequently apoptosis.

In conclusion I propose a mechanism of ventilation/stretch-induced apoptotic cell death in which an increase in ventilation/stretch-induced ceramide may act as a signaling molecule for the activation of autophagy, and an increase in autophagosomes/LC3B-II may lead to apoptotic pulmonary cell death (Fig. 22). The present study extends our mechanistic understanding of stretch-induced cell death and highlights the complex relationship between autophagy, apoptosis and their mutual mediators during mechanical ventilation. My data suggests that pharmacological inhibitors directed at autophagy, and at subsequent apoptosis activation, could be tested for potential as an adjunct therapy to help reduce the neonatal mortality and morbidity associated with chronic mechanical ventilation. Specifically, my data also suggests that manipulating ceramide signaling, by SMPD1 activity, may be a potential therapeutic target for the treatment of lung injury conditions such as BPD.

Overall, the studies conducted in this thesis accomplished the aims originally outlined. I believe that the results presented here extend the current knowledge regarding the molecular mechanisms involved in VILI, and also leads to areas of further investigation involved in the management of chronic lung disease in neonatal care.
Figure 22. Proposed mechanism of mechanical stretch-induced apoptosis.

Mechanical stretch may stimulate rapid generation of ceramide via SMPD1 activity leading to activation of autophagy. Accumulation of autophagic mediators may trigger cell death by apoptosis.
Chapter 6
Future Directions

6 Future directions

The results of this thesis begin to delineate the molecular mechanism(s) in response to injurious mechanical ventilation, showing that mechanical stretch induces a rapid increase in ceramide and activation of the autophagy pathway, which leads to apoptotic cell death. However, the molecular interactions between mediators of the induced cell fate pathways are unknown and further investigation is required to better understand the cellular response to lung injury.

6.1 What is the mechanism of ceramide-induced autophagy?

Work from this thesis illustrates the capacity of ceramide to induce the autophagy pathway during the stretch-induced response in premature lung epithelial cells. However, the exact mechanism is unknown and future studies are required to understand the means by which ceramide acts as a pro-autophagic mediator. Ceramide has been reported to act as a suppressor of Akt, a pro-survival molecule (Zhou et al., 1998). Thus, inhibition of mTOR signaling downstream of Akt may be a mechanism by which ceramide induces autophagy. Treatment with exogenous C2 ceramide or tamoxifen has been shown to suppress Akt signaling leading to autophagy activation in human colon and breast cancer cell lines (Scarlatti et al., 2004). Additionally, a study has demonstrated that amino acid starvation leads to increased ceramide levels, which inactivates mTOR signaling and induces autophagy (Taniguchi et al., 2012).

Disruption of the inhibitory BECN1:Bcl-2 complex has also been implicated as a mechanism in ceramide-induced autophagy. Treatment with C2 ceramide and tamoxifen has also been shown to enhance the expression of BECN1 to promote autophagy, demonstrating that ceramide can act on multiple targets in the autophagy pathway (Scarlatti et al., 2004). Intracellular ceramide can also liberate BECN1 for induction of autophagy via the JNK-mediated phosphorylation of Bcl-2 (Pattingre et al., 2009). Bcl-2 has notably emerged as a critical mediator between ceramide-induced autophagy and apoptosis. Specifically; the ceramide-dependent dephosphorylation of mitochondrial Bcl-2 promotes apoptosis while phosphorylation of ER-targeted Bcl-2 enhances
autophagy (Young et al., 2013). Ceramide is also a well-established activator of stress-activated kinase JNK and transcription factor c-Jun (Westwick et al., 1995; Coroneos et al., 1996). Ceramide mediated c-Jun has been demonstrated to positively regulate expression of LC3 (Ting Sun et al., 2011). ER stress has also been attributed to ceramide-mediated autophagy activation. For example, downregulation of ER-localized CERS2 results in accumulation of long-chain ceramides (C14 an C16) and the induction of ER stress-dependent autophagy (Spassieva et al., 2009). Previous studies have elucidated several targets of ceramide in the autophagy pathway, and it will be interesting to determine how ceramide activates autophagy in response to mechanical stretch. Therefore I propose immediate future studies that initially investigate whether induction of autophagy by ceramide is attributed to the PI3K/Akt/mTOR signaling pathway, disruption of the BECN1:Bcl 2 complex, or induction of ER stress.

6.2 How does autophagy induce apoptosis in lung injury?

The functional relationship between apoptosis and autophagy is complex in that autophagy may constitute a stress adaption that avoids cell death whereas in other cellular settings, autophagy may activate cell death mechanisms such as apoptosis or necrosis (Maiuri et al., 2007). Investigation into the crosstalk between apoptosis and autophagy has identified numerous mediators (Maiuri et al., 2007; Giansanti et al., 2011). For example, the Bcl-2 proteins (Bcl-2, Bcl-xL, and Mcl-1) do not only act as anti-apoptotic molecules, but also bind the BH3 domain of BECN1 to suppress PI3KC3 activity and autophagosome formation (Kroemer and Levine, 2008).

There is also increasing evidence that accumulation of autophagosomal membranes serve as platforms for caspase-8 activation and apoptotic cell death via the extrinsic death pathway (Young et al., 2012). ATG5 has been shown to interact with Fas-associated protein with death domain (FADD) in interferon (IFN)-gamma-induced autophagic cell death (Pyo et al., 2005). Interestingly, a recent study suggested that interaction between ATG5-12 and FADD recruits caspase 8 to promote caspase-dependent apoptosis during hyperautophagic conditions, averting necrosis and inflammation in vivo (Bell et al., 2008). The accumulation of damaged or misfolded proteins can lead to detrimental consequences within cells called proteotoxicity resulting in increased oligomerization and activation of caspase-8 at intracellular membranes (Pan et al., 2011). Oligomerization and activation of caspase-8 is enhanced via its interaction with LC3 in
response to proteotoxic stress (Pan et al., 2011). LC3B protein has also been shown to regulate apoptosis through interactions with caveolin-1 and FasL in a smoke-induced COPD model (Zhi-Hua Chen, Lam, Jin, Kim, Cao, Seon-Jin Lee, Ifedigbo, Parameswaran, Ryter, and Choi, 2010b).

Previous study in our lab demonstrated that mechanical ventilation induces apoptosis via the extrinsic death pathway (Kroon et al., 2013), and based on the results of this thesis, an accumulation of LC3B-II protein enhanced cleaved CASP-3 protein amounts. Therefore, for future studies, it would be important to further investigate the potential interaction between LC3B and a mediator of apoptosis, specifically within the Fas/FasL signaling pathway.

6.3 What other cell death mechanisms are involved in VILI?

Increased apoptotic activity in epithelial cells of preterm infants has been observed (Hargitai et al., 2001). However, the role of other forms of PCD such as necroptosis has yet to be investigated. Although fundamentally distinct, significant cross-talk between necroptosis, autophagy, and apoptosis has been described (Feng et al., 2007; Declercq et al., 2009). For instance, inhibition of autophagy by both pharmacological inhibitors and genetic knockdown of autophagy-specific genes rescued palmitic acid-induced programmed necrotic cell death in endothelial cells (Khan et al., 2012). A recent study demonstrated that cigarette smoke causes mitochondrial dysfunction and subsequent mitophagy, the autophagy-dependent elimination of mitochondria. Cigarette smoke also caused cell death by necroptosis that could be ameliorated by genetic deficiency of mitophagy regulator PINK1 (Mizumura et al., 2014). These results demonstrate a mechanistic explanation for how cigarette smoke can regulate lung cell death through the induction of mitophagy and necroptosis, which may contribute to the pathogenesis of COPD (Mizumura et al., 2014). The results from this thesis demonstrate an interconnected relationship between autophagy and apoptosis in response to injurious stretch, however, it is mostly likely that autophagy and apoptosis are not mutually exclusive and a network of PCD mechanisms are activated during injurious ventilation. Immediate future study must be conducted to better understand the network of cell death pathway mechanisms activated in response to lung injury. Specifically, it would be interesting to determine whether necroptosis is an active pathway during mechanical ventilation, and its interactions with other cell fate pathways.
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