ORIGINAL ARTICLE

Acid Sphingomyelinase Inhibition Attenuates Cell Death in Mechanically Ventilated Newborn Rat Lung

Behzad Yeganeh[1]*, Joyce Lee[1,2]*, Claudia Bilodeau[1,3], Irene Lok[1], Leonardo Ermini[1], Cameron Ackerley[1], Isabella Caniggia[4], Jeroen Tibboel[1,5], Andre Kroon[1,5], and Martin Post[1,2]

[1]Translational Medicine Program, Peter Gilgan Centre for Research and Learning, Hospital for Sick Children, Toronto, Ontario, Canada; [2]Institute of Medical Science and [3]Department of Laboratory Medicine and Pathobiology, University of Toronto, Ontario, Canada, [4]Mount Sinai Hospital, the Lunenfeld-Tanenbaum Research Institute, Toronto, Ontario, Canada; and [5]Department of Pediatrics, Erasmus MC–Sophia, Rotterdam, the Netherlands

Abstract

Rationale: Premature infants subjected to mechanical ventilation (MV) are prone to lung injury that may result in bronchopulmonary dysplasia. MV causes epithelial cell death and halts alveolar development. The exact mechanism of MV-induced epithelial cell death is unknown.

Objectives: To determine the contribution of autophagy to MV-induced epithelial cell death in newborn rat lungs.

Methods: Newborn rat lungs and fetal rat lung epithelial (FRLE) cells were exposed to MV and cyclic stretch, respectively, and were then analyzed by immunoblotting and mass spectrometry for autophagy, apoptosis, and bioactive sphingolipids.

Measurements and Main Results: Both MV and stretch first induce autophagy (ATG 5–12 [autophagy related 5–12] and LC3B-II [microtubule-associated proteins 1A/1B light chain 3B-II] formation) followed by extrinsic apoptosis (cleaved CASP8/3 [caspase-8/3] and PARP [poly(ADP-ribose) polymerase] formation). Stretch-induced apoptosis was attenuated by inhibiting autophagy. Coimmunoprecipitation revealed that stretch promoted an interaction between LC3B and the FAS (first apoptosis signal) cell death receptor in FRLE cells. Ceramide levels, in particular C16 ceramide, were rapidly elevated in response to ventilation and stretch, and C16 ceramide treatment of FRLE cells induced autophagy and apoptosis in a temporal pattern similar to that seen with MV and stretch. SMPD1 (sphingomyelin phosphodiesterase 1) was activated by ventilation and stretch, and its inhibition prevented ceramide production, LC3B-II formation, LC3B/first apoptosis signal interaction, caspase-3 activation, and, ultimately, FLRE cell death. SMPD1 inhibition also attenuated ventilation-induced autophagy and apoptosis in newborn rats.

Conclusions: Ventilation-induced ceramides promote autophagy-mediated cell death, and identifies SMPD1 as a potential therapeutic target for the treatment of ventilation-induced lung injury in newborns.

Keywords: mechanical ventilation; lung epithelial cells; autophagy; apoptosis; ceramides

Although mechanical ventilation (MV) is a critical component of modern neonatal intensive care, it remains a major risk factor for development of bronchopulmonary dysplasia (BPD) (1). Over the last 50 years, there has been a movement toward the use of gentler ventilation strategies designed to minimize volutrauma; however, the incidence of BPD has not decreased (2, 3). Hence, a better understanding of the pathophysiology of MV-induced newborn

(Received in original form March 28, 2018; accepted in final form October 12, 2018)

*These authors contributed equally to this work.

Supported by Canadian Institutes of Health Research grant FDN-143309 and Canadian Foundation of Innovation grant CFI-12156; J.L. was the recipient of an Ontario Graduate Scholarship and a Restracom award from the Hospital for Sick Children, and M.P. is the holder of a Canadian Research Chair in Fetal, Neonatal, and Maternal Health.

Author Contributions: B.Y., J. L., and M.P. conceived the project, designed the experiments, and wrote the manuscript; B.Y., J.L., and C.B. performed the cell experiments; I.L., J.T., and A.K. conducted all animal experiments; C.A. performed electron microscopy; L.E. performed the lipid analysis; I.C. provided expertise on autophagy and cell death.

Correspondence and requests for reprints should be addressed to Martin Post, Ph.D., 09.9705, Peter Gilgan Centre for Research and Learning, Hospital for Sick Children, 686 Bay Street, Toronto, ON, M5G 0A4 Canada. E-mail: martin.post@sickkids.ca.

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

Am J Respir Crit Care Med Vol 199, Iss 6, pp 760–772, Mar 15, 2019

Originally Published in Press as DOI: 10.1164/rccm.201803-0583OC on October 16, 2018
Internet address: www.atsjournals.org

At a Glance Commentary

Scientific Knowledge on the Subject: Mechanical ventilation (MV) of preterm infants is often associated with chronic lung injury. Ventilation triggers epithelial cell death and inhibits alveolarization. The contribution of autophagy and interplay between autophagy and apoptosis in ventilation-induced epithelial cell death is still elusive.

What This Study Adds to the Field: The present study shows that MV of newborn rat lung as well as cyclic stretch of fetal rat lung epithelial cells rapidly induces autophagy that then triggers extrinsic apoptosis. Ventilation (stretch)-induced ceramide generation is crucial for this interplay between autophagy and apoptosis. Blockage of ceramide production with SMPD-1 (sphingomyelin phosphodiesterase-1) inhibitors abrogates ventilation-induced autophagy and apoptosis in newborn rats, and identifies SMPD-1 as a potential therapeutic target for treatment of ventilation-induced lung injury.

lung injury, and possible therapeutic interventions, is warranted.

The exact biological mechanisms by which MV disrupts normal lung development are not known; however, studies suggest multifactorial causes (4, 5), including apoptosis (6, 7). Hargitai and colleagues (8) examined the incidence of apoptosis in various organs (lung, kidney, and brain) of 24 autopsy cases of ventilated, preterm infants who suffered respiratory distress. Although apoptotic activity was very low in stage I of BPD, elevated apoptosis was found in stages II, III, and IV of BPD among alveolar and bronchiolar cells (8). Mokres and colleagues (6) showed that prolonged MV of neonatal mice increased lung epithelial cell apoptosis that was accompanied by inhibition of alveolar development. Our group (7) reported increased cell death via the extrinsic, but not intrinsic, apoptotic pathway in lung epithelial cells as a result of MV. Hindering signaling via the FAS (first apoptosis signal) receptor using an anti-FAS ligand antibody markedly inhibited MV-triggered apoptosis. However, the mechanism leading to MV-induced extrinsic apoptosis is unknown, and the upstream signaling pathways of this event have yet to be elucidated.

Emerging evidence demonstrates autophagy to be upstream of apoptosis in cell fate decisions (9, 10). Autophagy is an evolutionarily conserved, tightly regulated catabolic process that serves to maintain homeostasis in response to cellular stress or development (11). It primarily functions as a cell survival mechanism, but under conditions of cellular stress, excessive or uncontrolled levels of autophagy can induce autophagy-dependent cell death (12). Although many studies have examined the interconnection between autophagy and apoptosis on cell fate in various disease models (10, 11, 13–15), evidence of cross-talk between autophagy and apoptosis in an MV setting is very limited. In human, prolonged MV has been shown to trigger activation of autophagy in the diaphragm (16), but apoptosis was not investigated. In adult mice, MV has been reported to increase autophagy, inflammation, and injury of the lung (17). Interestingly, mice lacking autophagin 1 or ATG4B (autophagy related 4B) (*Atg4b* knockout) display an ameliorated inflammatory response and decreased lung injury when ventilated (17). Despite these reports, the autophagy/apoptosis cross-talk in MV-induced cell death in newborns remains unknown.

The interplay between autophagy and apoptosis involves many regulatory molecules (11, 13), including sphingolipids (18, 19). Besides being a structural component of the membrane bilayer, sphingolipids, such as ceramide and sphingosine-1-phosphate (S1P), play an essential role in various cell-fate pathways (20). We and others reported increased ceramide levels in asthma, emphysema, and cystic fibrosis (21–24), as well as in oxygen-induced BPD-like injury in newborn rats (25). More recently, we have shown increased ceramide levels in tracheal aspirates of 122 preterm infants after the first day of exposure to MV (26). In rodent models, ceramide upregulation has been shown to be associated with VEGF (vascular endothelial growth factor) suppression (27) and decreased surfactant protein B levels (28), two molecules implicated in the development of BPD. Furthermore, we (25) and others (27) demonstrated that intervention with an S1P analog in murine models of BPD and emphysema, respectively, had a favorable effect on alveolar abnormalities. Ceramides are well known inducers of cell death pathways (20), and have been shown to act as an activation signal for autophagy (19, 29). However, the role of ceramides in mediating MV-induced apoptosis, and possibly autophagy, has yet to be investigated.

In the current study, we tested the hypothesis that ventilation-induced ceramides in newborns promote autophagy-mediated lung epithelial apoptosis. We examined the interrelationship between ceramide, autophagy, and apoptosis in regulating MV-induced cell death using *in vivo* and *in vitro* rat models of MV. We demonstrate that MV increases pulmonary ceramide levels that trigger autophagy and subsequent extrinsic apoptosis of lung epithelial cells. Moreover, prevention of ceramide generation confers protection against ventilation (stretch)-induced injury in newborn rats by attenuating autophagy and apoptosis.

Methods

For a detailed description of methods, consult the online supplement.

Animals

Timed pregnant Sprague-Dawley rats were obtained from Charles River Laboratories, and animal studies were conducted according to criteria established by the Canadian Council on Animal Care and approved by the Animal Care and Use Committee of the Hospital for Sick Children (Toronto, ON, Canada).

MV and BAL Fluid Collection

Rat pups (7 d old) were ventilated using a rodent ventilator (FlexiVent; Sci-req) with moderate V_T (inspired V_T = 7–9 ml/kg; respiratory rate = 150 min^{-1}; positive end-expiratory pressure = 2 cm H_2O) in room air for 4, 12, or 24 hours as previously described (7, 30). BAL fluid (BALF) was collected (31) and stored at −80°C before ceramide measurements. Lung tissues were collected, flash frozen, and stored at −80°C until further analysis or prepared for histology.

Mechanical Stretch of Cells

Day-19 fetal rat lungs were collected from timed-pregnant rats, and fetal rat lung epithelial (FRLE) cells were isolated and subjected to stretch, as previously described (7). Briefly, 24 hours after isolation, FRLE cells were seeded ($\sim 7.5 \times 10^5$ cells/well) onto silastic collagen type I–coated Bioflex six-well plates (Flexcell, BF3001C) and maintained in Eagle's minimum essential medium (MEM) plus 5% (vol/vol) FBS. After 24 hours of incubation, the medium was changed to MEM plus 0.5% (vol/vol) FBS for 1–2 hours and refreshed again with MEM plus 0.5% (vol/vol) FBS just before mounting in a Flexercell FX-4000 Strain Unit (Flexercell). Cyclic continuous radial elongations of 20% were applied at intervals of 30 cycles per minute for various times. Depending on experimental time points (15, 30, 45, and 60 min and 4, 8, and 12 h), stretched cells were collected and prepared for further analyses. Control cells were grown on silastic collagen type I–coated Bioflex plates and incubated in MEM plus 0.5% (vol/vol) FBS, but were not subjected to stretch.

Mass Spectral Analysis of Sphingolipids

Both cells and BALFs were processed for sphingolipid extraction and mass spectral analysis as previously described (25). The procedure of mass spectral analysis is detailed in the online supplement.

Western Blotting

Immunoblotting analysis was conducted as previously described (30). β-Actin was used as protein loading control. Antibodies and their concentrations are described in Table E1 in the online supplement.

Transmission Electron Microscopy

FRLE cells were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Samples were prepared and photographed using a JEOL JSM 6700F transmission electron microscope, as previously described (32).

Statistical Analysis

All values are presented as means (±SEM) of at least three separate experiments. Statistical analysis was done by unpaired student *t* test for two groups, or one-way ANOVA for more than two groups. GraphPad Prism Version 6.0 software (GraphPad Software Inc.) was used. Statistical significance was defined as P less than 0.05.

Results

MV Temporally Induces Autophagy and Apoptosis

First, we investigated the incidence of apoptosis in rat pups exposed to 12 and 24 hours of ventilation. Western blotting demonstrated that 24 hours of ventilation with moderate V_T induces apoptosis (i.e., significant increases in Cl.CASP3 [cleaved caspase-3] and Cl.PARP [cleaved poly(ADP-ribose) polymerase] levels in ventilated versus nonventilated lungs; Figure 1A, $P < 0.05$). Shorter ventilation (12 h) significantly increased Cl.CASP8, but not Cl. CASP3 and Cl.PARP, levels (Figure E1A, $P < 0.05$), suggesting a temporal activation of the extrinsic cell death pathway by ventilation. Next, we asked whether autophagy is involved in the apoptotic response of newborn rat lung to MV. Western blot analysis of autophagy-related proteins revealed significant increases in ATG5–12 (autophagy related 5–12) and LC3B-II (microtubule-associated proteins 1A/1B light chain 3B-II) that was accompanied by a decrease in p62/SQSTM1 (sequestosome1) in lungs of newborn rats ventilated for 12 hours compared with nonventilated controls (Figure 1B, $P < 0.05$). Immunohistochemistry using an anti-LC3B antibody corroborated the immunoblot findings (Figure E1B). Immunoblotting revealed no significant differences in ATG5–12, p62/SQSTM1 and LC3B-II levels after 24 hours of ventilation (Figure E1C), suggesting that MV-induced autophagy precedes apoptosis in the newborn rat lung.

Mechanical Stretch Temporally Triggers Autophagy and Apoptosis

To study the mechanism responsible for ventilation-induced autophagy and apoptosis in newborn rat lungs, we used primary isolated FRLE cells as an *in vitro* preterm model (7). The cells were subjected to cyclic stretch for up to 12 hours and apoptosis was assessed. We observed that Cl.CASP8 levels and activity peaked within 45 minutes of cyclic stretch and declined afterwards (Figure 2A, $P < 0.05$). The initial CASP8 activation was followed by cleavage and activation of downstream effector CASP3, as demonstrated by elevated Cl.CASP3 and Cl.PARP levels after 8 and 12 hours of cyclic stretch (Figure 2B, $P < 0.05$). Flow cytometry for annexin V supported these apoptotic findings (Figure E2A). Next, we determined whether *in vivo* ventilation-induced autophagy could be recapitulated in an *in vitro* setting and measured the autophagy marker, LC3B-II, in cell lysates from FRLE cells exposed to various times (0, 4, 8, 12 h) of cyclic stretch. No significant changes in LC3B-II levels were noted between these time points (Figure E2B). We then speculated that autophagy may be an earlier response to mechanical stretch and, therefore, measured autophagy proteins at 15-minute intervals after initiation of cyclic stretch (Figure 2C). A significant increase in autophagy-related proteins (ATG5–12, ATG7, and LC3B-II) and p62/SQSTM1 turnover were observed within the first hour of stretch, consistently reaching greatest levels after 30–45 minutes (Figure 2C, $P < 0.05$). Transmission electron microscopy, the gold standard for autophagy detection, confirmed the presence of autophagosomes in FRLE cells stretched for 45 minutes compared with static controls (Figure 2D). Assessment of LC3B-II expression and p62/SQSTM1 turnover in the presence and absence of bafilomycin A_1 (blocks autophagosome–lysosome fusion) (10) revealed significantly increased LC3B-II and p62/SQSTM1 levels in bafilomycin A_1–treated stretched cells compared with nontreated stretched cells (Figure E2C, $P < 0.05$), indicating that the elevated LC3B-II levels in stretched FRLE cells were due to rapid activation of the autophagy pathway.

Mechanical Stretch–induced Autophagy Mediates Apoptosis

We wondered whether the rapid activation of autophagy after ventilation (stretch) affected subsequent apoptosis. Therefore, we examined the effect of autophagy inhibition on apoptosis. Pretreatment of FRLE cells with various class III phosphatidylinositol-3-kinase inhibitors, 3-methyladenine, KU5593, and Gö6976 (33), before cyclic stretch, prevented the rapid (45-min) upregulation of LC3B-II by stretch (Figure 3A) and significantly reduced Cl.CASP3 levels in FRLE cells stretched for 12 hours (Figure 3B, $P < 0.05$). Knockdown of *Atg7* with siRNA abrogated the rapid (45-min) LC3B-II induction, as well as the increase in Cl. CASP3 after 12 hours of stretching relative to scrambled siRNA–treated

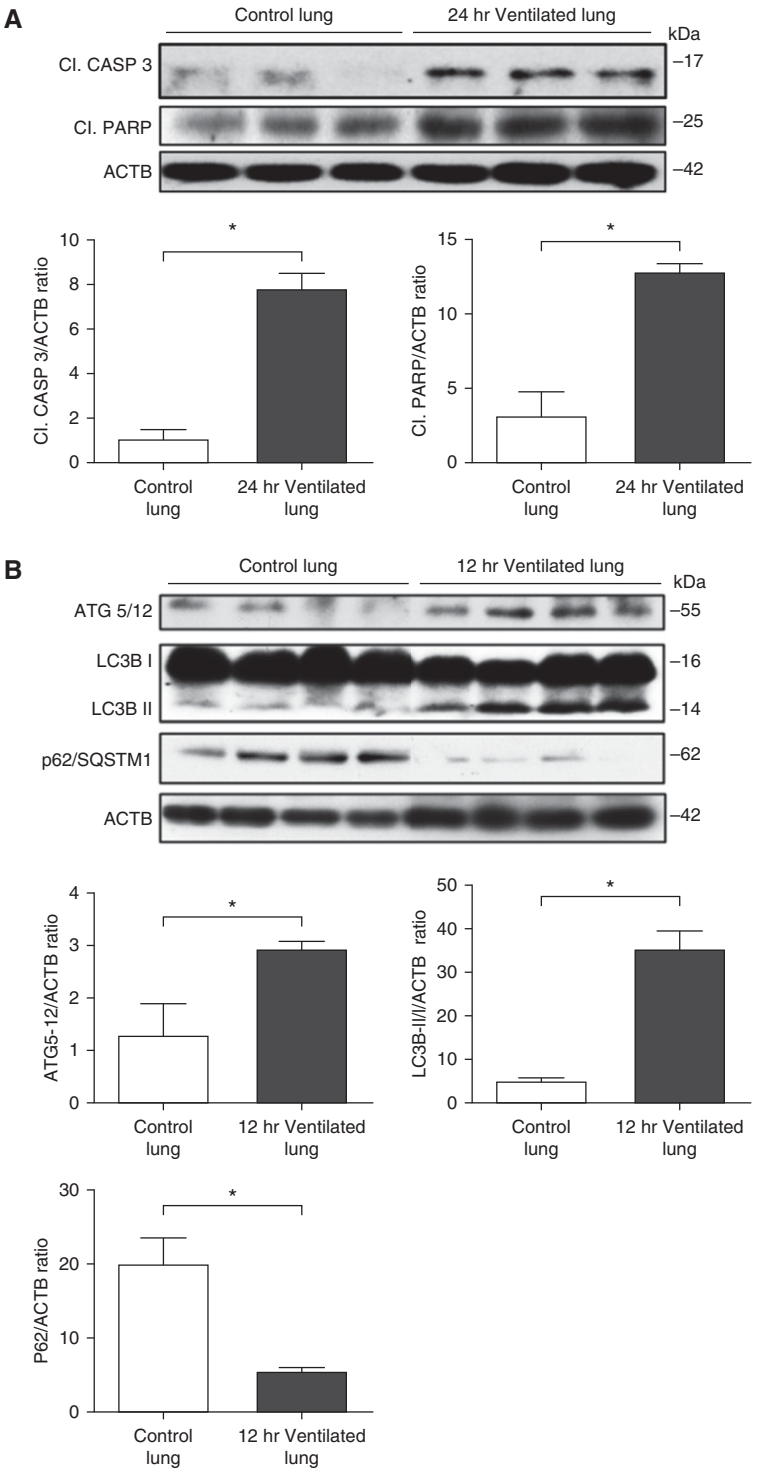

Figure 1. Mechanical ventilation (MV) of newborn rat induces pulmonary autophagy and apoptosis in a time-dependent manner. (*A*, top panel) Representative immunoblots of Cl.CASP3 (cleaved caspase-3) and Cl.PARP (cleaved PARP) in tissue lysates of newborn rat lung subjected to 24 hours of MV compared with nonventilated rat lung control. (*A*, bottom panels) Densitometric analysis of Cl.CASP3 and Cl.PARP proteins in ventilated versus control rat lung ($n = 3$). (*B*, top panel) Representative immunoblots for ATG5–12, LC3B-II, and p62/SQSTM1 in tissue lysates of newborn rat lung subjected to 12 hours of MV compared with nonventilated rat lung control. (*B*, bottom panels) Densitometric analysis of ATG5–12, LC3B-II, and p62/SQSTM1 (bottom panels) in ventilated versus control rat lung ($n = 4$). "*n*" indicates number of individual pups. *$P < 0.05$. ACTB = β-actin.

control cells (Figure 3C, $P < 0.05$). Next, we determined the effect of caspase inhibition on autophagy activation. Treatment of FRLE cells with Z-VAD-FMK (pan-caspase inhibitor) significantly decreased Cl.CASP3 levels in FRLE cells subjected to 8–12 hours of stretch compared with untreated stretched cells (Figure E3A, $P < 0.05$). However, Z-VAD-FMK did not affect the stretch-induced LC3B-II formation at 45–60 minutes (Figure E3A).

MV (Stretch) Induces Autophagy and Apoptosis via Ceramide

To explore the role of ceramides in MV-induced cell death, we first assessed whether ceramide levels in BALF of ventilated newborn rat lungs were altered. We found that ventilation of newborn rats for 12 and 24 hours significantly increased the BALF content of multiple ceramide species compared with control rat pups (Figures 4A and 4B, and Tables E2 and E3, $P < 0.05$). Particularly long-chain C16 ceramide was greatly elevated (Figures 4A and 4B, and Tables E2 and E3, $P < 0.05$). This ventilation-induced increase in ceramides was specific, as no changes were noted in BALF content of C16 sphingomyelin (Tables E2 and E3) or any lysophosphatidylcholine or phosphatidylcholine species (Table E4). In addition, BALF sphingosine, S1P, and dihydroceramide content was not increased by ventilation (Figure E3B). Therefore, further analysis focused solely on C16 ceramide.

Like ventilated newborn lungs, C16 ceramide was rapidly increased in FRLE cells subjected to stretch (Figure E4A, $P < 0.05$), just before the peak of LC3B-II expression that occurred after 45 minutes of stretch. Next, we investigated whether C16 ceramide could induce autophagy and apoptosis in FRLE cells. C16 ceramide stimulated p62/SQSTM1 degradation and increased LC3B-II levels within 45 minutes of incubation (Figure 4C, $P < 0.05$). CASP8 activation was increased after 45 minutes and peaked at 4 hours after treatment (Figure E4B, $P < 0.05$). At the latter time point we observed significant increases in Cl.CASP3 levels (Figure E4C, $P < 0.05$). These temporal induction patterns of LC3B-II, Cl.CASP8, and Cl.CASP3 after C16 ceramide treatment were strikingly similar to those observed in stretch-induced autophagy and apoptosis (Figures 2A and 2C). Furthermore, siRNA silencing of *Atg7*

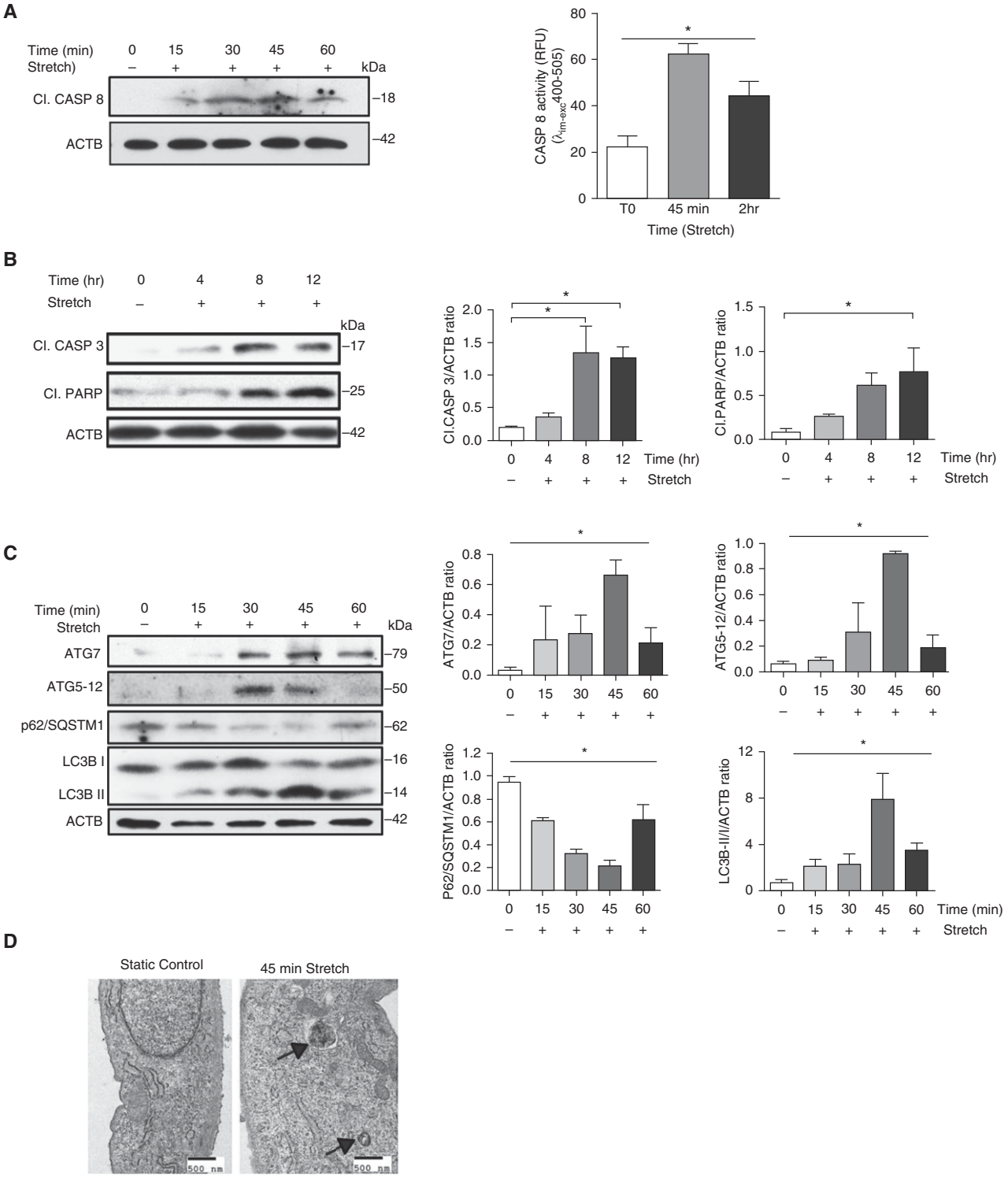

Figure 2. Mechanical stretch induces autophagy and apoptosis in fetal rat lung epithelial (FRLE) cells in a time-dependent manner. (*A*, left panel) Representative immunoblot for cleaved caspase (Cl.CASP)-8 protein in lysates from FRLE cells subjected to stretch for 0, 15, 30, 45, and 60 minutes. (*A*, right panel) CASP8 activity measured in whole-cell lysates by fluorometric assay ($n = 4$). (*B*, left panel) Representative immunoblots for Cl.CASP3 and cleaved PARP (Cl.PARP) proteins in lysates from FRLE cells subjected to stretch for 0, 4, 8, and 12 hours. (*B*, right panels) Densitometric analysis of Cl.CASP3 and Cl.PARP ($n = 3$). (*C*, top panel) Representative immunoblots for ATG7, ATG5–12, p62/SQSTM1, and LC3B-II in lysates of FRLE cells subjected to stretch for 0, 15, 30, 45, and 60 minutes. (*C*, bottom panels) Densitometric analysis of ATG7, ATG5–12, p62/SQSTM1, and LC3B-II ($n = 3$). (*D*) Representative transmission electron microscope images of an FRLE cell subjected to stretch for 45 minutes (right) compared with a static control cell (left). Autophagosomes are denoted by arrows. "*n*" indicates number of separate cell culture isolations. Scale bars = 500 nm; $^*P < 0.05$. ACTB = β-actin; RFU = relative fluorescent units.

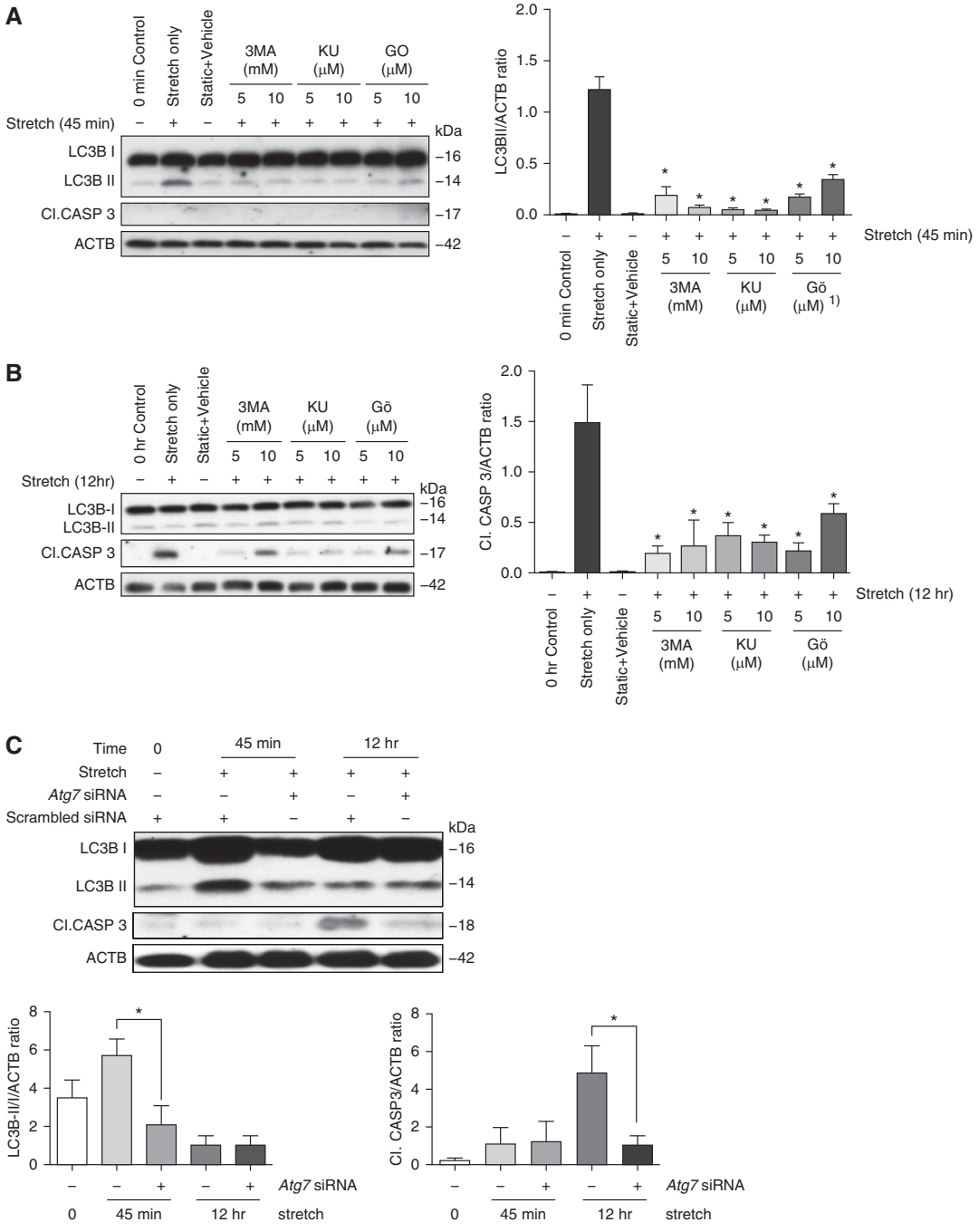

Figure 3. Mechanical stretch–induced autophagy mediates apoptosis in fetal rat lung epithelial (FRLE) cells. (*A*, left panel) Representative immunoblots for LC3B-II and cleaved caspase (Cl.CASP)-3 in lysates of FRLE cells subjected to stretch for 45 minutes in the presence of various class III phosphatidylinositol-3-kinase (PI3K) inhibitors. (*A*, right panel) Densitometric analysis of LC3B-II ($n = 3$). (*B*, left panel) Representative immunoblots for LC3B-II and Cl.CASP3 in lysates of FRLE cells treated with vehicle (control) or class III PI3K inhibitors and subjected to stretch for 12 hours ($n = 3$). (*B*, right panel) Densitometric analysis of Cl.CASP3 ($n = 3$). (*C*, top panel) Representative immunoblots for LC3B-II and Cl.CASP3 proteins in lysates of FRLE cells transfected with scrambled (control) or *Atg7* siRNA and subjected to stretch for 45 minutes or 12 hours. (*C*, bottom panels) Densitometric analysis of LC3B-II and Cl.CASP3 relative to scrambled siRNA control cells ($n = 3$). "*n*" indicates number of separate cell culture isolations. *$P < 0.05$. 3MA = 3-methyladenine; ACTB = β-actin; Gö = Gö6976; KU = KU5593.

in FRLE cells before exogenous C16 ceramide exposure ameliorated ceramide-induced LC3B-II and Cl.CASP3 (Figure 4D, $P < 0.05$).

We then investigated whether ventilation (stretch)-induced ceramide generation is responsible for subsequent autophagy and apoptosis. Pretreatment of FRLE cells with myriocin, an inhibitor of the ceramide *de novo* synthesis pathway (34), did not block the rapid LC3B-II induction in stretched FRLE cells (Figure E5A). Thus,

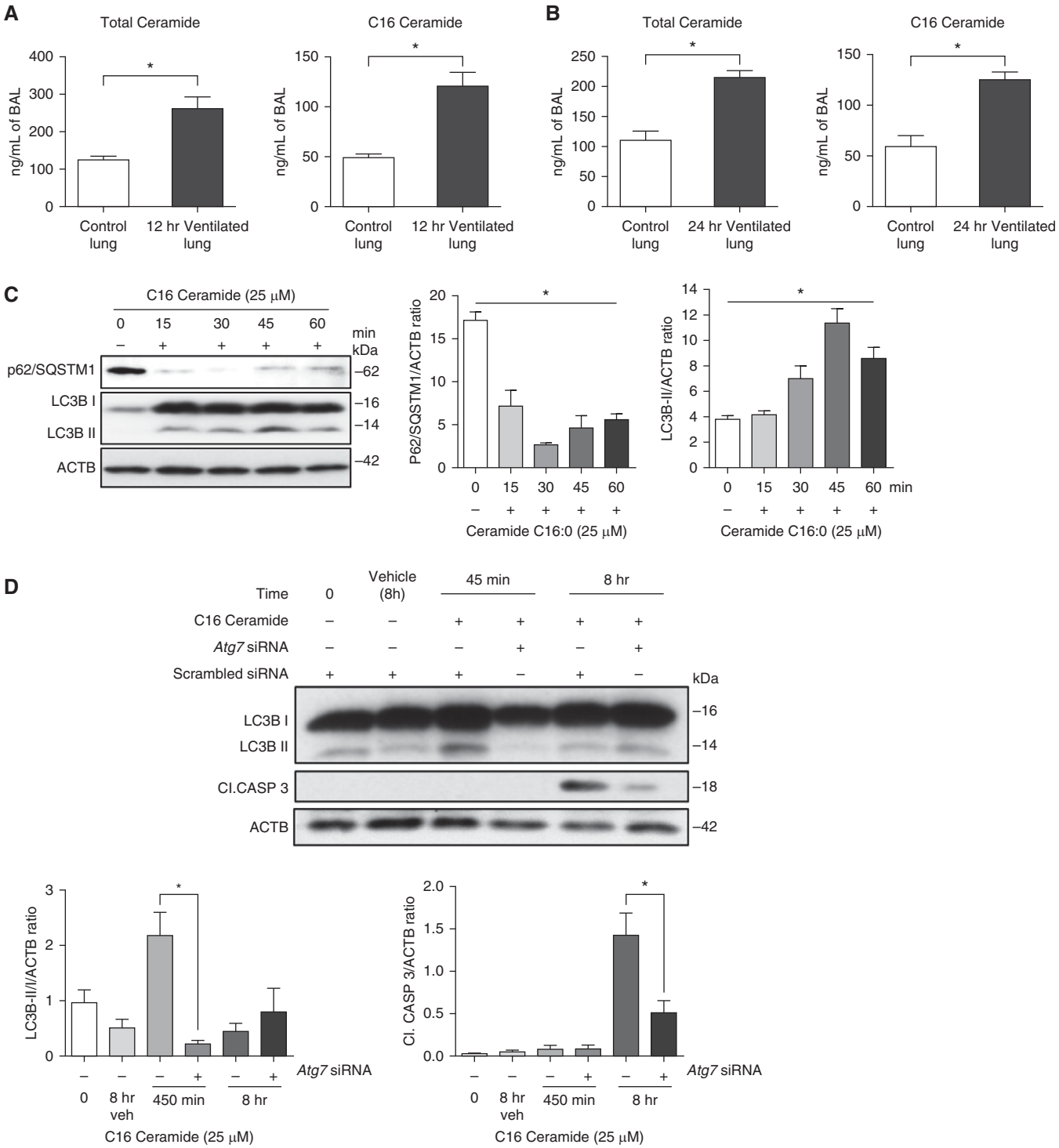

Figure 4. Mechanical ventilation (stretch)–induced ceramide generation triggers autophagy and apoptosis in fetal rat lung cells. (*A* and *B*) Total ceramide and C16 ceramide content in BAL fluid of newborn pups after 12 hours ($n = 8$) (*A*) and 24 hours ($n = 6$) (*B*) of ventilation compared with nonventilated ($n =$ 6–8) controls, respectively ($n =$ number of individual pups). (*C*) Representative immunoblots (left panel) and densitometric analysis (right panels) for p62/SQSTM1 and LC3B-II in lysates of fetal rat lung epithelial (FRLE) cells treated with C16 ceramide for 60 minutes ($n = 3$). (*D*, top panel) Representative immunoblots for LC3B-II and cleaved caspase (Cl.CASP)-3 of lysates of FRLE cells transfected with scrambled or *Atg7* siRNA and then treated with C16 ceramide for 45 minutes or 8 hours. (*D*, bottom panels) Densitometric analysis of LC3B-II after 45 minutes of C16 ceramide treatment and Cl.CASP3 after 8 hours of C16 ceramide treatment ($n = 3$). (*C* and *D*) "*n*" indicates number of separate cell culture isolations. $^*P < 0.05$. ACTB = β-actin.

we thought that the increase in ceramide may be due to breakdown of sphingomyelin to ceramide by acid sphingomyelinase (sphingomyelin phosphodiesterase [SMPD] 1). Indeed, SMPD1 activity was significantly increased in FRLE cells stretched for 30 minutes (Figure 5A, $P < 0.05$). To further assess the contribution of SMPD1 to increased ceramide levels and subsequent autophagy and apoptosis, FRLE cells were treated with inhibitors of SMPD1, fluoxetine (35) and desipramine (36), and subjected to mechanical stretch. Inhibition of SMPD1 significantly decreased LC3B-II levels and CASP8 activation after 45-minute stretch (Figures 5B and 5C, $P < 0.05$), as well as Cl.CASP3 levels after 8 hours of stretch compared with controls (Figure 5D, $P < 0.05$).

To extrapolate these *in vitro* findings to the *in vivo* setting, we first measured SMPD1 activity in lungs of ventilated newborn rats. SMPD1 activity was significantly increased in newborn rat lungs ventilated for 12 hours (Figure 6A, $P < 0.05$). To further study the effect of SMPD1 inhibition on MV-induced autophagy, newborn rats were administered desipramine before ventilation (7). BALF C16 ceramide was increased after 4 hours of ventilation. This increase was significantly reduced in rat pups pretreated with SMPD1 inhibitor (Figure 6B, $P < 0.05$). LC3B-II and ATG5–12 levels were also significantly reduced in the lungs of the desipramine-treated versus nontreated rat pups (Figure 6C, $P < 0.05$), demonstrating that inhibition of SMPD1 limits MV-induced autophagy in newborn rats. In line with our 12-hour ventilation data, no CASP3 activation was noted after 4 hours of ventilation.

Mechanical Stretch– and Ceramide-induced LC3B Interacts with FAS

To gain insight into the mechanism by which stretch/ceramide–induced autophagy leads to extrinsic apoptosis, we investigated the effect of autophagy inhibition on CASP8 activation. Treatment of FRLE cells with class III phosphatidylinositol-3-kinase inhibitors abolished stretch-induced Cl. CASP8 expression and activation (Figure 7A and Figure E5B, $P < 0.05$). A similar observation was made when FRLE cells were transfected with *Atg7* siRNA before a 45-minute stretch (Figure 7B, $P < 0.05$). Moreover, *Atg7* knockdown inhibited CASP8 cleavage and activation in FRLE cells that were treated with C16 ceramide (Figure 7B and Figure E5C, $P < 0.05$).

Autophagy interplays with apoptosis via an interaction between LC3B and the FAS cell death receptor, a major inducer of CASP8 activation (37, 38). Thus, we next examined whether LC3B interacts with FAS after exposure of FRLE cells to stretch or ceramide. Coimmunoprecipitation assays revealed binding of LC3B with FAS after 45-minute treatment with C16 ceramide (Figure 7C) or exposure to

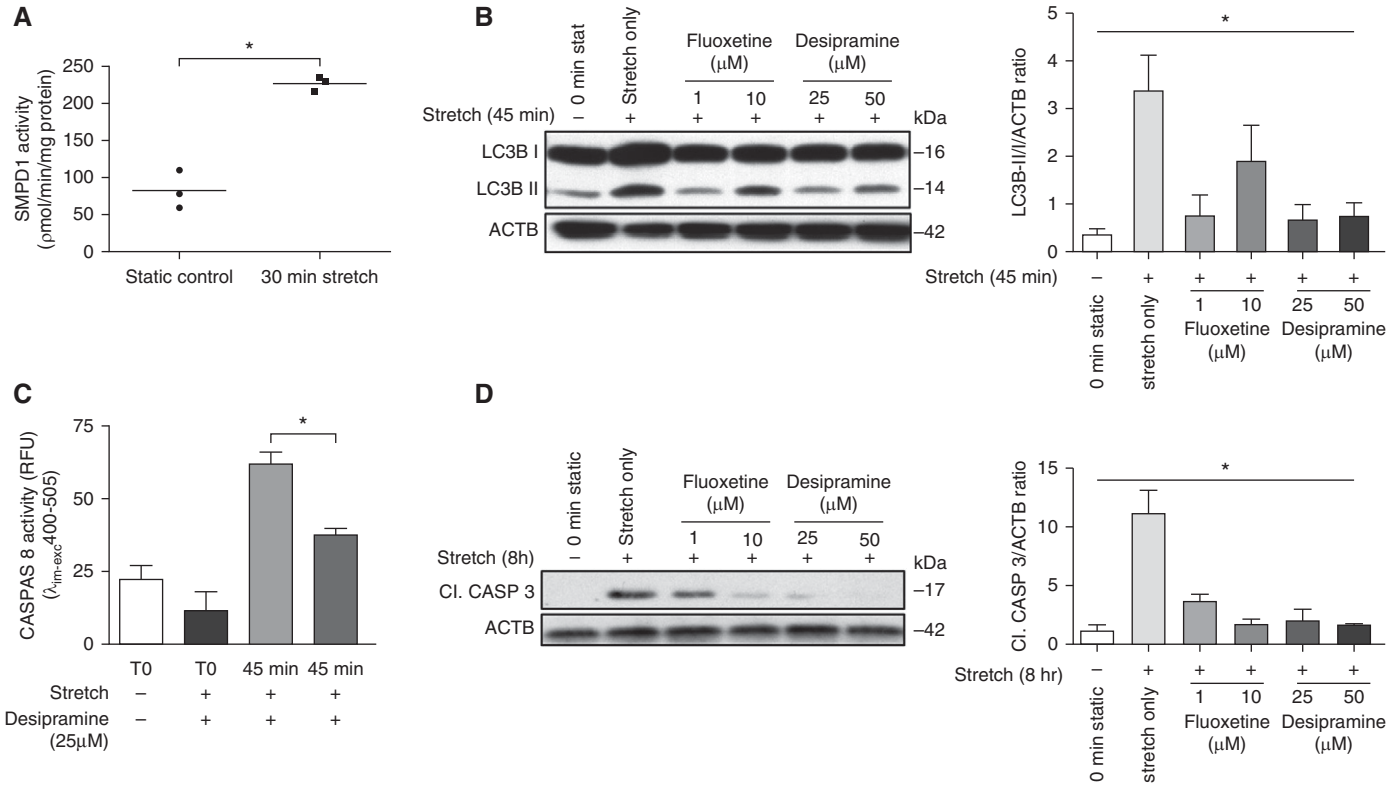

Figure 5. Inhibition of acid sphingomyelinase prevents mechanical stretch–induced autophagy and apoptosis in fetal rat lung epithelial (FRLE) cells. (*A*) SMPD-1 (sphingomyelin phosphodiesterase-1) activity in FRLE cells subjected to stretch for 30 minutes compared with static cultured FRLE cells. (*B*) Representative immunoblots (left panel) and densitometric analysis (right panel) for LC3B-II in lysates of FRLE cells subjected to stretch for 45 minutes in the presence and absence of acid sphingomyelinase (SMPD1) inhibitors, fluoxetine and desipramine ($n = 3$). (*C*) CASP8 (caspase-8) activity in FRLE cells subjected to stretch for 45 minutes in absence and presence of desipramine ($n = 4$). (*D*) Representative immunoblots (left panel) and densitometric analysis (right panel) for cleaved CASP (Cl.CASP)-3 in lysates of FRLE cells after 8 hours of stretch ($n = 3$). "*n*" indicates number of separate cell culture isolations. *$P < 0.05$. ACTB = β-actin; RFU = relative fluorescent units.

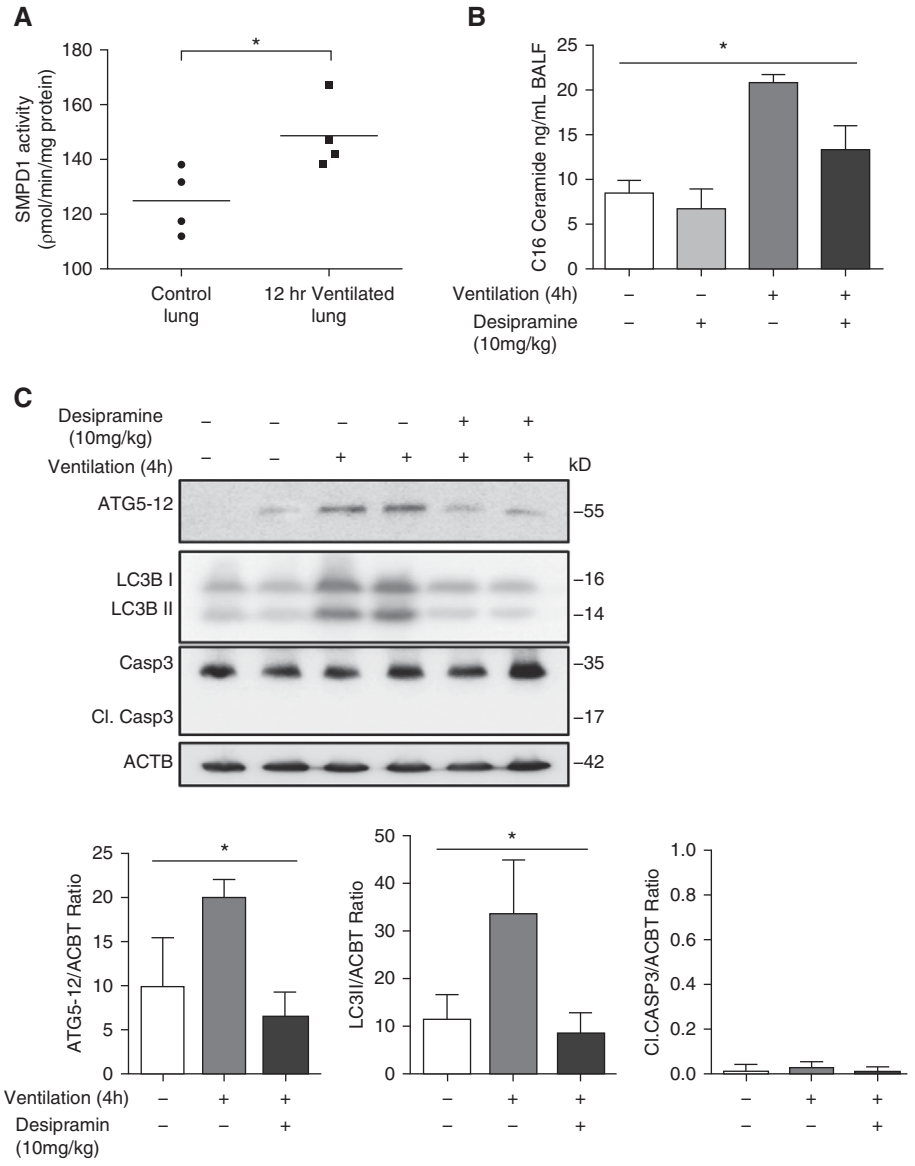

Figure 6. Inhibition of mechanical ventilation (MV)–induced ceramide production reduces autophagy and apoptosis in newborn rat lungs. (*A*) SMPD-1 (sphingomyelin phosphodiesterase-1) activity in tissue lysates of newborn rat lung subjected to 12 hours of MV ($n = 4$) compared with nonventilated newborn rat lungs. (*B*) C16 ceramide content in BAL fluid (BALF) of rat pups pretreated with or without desipramine (10 mg/kg) and ventilated for 4 hours ($n = 4$). (*C*, top panel) Representative immunoblots of ATG5–12, LC3B-II and Cl.CASP3 (cleaved caspase-3) in lung tissue lysates of two newborn rats pretreated with or without desipramine before 4 hours of MV ($n = 4$). (*C*, bottom panels) Densitometric analysis of ATG5–12, LC3B-II and Cl.CASP3. "*n*" indicates number of individual pups. $^*P < 0.05$. ACTB = β-actin.

stretch (Figure 7D). To determine whether stretch-induced C16 ceramide triggers this interaction between LC3B and FAS in FRLE cells, we performed coimmunoprecipitation assays on stretched FRLE cells in the presence and absence of desipramine. Desipramine abolished stretch-induced LC3B–FAS binding (Figure 7E), suggesting that stretch/ceramide–induced autophagy may activate the extrinsic death pathway via a LC3B and FAS interaction.

Discussion

Several studies have implicated apoptosis as a culprit for the adverse effects of MV on alveolar development (6, 7). Previous work from our laboratory showed that prolonged ventilation in newborn rats leads to cell cycle arrest (30) and apoptotic cell death via the FAS receptor (7). In the current study, we confirm the latter findings and reaffirm that both MV of newborn rats and cyclic stretch of FRLE cells induces extrinsic apoptosis by a mechanism that depends on activation of the autophagy pathway by ceramides in a time-dependent manner. MV-elevated ceramides induce autophagy that is upstream to FAS-mediated CASP8- and CASP3-dependent extrinsic apoptosis. Blocking ceramide generation by inhibiting SMPD1 activity during MV prevents autophagy and subsequent apoptotic cell death. These findings extend our mechanistic understanding of MV-induced cell death in newborn lungs as described in the following section.

MV Triggers Autophagy and Extrinsic Apoptosis in a Time-Dependent Manner

In the present study, we found a temporal activation of both autophagy and apoptosis after MV. *In vitro*, stretch activates autophagy in FRLE cells within 30–45 minutes, whereas *in vivo* MV triggers autophagy in newborn rat lung after 4 hours. The increase in autophagy was accompanied by augmented Cl.CASP8 activation that occurred before apparent increases in Cl.CASP3 and Cl.PARP, which were augmented after 8 hours of stretch and 24 hours of ventilation, respectively. These findings support the idea that MV (stretch)-induced autophagy precedes apoptosis. Increased autophagy has also been reported in lungs of hyperoxia-exposed newborn mice as well as in lung tissues of premature infants with BPD (39). Interestingly, autophagy was induced within 15 minutes of exposure of FRLE cells to mechanical stretch, whereas hyperoxia (39) required a much longer time to induce autophagy (>4 h). This suggests that autophagy induction pathways triggered by stretch and hyperoxia are likely different. Autophagy and apoptosis can be positively and negatively interconnected (11, 18, 38). Here, we found a requirement for autophagy in MV (stretch)-induced apoptosis as inhibition of autophagy reduced CASP8 and CASP3 cleavage. In contrast, stimulation of autophagy with rapamycin reduced apoptosis and improved lung architecture in hyperoxia-exposed newborn mice (39).

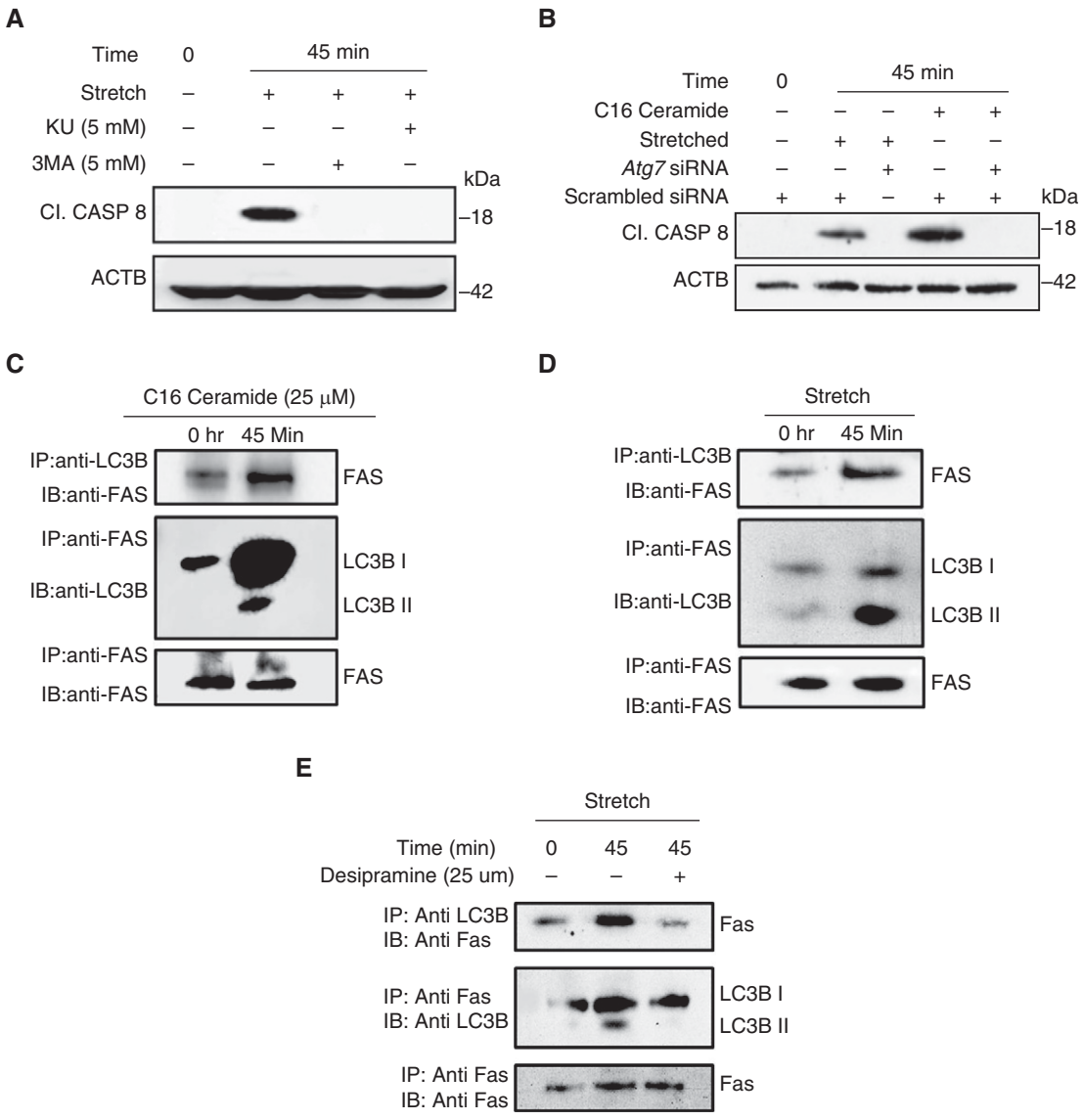

Figure 7. Mechanical stretch– and ceramide-induced autophagy stimulates CASP8 (caspase-8) cleavage via an interaction between LC3B and FAS (first apoptosis signal) in fetal lung epithelial cells. (*A*) Representative immunoblot for cleaved CASP (Cl.CASP)-8 in lysates of fetal rat lung epithelial (FRLE) cells pretreated for 60 minutes with 3-methyladenine (3MA) and KU5593 (KU), and then stretched for 45 minutes. (*B*) Representative immunoblots for Cl.CASP8 in lysates of FRLE cells transfected with scrambled (control) or *Atg7* siRNA and then treated with either C16 ceramide or subjected to stretch for 45 minutes. (*C* and *D*) Representative coimmunoprecipitation assays of LC3B and FAS in FRLE cell lysates treated with C16 ceramide (*C*) or stretched for 45 minutes (*D*). Representative coimmunoprecipitation assay of LC3B and FAS after inhibition of SMPD-1 (sphingomyelin phosphodiesterase-1) activity. FRLE cells were subjected to stretch for 45 minutes in the presence and absence of 25 μm desipramine. Lysates were immunoprecipitated (IP) with anti-LC3B or anti-FAS. IPs were then immunoblotted (IB) with anti-FAS or anti-LC3B, respectively. All blots are representative of two to three independent experiments. ACTB = β-actin.

These opposite findings underscore that autophagy-apoptotic relations are highly context dependent.

MV-induced Autophagy Triggers Extrinsic Apoptosis via LC3B–FAS Interaction

In the current study, we detected an MV (stretch)-induced association between LC3B-II and FAS. Because LC3B has been reported to mediate CASP8 activation (40, 41), we speculate that the LC3B–FAS interaction rapidly activates CASP8 and thereby the extrinsic cell death pathway. Our observation that inhibition of LC3B-II formation attenuated stretch-induced CASP8 activation, and downstream Cl.CASP3 signaling supports this speculation.

It is also possible that the LC3B–FAS interaction results in necroptosis, a nonapoptotic form of cell death, because it is generally triggered by engagement of TNF-α with TNF receptor superfamily members, such as FAS and Toll-like receptors (42). Emerging evidence suggests that autophagy regulates

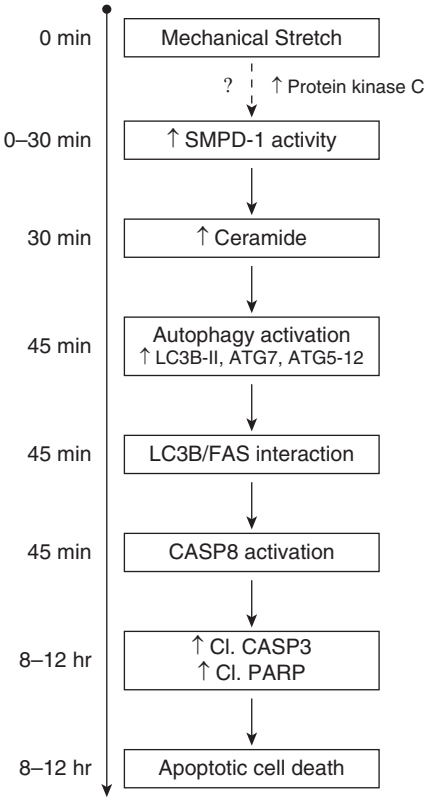

Figure 8. Proposed mechanism of mechanical stretch–induced apoptosis. Mechanical ventilation elicits generation of ceramide via SMPD-1 (sphingomyelin phosphodiesterase-1) activation, leading to activation of autophagy. Autophagic protein LC3B subsequently interacts with FAS (first apoptosis signal), which initiates extrinsic apoptosis via activation of CASP8 (caspase 8) followed by cleavage of CASP3 (Cl. CASP3) and PARP (Cl. PARP) and ensuing completion of apoptotic cell death.

necroptosis (43), but, to our knowledge, nobody has yet reported the effect of MV on this cell death pathway. The linkage of MV (stretch)-induced autophagy to apoptosis via LC3B–FAS is further supported by recent studies showing that activation of extrinsic apoptosis in lung epithelial cells after hyperoxia and cigarette smoke exposure involves an interaction between LC3B and FAS that is mediated by Cav-1 (caveolin-1) (37, 38). However, the outcome of the LC3B–FAS interaction is context dependent. Like our present study, silencing of LC3B in cigarette smoke–induced cell death results in inhibition of apoptosis by increasing Cav-1–dependent FAS sequestration (38). In contrast, in hyperoxia-induced cell death, LC3B silencing promotes apoptosis, whereas overexpressing LC3B confers cytoprotection (37). These different outcomes may be due to different binding sites and/or affinities between LC3B and FAS.

MV-induced Ceramide Generation Activates Autophagy

Sphingolipids are important mediators of lung injury in various respiratory disorders (20–24, 27, 28). Here, we observed that ceramides, specifically C16 ceramide, were increased in ventilated newborn rat lung. C16 ceramide levels increased in BALFs of lungs ventilated up to 12 hours, but remained constant thereafter, suggesting rapid ceramide generation during the first 12 hours of ventilation with no further upregulation. We have recently measured ceramide levels in 122 infants born 32 weeks or less of gestational age exposed to MV (26). All infants displayed an increase in ceramides in their tracheal aspirates after the first day of intubation (26), in line with our newborn rat findings. Ceramide is a well-established apoptosis inducer (21, 23, 44); however, recent studies have also implicated ceramide in the induction of autophagy (19, 29). We found that exogenous C16 ceramide activated both autophagy and apoptosis in FRLE cells in similar temporal patterns as that seen in the stretch-activated response. When we performed siRNA knockdown of *Atg7* before C16 ceramide exposure, autophagy was inhibited, whereas apoptosis was partially, but significantly, reduced. Thus, C16 ceramide likely induces apoptosis in immature lung epithelial cells via the autophagy pathway. C16 ceramide has recently been shown to trigger necroptosis in lung epithelial cells exposed to cigarette smoke (45), but it is unknown whether this cell death pathway is activated by MV.

Blockage of SMPD1 Inhibits MV-induced Autophagy and Extrinsic Apoptosis

In the present study, inhibition of the ceramide *de novo* synthesis pathway by myriocin did not affect stretch-induced autophagy, suggesting that elevated ceramide levels could be due to increased sphingomyelinase activity. Sphingomyelinases convert sphingomyelin to ceramide, and are characterized by their pH optimum as acid, neutral, and alkaline isoforms. Here, we focused on SMPD1, an acid spingomyelinase found mainly in lysosomes and in secretory form (46, 47). Its activation above homeostatic levels has been implicated in stress-induced autophagy (48, 49). In the present study, we found increased SMPD1 activity, which we contribute to lysosomal SMPD1, in ventilated rat lung and stretched FRLE cells compared with their respective controls. We have previously shown that cyclic stretch rapidly (<5 min) induces the phospholipase C-γ–protein kinase C (PKC) pathway in fetal lung cells (50). Because SMPD1 is activated by PKCδ phosphorylation (51), it is plausible that MV (stretch)-induced SMPD1 activity is transduced via PKC-dependent phosphorylation. Importantly, we demonstrated that inhibition of SMPD1 activity ameliorated stretch-induced autophagy and apoptosis. In addition, SMPD1 inhibition interrupted the interaction between LC3B-II and FAS. This supports the idea that stretch-induced association between LC3B-II and FAS is mediated via stretch-stimulated ceramide generation. A limitation of our neonatal ventilation model is that histological lung damage is only notable after more than 8 hours of ventilation (30). Because we were interested in the pathological pathways initiating the injury, we ventilated for up to 4 hours to assess the SMPD1 intervention. Although SMPD1 inhibition prevented activation of cell death pathways within these 4 hours, it remains to be investigated whether this intervention also reduces the histological damage seen at later time points.

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. Previous studies have shown that stimulation of *de novo* ceramide synthesis leads to phosphorylation of BCL-2 (B-cell lymphoma 2), and its subsequent dissociation from BECN1 (beclin 1) results in autophagy activation (19). Ceramide has also been reported to suppress Akt signaling, resulting in negative regulation of mTOR (mammalian target of rapamycin) and autophagy activation (29). Taken

together, our results show that ventilation-induced C16 ceramide buildup in lung epithelial cells activates autophagy, resulting in autophagosome/LC3B-II accumulation that then triggers extrinsic apoptotic epithelial cell death via LC3B–FAS (Figure 8). Our *in vivo* data show that blockage of SMPD1 reduces ventilation-induced ceramide generation and autophagy in newborn rats. Future studies should investigate whether inhibition of ceramide production in ventilation-induced newborn lung injury has potential as a treatment strategy to prevent or limit BPD. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank Jinxia Wang, Irene Tseu, and Angie Griffin for technical assistance, and the Analytical Facility for Bioactive Molecules at the Hospital for Sick Children for their assistance with the lipidomic analysis.

References

1. Jobe AH. The new bronchopulmonary dysplasia. *Curr Opin Pediatr* 2011; 23:167–172.
2. Stoll BJ, Hansen NI, Bell EF, Walsh MC, Carlo WA, Shankaran S, *et al*.; Eunice Kennedy Shriver National Institute of Child Health and Human Development Neonatal Research Network. Trends in care practices, morbidity, and mortality of extremely preterm neonates, 1993–2012. *JAMA* 2015;314:1039–1051.
3. Cools F, Offringa M, Askie LM. Elective high frequency oscillatory ventilation versus conventional ventilation for acute pulmonary dysfunction in preterm infants. *Cochrane Database Syst Rev* 2015;(3): CD000104.
4. Uhlig U, Uhlig S. Ventilation-induced lung injury. *Compr Physiol* 2011;1: 635–661.
5. Carvalho CG, Silveira RC, Procianoy RS. Ventilator-induced lung injury in preterm infants. *Rev Bras Ter Intensiva* 2013;25:319–326.
6. Mokres LM, Parai K, Hilgendorff A, Ertsey R, Alvira CM, Rabinovitch M, *et al*. Prolonged mechanical ventilation with air induces apoptosis and causes failure of alveolar septation and angiogenesis in lungs of newborn mice. *Am J Physiol Lung Cell Mol Physiol* 2010;298: L23–L35.
7. Kroon AA, Delriccio V, Tseu I, Kavanagh BP, Post M. Mechanical ventilation–induced apoptosis in newborn rat lung is mediated via FasL/Fas pathway. *Am J Physiol Lung Cell Mol Physiol* 2013;305: L795–L804.
8. Hargitai B, Szabó V, Hajdú J, Harmath A, Pataki M, Farid P, *et al*. Apoptosis in various organs of preterm infants: histopathologic study of lung, kidney, liver, and brain of ventilated infants. *Pediatr Res* 2001; 50:110–114.
9. Cui Q, Tashiro S, Onodera S, Minami M, Ikejima T. Autophagy preceded apoptosis in oridonin-treated human breast cancer MCF-7 cells. *Biol Pharm Bull* 2007;30:859–864.
10. Yeganeh B, Ghavami S, Rahim MN, Klonisch T, Halayko AJ, Coombs KM. Autophagy activation is required for influenza A virus–induced apoptosis and replication. *Biochim Biophys Acta* 2018;1865: 364–378.
11. Mariño G, Niso-Santano M, Baehrecke EH, Kroemer G. Self-consumption: the interplay of autophagy and apoptosis. *Nat Rev Mol Cell Biol* 2014;15:81–94.
12. Liu Y, Levine B. Autosis and autophagic cell death: the dark side of autophagy. *Cell Death Differ* 2015;22:367–376.
13. Luo S, Rubinsztein DC. Atg5 and Bcl-2 provide novel insights into the interplay between apoptosis and autophagy. *Cell Death Differ* 2007; 14:1247–1250.
14. Kabbage M, Williams B, Dickman MB. Cell death control: the interplay of apoptosis and autophagy in the pathogenicity of *Sclerotinia sclerotiorum*. *PLoS Pathog* 2013;9:e1003287.
15. Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, *et al*. Bcl-2 antiapoptotic proteins inhibit Beclin 1–dependent autophagy. *Cell* 2005;122:927–939.
16. Hussain SN, Mofarrahi M, Sigala I, Kim HC, Vassilakopoulos T, Maltais F, *et al*. Mechanical ventilation–induced diaphragm disuse in humans triggers autophagy. *Am J Respir Crit Care Med* 2010;182:1377–1386.
17. López-Alonso I, Aguirre A, González-López A, Fernández AF, Amado-Rodríguez L, Astudillo A, *et al*. Impairment of autophagy decreases ventilator-induced lung injury by blockade of the NF-κB pathway. *Am J Physiol Lung Cell Mol Physiol* 2013;304:L844–L852.
18. Young MM, Kester M, Wang HG. Sphingolipids: regulators of crosstalk between apoptosis and autophagy. *J Lipid Res* 2013;54:5–19.
19. Pattingre S, Bauvy C, Carpentier S, Levade T, Levine B, Codogno P. Role of JNK1-dependent Bcl-2 phosphorylation in ceramide-induced macroautophagy. *J Biol Chem* 2009;284:2719–2728.
20. Lee J, Yeganeh B, Ermini L, Post M. Sphingolipids as cell fate regulators in lung development and disease. *Apoptosis* 2015;20: 740–757.
21. Petrache I, Natarajan V, Zhen L, Medler TR, Richter AT, Cho C, *et al*. Ceramide upregulation causes pulmonary cell apoptosis and emphysema-like disease in mice. *Nat Med* 2005;11:491–498.
22. Ammit AJ, Hastie AT, Edsall LC, Hoffman RK, Amrani Y, Krymskaya VP, *et al*. Sphingosine 1-phosphate modulates human airway smooth muscle cell functions that promote inflammation and airway remodeling in asthma. *FASEB J* 2001;15:1212–1214.
23. Teichgräber V, Ulrich M, Endlich N, Riethmüller J, Wilker B, De Oliveira-Munding CC, *et al*. Ceramide accumulation mediates inflammation, cell death and infection susceptibility in cystic fibrosis. *Nat Med* 2008;14:382–391.
24. Tibboel J, Reiss I, de Jongste JC, Post M. Ceramides: a potential therapeutic target in pulmonary emphysema. *Respir Res* 2013;14:96.
25. Tibboel J, Joza S, Reiss I, de Jongste JC, Post M. Amelioration of hyperoxia-induced lung injury using a sphingolipid-based intervention. *Eur Respir J* 2013;42:776–784.
26. van Mastrigt E, Zweekhorst S, Bol B, Tibboel J, van Rosmalen J, Samsom JN, *et al*. Ceramides in tracheal aspirates of preterm infants: marker for bronchopulmonary dysplasia. *PLoS One* 2018;13: e0185969.
27. Yasuo M, Mizuno S, Allegood J, Kraskauskas D, Bogaard HJ, Spiegel S, *et al*. Fenretinide causes emphysema, which is prevented by sphingosine 1-phoshate. *PLoS One* 2013;8:e53927.
28. Sparkman L, Chandru H, Boggaram V. Ceramide decreases surfactant protein B gene expression via downregulation of TTF-1 DNA binding activity. *Am J Physiol Lung Cell Mol Physiol* 2006;290:L351–L358.
29. Scarlatti F, Bauvy C, Ventruti A, Sala G, Cluzeaud F, Vandewalle A, *et al*. Ceramide-mediated macroautophagy involves inhibition of protein kinase B and up-regulation of beclin 1. *J Biol Chem* 2004; 279:18384–18391.
30. Kroon AA, Wang J, Kavanagh BP, Huang Z, Kuliszewski M, van Goudoever JB, *et al*. Prolonged mechanical ventilation induces cell cycle arrest in newborn rat lung. *PLoS One* 2011;6:e16910. [Published erratum appears in *PLoS One* 6(3).]
31. Kroon AA, Wang J, Huang Z, Cao L, Kuliszewski M, Post M. Inflammatory response to oxygen and endotoxin in newborn rat lung ventilated with low tidal volume. *Pediatr Res* 2010;68:63–69.
32. Ridsdale R, Tseu I, Wang J, Post M. CTP:phosphocholine cytidylyltransferase alpha is a cytosolic protein in pulmonary epithelial cells and tissues. *J Biol Chem* 2001;276:49148–49155.
33. Farkas T, Daugaard M, Jäättelä M. Identification of small molecule inhibitors of phosphatidylinositol 3-kinase and autophagy. *J Biol Chem* 2011;286:38904–38912.
34. Hanada K, Nishijima M, Fujita T, Kobayashi S. Specificity of inhibitors of serine palmitoyltransferase (SPT), a key enzyme in sphingolipid biosynthesis, in intact cells: a novel evaluation system using an SPT-defective mammalian cell mutant. *Biochem Pharmacol* 2000;59: 1211–1216.
35. Gulbins E, Palmada M, Reichel M, Lüth A, Böhmer C, Amato D, *et al*. Acid sphingomyelinase-ceramide system mediates effects of antidepressant drugs. *Nat Med* 2013;19:934–938.

36. Göggel R, Winoto-Morbach S, Vielhaber G, Imai Y, Lindner K, Brade L, *et al*. PAF-mediated pulmonary edema: a new role for acid sphingomyelinase and ceramide. *Nat Med* 2004;10:155–160.
37. Tanaka A, Jin Y, Lee SJ, Zhang M, Kim HP, Stolz DB, *et al*. Hyperoxia-induced LC3B interacts with the Fas apoptotic pathway in epithelial cell death. *Am J Respir Cell Mol Biol* 2012;46:507–514.
38. Chen ZH, Lam HC, Jin Y, Kim HP, Cao J, Lee SJ, *et al*. Autophagy protein microtubule-associated protein 1 light chain-3B (LC3B) activates extrinsic apoptosis during cigarette smoke–induced emphysema. *Proc Natl Acad Sci USA* 2010;107:18880–18885.
39. Sureshbabu A, Syed M, Das P, Janér C, Pryhuber G, Rahman A, *et al*. Inhibition of regulatory-associated protein of mechanistic target of rapamycin prevents hyperoxia-induced lung injury by enhancing autophagy and reducing apoptosis in neonatal mice. *Am J Respir Cell Mol Biol* 2016;55:722–735.
40. Pan JA, Ullman E, Dou Z, Zong WX. Inhibition of protein degradation induces apoptosis through a microtubule-associated protein 1 light chain 3–mediated activation of caspase-8 at intracellular membranes. *Mol Cell Biol* 2011;31:3158–3170.
41. Young MM, Takahashi Y, Khan O, Park S, Hori T, Yun J, *et al*. Autophagosomal membrane serves as platform for intracellular death-inducing signaling complex (iDISC)–mediated caspase-8 activation and apoptosis. *J Biol Chem* 2012;287:12455–12468.
42. Hanson B. Necroptosis: a new way of dying? *Cancer Biol Ther* 2016;17: 899–910.
43. Goodall ML, Cramer SD, Thorburn A. Autophagy complexes cell death by necroptosis. *Oncotarget* 2016;7:50818–50819.
44. Petrache I, Natarajan V, Zhen L, Medler TR, Richter A, Berdyshev EV, *et al*. Ceramide causes pulmonary cell apoptosis and emphysema: a role for sphingolipid homeostasis in the maintenance of alveolar cells. *Proc Am Thorac Soc* 2006;3:510.
45. Mizumura K, Justice MJ, Schweitzer KS, Krishnan S, Bronova I, Berdyshev EV, *et al*. Sphingolipid regulation of lung epithelial cell mitophagy and necroptosis during cigarette smoke exposure. *FASEB J* 2018;32:1880–1890.
46. Jenkins RW, Canals D, Hannun YA. Roles and regulation of secretory and lysosomal acid sphingomyelinase. *Cell Signal* 2009;21:836–846.
47. Yang Y, Uhlig S. The role of sphingolipids in respiratory disease. *Ther Adv Respir Dis* 2011;5:325–344.
48. Patschan S, Chen J, Polotskaia A, Mendelev N, Cheng J, Patschan D, *et al*. Lipid mediators of autophagy in stress-induced premature senescence of endothelial cells. *Am J Physiol Heart Circ Physiol* 2008;294:H1119–H1129.
49. Lee JK, Jin HK, Park MH, Kim BR, Lee PH, Nakauchi H, *et al*. Acid sphingomyelinase modulates the autophagic process by controlling lysosomal biogenesis in Alzheimer's disease. *J Exp Med* 2014;211: 1551–1570.
50. Liu M, Xu J, Liu J, Kraw ME, Tanswell AK, Post M. Mechanical strain–enhanced fetal lung cell proliferation is mediated by phospholipase C and D and protein kinase C. *Am J Physiol* 1995; 268:L729–L738.
51. Zeidan YH, Hannun YA. Activation of acid sphingomyelinase by protein kinase Cdelta-mediated phosphorylation. *J Biol Chem* 2007;282: 11549–11561.