

Ventilation-induced Alterations in Lung Development

*Veranderingen in long ontwikkeling
veroorzaakt door ventilatie*

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Veranderingen in long ontwikkeling
veroorzaakt door ventilatie

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Chapter 1

General introduction



Mechanical ventilation is a lifesaving treatment in critically ill neonates. However, mechanical ventilation is also one of the most important risk factors (Table 1) of Bronchopulmonary dysplasia (BPD), the most common chronic lung disease in infancy with long-term pulmonary and neurological complications (1). Exposure of immature lungs to positive pressure ventilation results in oxidative stress and ventilator-induced lung injury. The resulting injury and inflammation lead to abnormal developmental and reparative processes in the lung.

This general introduction outlines the current knowledge regarding Bronchopulmonary dysplasia related to lung development, mechanisms of ventilator-induced lung injury, mechanosensing and mechanotransduction and pathways involved in the pathogenesis of BPD related to mechanical ventilation.

Table 1. Risk factors for development of BPD, related to gestational age in human.

Gestational age	Stage of lung development	Risk factors
24 - 32 weeks GA	Canalicular - Saccular	Genetic disposition, male gender, no antenatal steroids, fetal inflammation, chorioamnionitis
0 - 2 hrs postnatal	Saccular - Alveolar	High V_T , reactive oxygen species, no PEEP, no exogenous surfactant
> 2 hrs	Alveolar arrest in development	Baro/volutrauma, reactive oxygen species, patent ductus arteriosus, fluid therapy, (mal)nutrition, infections

BRONCHOPULMONARY DYSPLASIA AND LUNG DEVELOPMENT

In the mid-1960's mechanical ventilation was introduced to manage premature infants with respiratory insufficiency because of respiratory distress syndrome (RDS). Soon, radiographic and pathologic abnormalities were observed, implicating barotrauma and oxygen toxicity as the primary causal factor of these abnormalities. Northway *et al.* were the first to describe this lung injury in preterm infants following mechanical ventilation. (2). They called it bronchopulmonary dysplasia (BPD). The infants ranged from 1474 to 3204 g birth weight and 30 to 39 weeks gestational age. Their chest radiographs revealed over-inflation and cystic emphysema and pulmonary histopathology consisted of emphysema, atelectasis, fibrosis, marked epithelial squamous metaplasia and smooth muscle hypertrophy in the airways and the pulmonary vasculature. These changes were associated with severe respiratory failure with airway obstruction, pulmonary hypertension, and cor pulmonale. The lung damage in these infants was attributed primarily to the use of aggressive mechanical ventilation and high inspired oxygen

concentrations. In 1969, Pusey *et al.* described diffuse interstitial fibroplasia associated with mechanical ventilation in newborns including patients without RDS. There was no clear relationship demonstrated between the use of high oxygen concentration and BPD, thus suggesting barotrauma as the primary cause (3).

Later advances in neonatal therapies, including exogenous surfactant, antenatal corticosteroids, newer modes of mechanical ventilation, improved nutritional management and better nursing techniques led to improved survival of infants born more immature than those described by Northway *et al.* and the histopathological features of BPD changed dramatically. Besides dysmorphic vasculature, the consistent lesion seen in this 'new BPD' is alveolar simplification and enlargement; it results from impairment in postnatal alveolarization in an extremely immature lung following premature birth (4-7). The extent of lung development between 24 and 26 weeks gestation and 30 and 32 weeks gestation is substantive; extensive vasculogenesis within the developing terminal saccules, followed by secondary crests formation along with interstitial extracellular matrix loss and remodeling. This tissue remodeling requires coordinate regulation of cell proliferation and apoptosis. Although alveoli are present in some infants at 32 weeks, they are not uniformly present until 36 weeks during the alveolar stage of development. Premature birth and initiation of pulmonary gas exchange interrupts this normal alveolar and distal vascular development.

Although the characteristics of BPD have changed, the incidence of BPD has remained the same (8, 9) or has increased and the disorder is no longer limited only to the most severely ill preterm infants (10, 11). BPD rates are slightly higher in very-low birth-weight infants and decrease incrementally with increased gestational weight; with >50% of infants less than 750 g, 15% of infants greater than 1000 g, and about 7% of infants greater than 1250 g (12, 13).

The pathogenesis of BPD is multi factorial (Fig. 1). Animal models have contributed greatly to the understanding of the etiologic agents of BPD. The main risk factors predisposing patients to BPD include prematurity, mechanical ventilation, oxygen therapy, infection and patent ductus arteriosus (Table 1). Invasive mechanical ventilation is one of the major contributors to the development of BPD in infants. There is currently no ideal ventilation strategy that minimizes the risks of pulmonary sequelae in premature infants.

Unfortunately mechanical ventilation is an inevitable intervention in the neonatal intensive care unit and thus BPD may not entirely avoidable. Understanding the pulmonary injury sequence is important to the realisation that some lung damage may be minimised by using lung protective strategies and to develop new therapies to minimise or prevent ventilator-induced lung injury in the premature infant.

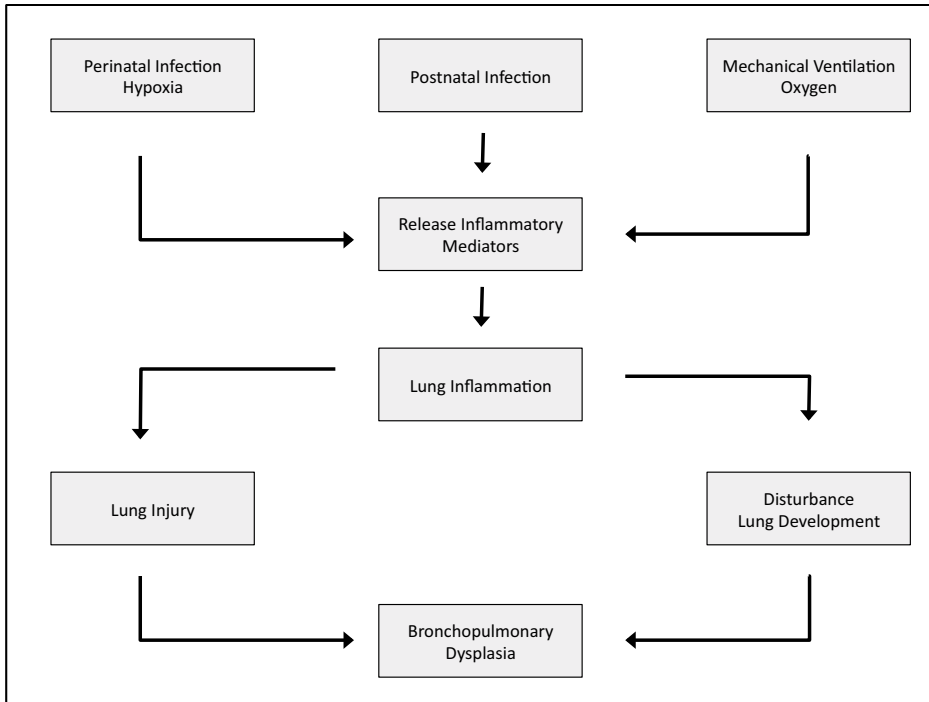


Figure 1. Concept of pathogenesis of BPD

MECHANISMS OF VENTILATOR-INDUCED LUNG INJURY

Barotrauma

Barotrauma is pressure-induced lung injury. Mechanical ventilation may cause pulmonary barotrauma through positive pressure. It can have a variety of clinical manifestations, including pneumothorax, pulmonary interstitial emphysema, pneumomediastinum and pneumopericardium. Barotrauma was suggested to be the most important factor in the pathogenesis of BPD until it was demonstrated that ventilation with high pressures without high volumes was not associated with increased lung oedema, in contrast to ventilation with low pressures and high volume (14). High pressure ventilation of premature rabbits did only result in increased microvascular permeability in the lung if chest expansion was not limited by a cast (15). Although these data indicate that volume distension of the lung rather than high peak pressures per se are more responsible for lung injury (16), barotrauma is still considered to be an important risk factor for the development of BPD (17).

Volutrauma

Volutrauma is injury caused by excessive tidal volume delivery. Ventilation with high tidal volumes leads to regional overdistension of alveoli and airways. In newborn animals this results in damage to the pulmonary capillary endothelium, alveolar and airway epithelium and the basement membranes (18, 19). The result is a leakage of fluid, protein and blood into the airways, alveoli and the lung interstitium, interfering with lung mechanics, inhibiting surfactant function and promoting lung inflammation (20-22). Adding positive end-expiratory pressure (PEEP) diminishes the severity of tissue injury (20, 23).

Atelectotrauma

Atelectotrauma is injury caused by repeated opening and closing of lung units. Cyclic opening and collapse of alveoli increases stretch and shear forces, resulting in lung injury and surfactant dysfunction. In an *ex vivo* rat lung model cyclic opening and closing from zero end-expiratory pressure (ZEEP) led to increased cytokine levels in the broncho-alveolar lavage fluid (24). Lung compliance decreased dramatically after ventilating rat lungs with a PEEP below the lower inflection point of the inspiratory pressure-volume curve compared to ventilation with PEEP levels above the inflection point. Histological analysis showed epithelial denudation and/or necrotic debris in the airways (25). PEEP above the lower inflection point minimized the influx of activated neutrophils into the lung of a surfactant-depleted piglet model (26). Possibly only the deflation limb of the pressure-volume curve provides the right information about the level of PEEP to prevent collapse of the alveoli during expiration (27, 28).

Biotrauma

Biotrauma is caused by the release of pro-inflammatory cytokines secondary to epithelial injury in the lung which contributes to a systemic inflammatory cascade of both pro-inflammatory and anti-inflammatory mediators (29). Especially high tidal volume ventilation and high inspiratory pressures can trigger an inflammatory response (30, 31). But even ventilation with low tidal volumes caused lung inflammation with impaired alveolarization and capillary development in a premature baboon model (32). Inflammation is a response to injury, resulting in recruitment of blood leucocytes, activation of tissue macrophages and production of inflammatory mediators. The inflammatory mediators are released by bronchial, bronchiolar and alveolar cells and by alveolar macrophages and neutrophils (33, 34). The inflammatory response may lead to cell regeneration and healing or progression of the inflammatory response, leading to a systemic inflammation (35). The immune response depends on the balance between pro-inflammatory, like tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6 and IL8, and anti-inflammatory cytokines, like IL-10 and IL-13 (36). TNF- α and IL-1 β cause up-

regulation of vascular adhesion molecules, resulting in adhering of blood neutrophils to endothelial cells, after which they extravasate (37). Especially CXC chemokines and IL-8 activate and attract neutrophils into interstitial and alveolar spaces of the lung (38). This activation and attraction of leukocytes may play a very important role in the pathogenesis of BPD (39, 40). Inhibition of neutrophil influx into the lung increased alveolar formation in a BPD model using newborn rats (40). NF- κ B plays a central role in the generation of the pro-inflammatory mediators. Anti-inflammatory IL-10 and IL-13 inhibit NF- κ B activation (41). Elevated concentrations of IL-1 β , IL-6 and IL-8 in amniotic fluid and bronchoalveolar lavage fluid (BALF) have been associated with BPD (42, 43).

MECHANOSENSING AND MECHANOTRANSDUCTION

Mechanosensing

Lung cells sense and integrate information from mechanical distortion. Detecting the mechanical stimuli on the cell and converting it into a biological signal is called mechanosensing (44). Mechanosensing is essential for the development and physiology of the lung cell (45), but is also suggested to play a role in the development of ventilator-induced lung injury (31). Integrins, the cytoskeleton, stretch-activated ion channels, growth factor receptors and cell-cell adhesion molecules are likely all involved in mechanosensing (44, 46). Integrins are transmembrane proteins, which anchor the cell on the basement membrane and extracellular matrix. They are in close contact with other proteins like cytoskeletal proteins and tyrosine kinases which are important for anchoring the cytoskeleton to the plasma membrane (47). The cytoskeleton itself may also sense cellular deformation (48). Mechanical stretch can affect the permeability of the plasma membrane to various ions. These changes in the concentration of specific ions in the cell lead to conversion of the physical stimulus into an electrical or chemical signal (44). The role of these channels in the pathogenesis of ventilator-induced lung injury is still unknown. Studies suggest that vascular endothelial growth factor (VEGF) receptor-2, receptor tyrosine kinases and platelet endothelial cell adhesion molecules-1 (PECAM-1) are involved in sensing mechanical stress in endothelial cells (49, 50).

Mechanotransduction

Mechanotransduction is the conversion of mechanical stimuli into biochemical and biomolecular signals (46). It has become evident that the mitogen-activated protein kinase (MAPK) has a key position in the process of mechanotransduction (51-53). After cellular deformation is sensed by the mechanosensor apparatus, the MAPK pathway is activated. As a result the mechanical signal is transduced to the nucleus and gene transcription is stimulated by activation of transcription factors (54). Most described

MAPK members are the extracellular signal-regulated kinase-1 and -2 (ERK1/2), the p38 MAPKs and the c-jun-N-terminal kinases (JNKs) (55). The MAPK pathway can be activated by a variety of extracellular signals including growth factors, cytokines, chemical and physical stress (54). The MAPK signaling cascade controls essential cellular functions, such as cell proliferation, programmed cell death and cell motility (54). Important transcription factor increased by cyclic stretch are IL-8, IL-6 and Egr-1 (53, 56, 57), which are associated with ventilator-induced lung injury (42, 43, 58). Another pathway activated by stretch is the NF- κ B pathway (52, 53, 59).

HOW MECHANICAL VENTILATION CAN INFLUENCE LUNG DEVELOPMENT

Proliferation

Normal lung development requires coordinated proliferation and differentiation of epithelial and mesenchymal cells. Cell proliferation and differentiation are controlled by cell-cycle progression, a highly organized and tightly regulated process. Because proliferating and non-proliferating epithelial or mesenchymal cells lay beside each other, both growth stimulatory and inhibitory pathways are likely to be activated at the same time.

Disruption of the cell cycle regulation can result in developmental abnormalities. The cell cycle of the standard eukaryotic cell is divided into four non-overlapping phases (Fig. 2), with DNA synthesis during the S phase and mitosis during the M phase. These two phases are separated by G_1 and G_2 phases during which mRNAs and proteins accumulate continuously. In G_1 phase the cell is preparing for DNA synthesis (60, 61). In the subsequent S phase, DNA duplication occurs. Before cells undergo mitosis they continue in the G_2 phase with cell growth, preparing for cell division in M phase. Cell which are in G_0 phase are not actively cycling (60). Each phase of the cell cycle contains checkpoints that allow the arrest of the cell cycle progression and activation of repair mechanisms or even the apoptotic cascade, leading to cell death. After passing these checkpoints cells go irreversible into the next phase (62). The key regulatory proteins, which allow the transition from one cell cycle phase to another, are called cyclin-dependent kinases (CDKs), a family of serine/threonine protein kinases, which are activated at specific points during cell cycle (60, 63). Positive regulation of CDK activity occurs through association with a cyclin and by phosphorylation via a CDK-activating kinase (62). Cyclins are produced at each of these phases and form a complex with their CDK partner. The level of activating cyclins in different stages of the cell cycle differ, whereas CDK protein levels remain constant (63). During the G_0 to S phase transition, cyclins D1, D2, D3 and C get activated. Cyclins D1, D2 and D3 bind to CDK4 and CDK6. These cyclins promote entry into the G_1 phase. Cyclin C binds to

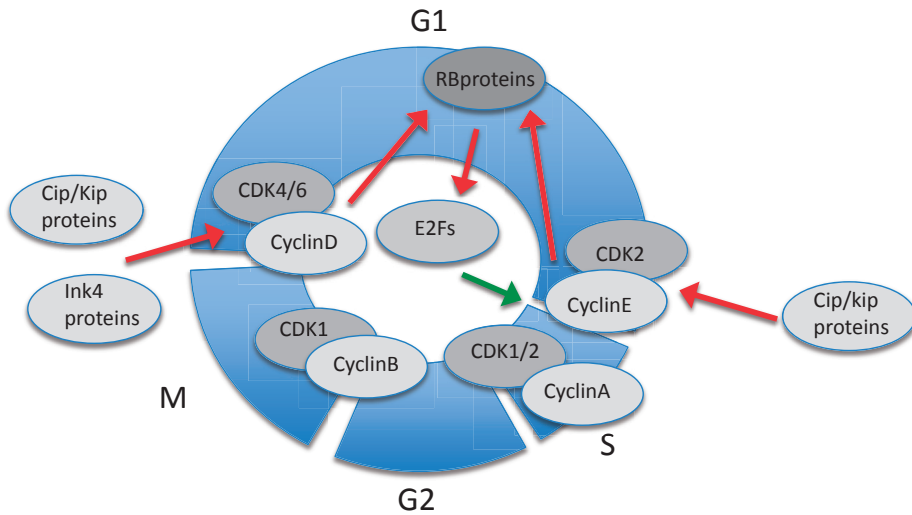


Figure 2. How different proteins influence cell cycle regulation. The cell cycle is divided in four phases: the G1, S, G2 and M phase. Cyclin-dependent kinases (CDKs) form subunits with cyclins, promoting entry into the next phase by phosphorylating and inactivating retinoblastoma protein (RB) (red arrow). This results in expression of E2F inducible genes, leading to entry into the S-phase (green arrow). Cip/Kip (p21^{WAF1/Cip1}, p27^{Kip1} and p57^{Kip2}) and Ink4 proteins (p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c} and p19^{Ink4d}) inhibit the formation of cyclin-CDK complexes (red arrows).

CDK8. Cyclin E activates CDK2 and is associated with transition during G1 to S phase. Cyclin A gets activated during the S phase transition and binds to CDK1 and CDK2. B type cyclins are present during G₂ and M phases and are associated with CDK1 (64). G and T type cyclins are associated with CDK5 and CDK9, respectively (65). Active CDK4/6-cyclin D complexes phosphorylate retinoblastoma susceptibility protein (RB) (66, 67). RB reacts with a wide array of proteins that are mostly involved in transcription control. Among these proteins transcription factors E2Fs are very important. They are central regulators of cell cycle gene expression, and directly regulate the expression of genes involved in DNA replication, DNA repair, and G₂/M progression. Sufficient phosphorylation of RB proteins inactivates its transcriptional repressor function, thus allowing expression of E2F target genes, whose activity is essential for entry into S phase (68). CDK's are also tightly regulated by inhibitory phosphorylation, mediated by the kinase Wee1 and MYTC1 (62) and by two families of inhibitors: Ink4 proteins and the Cip/Kip family. Ink4 proteins include p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c} and p19^{Ink4d}. Ink4 proteins can inhibit cyclin D-associated kinase activity via binding to the CDK4 subunit (69, 70). Cip/Kip family proteins include p21^{WAF1/Cip1}, p27^{Kip1} and p57^{Kip2}. Cip/Kip proteins bind to and inhibit cyclin-CDK complexes. Preferentially they act on cyclin E-CDK2 complexes *in vivo* (71). Over expression of these two families of inhibitors blocks cell proliferation (72-76).

Fetal lung cell growth

Fetal lung growth is dependent on several mechanical forces, including sufficient amniotic fluid and normal fetal breathing movements (FBM) (77, 78). The fetal lung actively secretes fluid into the lung lumen, creating a constant transpulmonary pressure in the potential airway and air spaces (79). The volume of this lung fluid is dependent on the diaphragmatic contractions associated with FBM (80). In the first trimester of intra-uterine life the fetus makes episodic breathing movements, which increase in frequency to 30% of time by birth, (81). Abolishing FBM by sectioning the upper cervical cord or phrenic nerves resulted in reduced lung growth and maturation (82, 83). The critical stimulatory role of mechanical stretch on lung growth, secondary to increased intratracheal pressure has extensively been investigated in tracheal occlusion (TO) studies (84-87). Tracheal occlusion demonstrated a significant influence on proliferation of alveolar type II cells (85, 87). Alveolar Type II cells are of critical clinical importance. They produce and secrete surfactant and are progenitors of the gas-exchanging type I cells (88). This stretch-related proliferation of alveolar type II cells is dependent on timing and duration of stretch. In a fetal rabbit model, a significant increase in alveolar type II cells proliferation was noted after TO in the late pseudoglandular stage, whereas TO in the late canalicular stage had no significant effect (87). Fetal sheep, exposed to 2, 4 or 10 days of TO in the alveolar stage of lung development, showed an increase in the number of alveolar type II cells only during days 2 to 4 (85). Fetal sheep exposed to TO in the early canalicular stage of lung development showed increased lung growth, but a decrease in alveolar type II cells (86). After 10 days of TO this decrease in alveolar type II cells coincides with an increase in alveolar type I cells, suggesting induced differentiation of alveolar type II cells in type I cells by sustained lung expansion (89). This gestation-dependent proliferative response of fetal lung cells to stretch was also demonstrated *in vitro*. Intermittent stretch (simulating normal FBM) for 48 hours of fetal rat lung cells (epithelial cells and fibroblasts) stimulated lung cell proliferation at 19 days of gestation (90). In contrast, intermittent mechanical stretch for 24 hours of fibroblasts isolated from fetal rat lungs at 19 days of gestation decreased proliferation by 42% compared to unstretched controls (91). Only stretching of fetal lung cells isolated on day 19 (canalicular stage of lung development) of gestation showed these effects. Stretching of cells isolated on day 18 and 20 did not alter the number of proliferating cells. These data from *in vivo* and *in vitro* studies indicate that mechanical stretch is essential for normal lung development and that responsiveness of lung cells to mechanical stretch is dependent on stage of lung development and duration of stretch. Very little is known how mechanical stretch influences the cell-cycle regulation of lung cells with regard to the proliferation. The involvement of the cyclin system has mainly been studied in oxidative stress models. Hyperoxia induced the expression of p21^{WAF1/Cip1} in newborn mice (92) and in lung epithelial cells (93) after at least 48 hours. Because

hyperoxia induces a BPD-like pathology (86, 94), this induction of p21^{WAF1/Cip1} may be responsible for disrupting coordinated cell proliferation and alveolarization. Indeed, in a premature born baboon model of BPD, baboons showed a threefold increase in p21^{WAF1/Cip1} expression. In contrast to normal lung development, during disease progression the proportion of proliferating type II cells increased and there was a temporal shift in p21^{WAF1/Cip1} expressing cells from type II cells at day 4-6 to mesenchymal cell at 14-21 days. The animals received ventilator support and oxygen as needed to achieve normal blood-gas measurements (95). In the same model increased expression of Cyclin D1, Cyclin E and Cdk4 was demonstrated at day 6-14, while that of Cyclin A and RB protein was decreased (96). Since the animals had lung injury, it is possible that ongoing lung repair demands an increased proliferation of cells at this time point. A possible candidate for the increased expression of p21^{WAF1/Cip1} is transforming growth factor- β (TGF- β). This cytokine is induced by hyperoxia (97) and can increase the transcription of p21^{WAF1/Cip1} (98).

Apoptosis

Cells can die in three different ways, by programmed cell death, oncosis or autophagy (99). Programmed cell death is a process of controlled cell death and is important in the development and remodeling of tissues that occur during the normal repair process (91, 100). Apoptosis is only one of several types of programmed cell death (101). Apoptotic cells are characterized by cytoplasmic, nuclear shrinkage and the formation of apoptotic bodies. The cellular membrane of apoptotic cells remain intact (102). The result is the complete elimination of cellular debris without induction of an inflammatory response (103, 104). Apoptosis is genetically determined during the course of development, but can also be triggered by external stimuli, such as inflammation and intrinsic stimuli, resulting from alteration of cellular function and metabolism. During apoptosis proteases are activated. Of these proteases, caspases are the most extensively studied. The human genome encodes 13 distinct caspases of which seven have been suggested to participate in apoptosis as initiator (caspase-2, -8, -9 and 10) or effector (caspase-3, -6 and 7) (105). Caspases can be activated by either the extrinsic pathway or by the intrinsic pathway (106, 107). The extrinsic pathway is triggered by the ligation of cell surface death receptors and their ligands, such as tumor necrosis factor (TNF), Fas ligand (FasL) and TNF-related apoptosis-inducing ligand. When the Fas receptor binds its ligand, this recognition event is translated into intracellular signals that eventually lead to caspase-3 activation, through caspase-8 activation (106). Activated caspase-3 further triggers enzymes responsible for apoptosis, resulting in nuclear condensation and fragmentation (105, 108). The intrinsic pathway is characterized by changes in mitochondrial outer membrane integrity, which is regulated by Bcl-2 family proteins. In this family both anti-apoptotic and pro-apoptotic proteins are recognized (108). The

balance between these two groups of proteins determines the integrity of the mitochondrial outer membrane. Permeabilization of the mitochondrial outer membrane will lead to release of mitochondrial molecules, of which cytochrome *c* is the most critical factor in the intrinsic pathway. When cytochrome *c* is released in the cytosol a cascade of different caspases will be activated. Finally, caspase-3 will be activated, leading to cell death. In certain conditions the extrinsic and intrinsic pathway are interlinked by caspase-8 (108). Besides caspase activation as executioner of programmed cell death, there is increasing evidence of the existence of caspase-independent pathways leading to apoptosis (109, 110).

Oncosis is characterized by a progressive loss of cytoplasmic membrane integrity, cytoplasmic swelling and both lysosomal and mitochondrial swelling and disruption, leading to cellular fragmentation and leakage of lysosomal and granular contents into the surrounding extracellular space (111). Calpains, a family of Ca^{2+} -activated neutral cysteine proteases, are considered to play a role in this type of cell death (112). They are activated by the increased cytoplasmic concentration of free calcium. In contrast to apoptosis, oncosis is often associated with inflammation in the adjacent tissues (99). Usually oncosis is associated with ischemia and sudden fall in tissue oxygenation. Bacterial exotoxins and mechanical stretch also may cause oncosis (113).

Autophagy is a process in which cells digest their own organelles and macromolecules to generate energy and metabolites to survive during a period of starvation or when it is deprived of growth factors. Cells that digest all available substrates will die eventually. This type of cell death is characterized by vacuolation and degradation of cytoplasmic components (114). Autophagy is an adaptive response to sublethal stress, such as nutrient limitation, heat, oxidative stress and/or the accumulation of damaged or excess organelles and abnormal cellular components (115, 116). It also has a role in the suppression of tumor growth, deletion of toxic misfolded proteins, elimination of intracellular microorganisms and antigen presentation (116). Malfunction of autophagy contributes to a variety of diseases including cancer, neurodegeneration, cardiovascular diseases and microbe infections (114). However, whether autophagy is involved in ventilator-induced lung injury remains to be demonstrated.

Apoptosis and stretch of fetal lung cells

During lung development and maturation alveolar type II cells undergo apoptosis. Three peaks of apoptosis have been described: during transition from the canalicular to sacular stages of lung development, during birth and during the third postnatal week (100, 117-119). The last peak coincides with alveolar septal formation. Peaks of apoptosis are preceded by peaks in proliferation of lung cells, indicating the significance of apoptosis during lung development by removal of excess lung cells (117-119). Apoptosis is also responsible for restoration of the alveolar epithelium after lung injury (120). The role of

stretch-induced apoptosis in lung development was first demonstrated in a fetal rabbits after TO. Ligation of the trachea caused a depletion of alveolar type II cells. Different *in vitro* studies have demonstrated that cyclic stretch of day 19 fetal rat type II cells and fibroblasts for 24 hours increased apoptosis (91, 121). These cells were exposed to 5% elongation to mimic FMB. These data demonstrate the important role of physiological stretch induced-apoptosis for lung remodeling.

However the mechanisms by which excessive stretch of fetal lung cells contributes to the development of BPD are not well defined. Exposure of fetal lung cells to 20% elongation to simulate ventilation-induced lung injury increased the number of apoptotic cells compared to cells exposed to 5% elongation (121). A relation between increased apoptosis and reduced alveolar formation was recently demonstrated (122). Mechanical ventilation for 24 hours of newborn mice led to a 5-fold increase of apoptotic lung cells. *In utero* ventilation of fetal sheep for 6 hours increased apoptosis of mainly interstitial cells (123). The increases in apoptosis coincided in both studies with an significant reduction in alveolarization. Only one study has reported the effect of ventilation on apoptosis in infants with BPD. In lung sections from ventilated preterm infants delivered at 22-36 weeks the number of apoptotic epithelial cells increased significantly compared to still born foetuses (104). Besides ventilation, these infants were also treated with additional oxygen.

That apoptosis of fetal lung cells is triggered by mechanical stretch is now widely recognized. However, which signaling pathways are involved remain to be elucidated. Stretch induces the activation of caspases (91, 122). It has been suggested that activation of the Fas/FasL system is pivotal in activating the caspases (124). Sustained lung expansion in fetal rabbits resulted in a time-specific increase of FasL protein in alveolar type II cells, synchronous with alveolar type II cell apoptosis (125). This time-specific activation of FasL protein was also demonstrated in fetal lung fibroblasts (91). Stretching of day 19 fetal rat fibroblasts induced apoptosis without detection of FasL. Obviously the Fas/FasL system is only activated by mechanical stretch during the late-gestational stage of lung development, leading to alveolar type II cell apoptosis. This is plausible considering the time-specific up-regulation of FasL mRNA and protein during lung development (126). Thus, signaling pathways independent of FasL must be involved in stretch-induced apoptosis of alveolar type II cells during earlier stages of lung development and apoptosis of lung fibroblasts. TNF- α and its receptors represent another major extrinsic cell death pathway. Mechanical ventilation up-regulates TNF- α (127). But if this pathway is important in stretch-induced apoptosis besides FasL is not known. Other up-regulated pro-inflammatory cytokines/chemokines by mechanical ventilation are IL-1 β , IL-6, IL-8, and TGF- β (121, 128, 129). Some of these inflammatory mediators have also pro-apoptotic properties. The role of TGF- β in the pathogenesis of BPD has more extensively been studied. Exposure of alveolar type II cells to hyperoxia increased

the sensitivity of these cells to TGF β -induced apoptosis (130). Prolonged ventilation of newborn mice increased Smad2 protein, indicating increased TGF- β signaling (122). Smad2 is a downstream effector molecule of TGF- β signaling (131). TGF- β induces apoptosis by enhancing the Fas/FasL system via caspase-3 activation, but also via down regulation of p21^{Cip1/Waf1} expression (124, 132). Expression of p21^{Cip1/Waf1} causes not only cell cycle inhibition but also suppresses apoptosis (133), which makes the role of p21^{Cip1/Waf1} in the pathogenesis of BPD complicated.

The extra cellular matrix

The extracellular matrix (ECM) plays an important role in lung development and the biomechanical behaviour of the lung. The macro molecules that constitute the ECM are: (1) fibrous proteins (collagen and elastin); (2) structural or adhesive proteins; and (3) proteoglycans and glycosaminoglycans (134). Elastic fibers are required for the vascular, conducting airway and terminal airspace compartments of the lung. Together with interstitial collagen, they form an elastic architecture that can undergo repeated deformation and recoil. For this property the orientation, thickness and length of the elastin fibers are important (135).

Initially, elastin is produced during the pseudoglandular stage of lung development (136). Alveolar elastin is produced primarily by α -smooth muscle actin-expressing cells of the alveolar wall (137). Elastogenesis is an essential step to the process of alveolar septation (138). Formation of new alveoli during the first phase of alveolarization starts with the development of new septa from immature preexisting septa. Elastin is organized into elastic fibers, which are composed of elastin and microfibrils. The microfibrils act as a scaffold on which elastin is deposited. Elastin is synthesized as tropoelastin. Fibulin5 and lysyl oxidase (LOX) play key roles during the assembly of tropoelastin into elastin fibers. Fibulin5 potentially induces elastic fiber assembly and maturation, by alignment of tropoelastin on microfibrils and facilitating cross-linking of tropoelastin by lysyl oxidase (139, 140). Fibulin5 and elastin null mice exhibit abnormal alveolar development (138, 141). Lungs of LOX null mice display impaired development of distal and proximal airways with dilatation of the alveolar walls, sacs and primitive alveolar structures (141).

Extracellular matrix glycoprotein tenascin-C (TN-C) plays a morpho-regulatory role during development and tissue remodeling. It is expressed in the human lung during all developmental periods (142). Fetal lungs of TN-C deficient mice have enlarged airspaces (143). Antiserum to TN-C inhibited branching morphogenesis of fetal rat lung explants (144). Once the lung has developed, the expression of TN-C is absent or very low, but reappears under pathological conditions caused by infections, inflammation or during tumorigenesis (145). The expression of TN-C is regulated by different growth factors, including TGF- β 1 (145, 146).

Blood vessels develop at the same time as the airways. Two processes of vascular growth have been described: angiogenesis and vasculogenesis. Angiogenesis is abluminal sprouting of new vessels from preexisting ones. This process concerns the formation of the large pulmonary vessels. The pulmonary capillary bed is formed by vasculogenesis. Vasculogenesis is the de-novo formation of vessels from precursor cells (angioblasts and endothelial precursors) in the mesenchyme. The airway acts as a template for the pulmonary vessels during this process (147). Hence, it is not surprising that different models of lung hypoplasia have shown that disrupted alveolarization is associated with abnormalities of the pulmonary circulation (148-150). The same observation has been made in diseases like BPD and congenital diaphragmatic hernia (151-154). Growth factor signaling, especially that of VEGF and PDGF, is important for regulating both the formation of alveoli and blood vessels. Inhibition of VEGF signaling using a receptor blocker decreased alveolarization in neonatal rats and mice (155, 156), while VEGF treatment enhances alveolarization (157, 158). The importance of PDGF-A in postnatal lung development has been demonstrated in PDGF-A deficient mice. These mice acquire pulmonary emphysema from failed alveolarization (159, 160). PDGF- α receptor signaling has been shown to contribute to the growth of pulmonary myofibroblasts, which play an important role in septal formation (161). Generally it is presumed that disruption of alveolarization will cause failure of lung vascular growth and that disruption of normal angiogenesis can also lead to impaired alveolarization. In which sequence mechanical ventilation interferes with alveolarization and vascularisation is yet unclear.

The extra cellular matrix and stretch

Mechanical forces have been shown to induce expression of tropoelastin *in vitro* (162) and *in vivo* (163). The increase in tropoelastin mRNA expression coincided with an increase in elastin deposition, predominantly at the tips of secondary septal crests, and an increase in alveolar numbers (163). Sustained reduction led to decreased tropoelastin expression with disorderly elastin deposition in the alveolar walls with less secondary septal crests. A decrease in lung tropoelastin mRNA expression and lung elastin was also demonstrated in hypoplastic fetal rat and human lung (164, 165). This indicates that non-injurious stretch of the fetal lung is important for correct deposition of elastin and for alveolar formation. Prolonged ventilation with room air of 2-4 days old mice and fetal sheep resulted in increased, dysregulated elastic fiber deposition (122, 123). Prolonged ventilation with 40% oxygen did decrease the expression of fibulin5 and emilin1, while the expression of other genes regulating elastic fiber assembly was not changed (166).

Cyclic mechanical strain of human airway smooth muscle cells has been shown to increase TN-C expression (167). Furthermore, TN-C expression is down-regulated in

murine model of pulmonary hypoplasia with co-existent diaphragmatic hernia (168). Interestingly, TN-C expression is decreased after ventilating newborn mice (169), while TN-C is highly expressed in autopsied babies with BPD (170). It is likely that down-regulation of TN-C after (non-injurious) ventilation may negatively influence alveolar development and that the increase in TN-C expression fits with a lung injury repair response. Recent analysis of gene expression in repairing lungs of naphthalene-exposed mice showed a transient increase of TN-C expression in the lung whereas abortive repair in a transgenic model allowing ablation of all reparative cells resulted in a progressive accumulation of TN-C (171).

Infants with BPD have dysmorphic capillaries and a decreased expression of VEGF in the lungs (154). In the BALF of preterm infants developing BPD the level of VEGF was also decreased (172). The same findings were reported in premature baboons, mice and lambs developing BPD. Pulmonary expression of VEGF and one or more VEGF receptors were decreased (169, 173-175). In all these studies mechanical ventilation was combined with oxygen. Ventilation with air for 8 hours did not alter the expression of VEGF or its receptor in newborn mice (169). In contrast the expression of PDGF-A was decreased after ventilation with oxygen as well as after ventilation with room air (169). This may indicate that PDGF signaling is more related to stretch-induced lung injury than VEGF signaling.

SYNOPSIS

Mechanical ventilation is a major contributor to the complex and multifactorial pathogenesis of BPD. It initiates a pulmonary fetal inflammatory response. Often this lung inflammation is superimposed onto an intra-uterine inflammation and is enhanced by exposure to supplemental oxygen. Stretch alone or in combination with these initiators of lung injury dysregulate the precise temporal and spatial orchestration of molecular interactions, necessary for normal lung development and lung repair. This molecular dysregulation results in an arrest in alveolarisation and vascular development. The underlying mechanisms and pathways by which mechanical ventilation causes the abnormal lung development that characterize BPD, however, remains largely unknown.

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Chapter 2

Scope and outline of this thesis



The clinical relevance of Bronchopulmonary dysplasia

BPD is most commonly defined as an ongoing requirement for supplemental oxygen and/or respiratory support at 36 weeks post-menstrual age (1). It is a chronic respiratory disease that develops as a consequence of neonatal lung injury. The inciting factor of BPD is often pre-maturity with respiratory distress syndrome (RDS) from surfactant deficiency. Mechanical ventilation is one of the most important risk factors. Patients with BPD remain oxygen dependent for many months and in the first two years they frequently require hospital readmission and use more medication than patients without BPD (2, 3). The most severely affected children may remain symptomatic and have evidence of airway obstruction as adults (4). Besides respiratory morbidity, BPD is frequently associated with other conditions related to preterm birth, including growth retardation, feeding difficulties, pulmonary hypertension, neurodevelopmental delay, hearing defects, and retinopathy of prematurity. Financial and emotional costs of BPD are tremendous (5, 6). Despite ongoing researches for the optimal ventilation strategy and drug therapies to prevent BPD or to mitigate its course, BPD is still a major complication of premature birth.

Why basic research?

As mentioned above, it may be evident that greater insight into molecular pathways is needed to develop new strategies or therapies. The underlying mechanisms by which risk factors such as mechanical ventilation, inflammation and oxygen, contribute to the pathogenesis of BPD are difficult to study in the clinical setting, where the role of each risk factor cannot be easily isolated from each other. Moreover, lung material of preterm infants to study molecular pathways involved in the pathogenesis of BPD is very limited. In this thesis we used a newborn rat model. This experimental approach takes advantage of the fact that rat lungs at birth have a saccular appearance, similar to the preterm neonate, and alveolarization in rats occurs postnatally between day 4 and day 21 (7). The newborn rat model enables us to examine the impact of each risk factor alone or in combination on genes and proteins that regulate lung growth and development.

This thesis

Lungs of preterm infants are in the early phases of lung development (canalicular and saccular phases of lung development). The alveolar and capillary development is inhibited following exposure to the injurious effects of mechanical ventilation, oxygen exposure, and inflammation. Insight in molecular pathways leading to arrest of alveologenesis and vasculogenesis, the hallmark of BPD, may have therapeutic potential for the prevention -or treatment- of BPD.

The major aims of this thesis are:

- To investigate the effect of different tidal volumes on the cytokine/chemokine production in newborn rat lungs.
- To investigate if mechanical ventilation with 50% oxygen superimposed on a systemic infection will enhance the cytokine/chemokine production in comparison with ventilation with room air without a systemic infection.
- To study which apoptotic pathways are induced *in vivo* by (prolonged) mechanical ventilation and *in vitro* by cell stretch.
- To determine the effect of (prolonged) mechanical ventilation on lung cell cycle regulators, proliferation and alveolarization *in vitro* and *in vivo*.
- To investigate how (prolonged) mechanical ventilation influences the expression of angiogenic and matrix molecules *in vivo*.

Stretching and alveolar collapse and re-expansion of alveoli is related to lung injury. **In chapter 3** we assessed the effect of low, moderate and high tidal volume (V_T) ventilation on cytokine/chemokine production. We hypothesized that continuous cyclic (over)stretching of the primitive airsacs would adversely affect cytokine/chemokine production and the adverse effect would be stretch-amplitude dependent. Secondly, we studied the effect of ventilation with controlled oxygen superimposed on a systemic inflammation on this cytokine/chemokine production.

Increased pro-inflammatory cytokines/chemokines production, induced by mechanical ventilation, hyperoxia and inflammation, is associated with activation of apoptotic pathways. Apoptosis is a highly orchestrated form of cell death. Lung cells undergo apoptosis as part of normal lung development and repair after lung injury. Several *in vivo* and *in vitro* studies suggest contribution of apoptosis to the pathophysiology of BPD due to an imbalance between anti-apoptotic and pro-apoptotic signaling pathways. **Chapter 4** is a review about the timing and apoptotic events during lung development and the pathogenesis of BPD with particular focus upon apoptotic pathways activated by mechanical ventilation, hyperoxia and inflammation. **Chapter 5** describes more in detail how apoptotic pathways are activated in newborn rats by prolonged mechanical ventilation and in isolated epithelial and fibroblast cells, subjected to continuous cyclic stretch. **Chapter 6** continues with the topic on lung cell proliferation. Besides apoptosis, tissue remodeling during lung development and after injury also requires well-coordinated regulation of cell proliferation. There is ample evidence that dysregulated cell proliferation, resulting from disruption of cell cycle control, promotes tumor progression in many cancers. If dysregulated cell proliferation is also involved in the pathogenesis of BPD is mostly unknown.

Also interactions between epithelial and mesenchymal cells play an important role both in lung development and in fibrotic disorders, including BPD. It is evident that

cytokines control these processes. Cytokines control the expression of several extracellular matrix (ECM) matrix molecules. In **Chapter 7** we investigate the influence of (prolonged) mechanical ventilation on the expression of angiogenic molecules (VEGF, PDGF, PECAM) and its receptors that regulate alveolarization and matrix molecules, principally the ones that regulate elastin synthesis and assembly (tropoelastin, fibulin5, lysyl oxidase). Finally the results of our findings are discussed and summarized in **Chapter 8 and 9**.

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Chapter 3

Inflammatory Response to Oxygen and Endotoxin in Newborn rat Lung ventilated with Low Tidal Volume

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ABSTRACT

Herein we determined the contribution of mechanical ventilation, hyperoxia and inflammation, individually or combined, to the cytokine/chemokine response of the neonatal lung. Eight day old rats were ventilated for 8 hours with low (~ 3.5 ml.kg⁻¹), moderate (~ 12.5 ml.kg⁻¹) or high (~ 25 ml.kg⁻¹) tidal volumes (V_T) and the cytokine/chemokine response was measured. Next, we tested whether low V_T ventilation with 50% oxygen or a pre-existing inflammation induced by lipopolysaccharide (LPS) would modify this response. High, moderate and low V_T ventilation significantly elevated CXCL-2 and IL-6 mRNA levels. Low V_T ventilation with 50% oxygen significantly increased IL-6 and CXCL-2 expression vs. low V_T ventilation alone. LPS pre-treatment combined with low V_T ventilation with 50% oxygen amplified IL-6 mRNA expression when compared to low V_T alone or low V_T +50% O₂ treatment. In contrast, low V_T -upregulated CXCL-2 levels were reduced to non-ventilated levels when LPS-treated newborn rats were ventilated with 50% oxygen. Thus, low V_T ventilation triggers the expression of acute phase cytokines and CXC chemokines in newborn rat lung, which is amplified by oxygen but not by a pre-existing inflammation. Depending on the individual cytokine or chemokine, the combination of both oxygen and inflammation intensifies or abrogates the low V_T -induced inflammatory response.

INTRODUCTION

Bronchopulmonary dysplasia (BPD) remains the most important cause of respiratory morbidity in very low birth weight infants. Mechanical ventilation (MV), intra-uterine infections and oxidative stress upregulate pro-inflammatory cytokines/chemokines including interleukin(IL)-1 β , IL-6 and IL-8 (1). Elevated concentrations of these cytokines/chemokines in amniotic fluid and bronchoalveolar lavage fluid (BALF) have been associated with BPD (2, 3). The contribution of each risk factor, alone or combined, to the inflammatory response remains to be determined.

Ample animal studies have suggested that high frequency oscillatory ventilation (HFOV) is less injurious compared to conventional ventilation (4, 5). However, in the baboon model of BPD impaired alveolarization and capillary development occurred in spite of appropriate oxygenation and use of HFOV (4). MV with moderate and high tidal volumes increased lung cytokine/chemokine response to systemic endotoxin in rabbits (6) and newborn rats (7). Oxidant injury alone can produce the pathologic features of BPD (8). Inflammatory cells such as monocytes and neutrophils are primary contributors to the oxygen-induced lung injury (9, 10). Other animal studies have investigated the contributions of oxygen exposure and MV alone or in combination. In term ventilated piglets hyperoxia caused less lung damage than hyperoxia combined with hyperventilation, but more than hyperventilation alone (11). Premature baboons ventilated with the minimum necessary supplemental oxygen had significant less damage than those ventilated with 100% oxygen (12), but alveolarization and capillary development was still impaired (4).

To our knowledge, no previous study has evaluated the combination of MV, hyperoxia and inflammation. Therefore, we first assessed the effect of low, moderate and high tidal volume (V_T) ventilation on cytokine/chemokine production. To mimic the clinical situation we used a newborn rat model (7). Rat lungs at birth have a sacular appearance, similar to the preterm neonate, and alveolarization in rats occurs postnatally between P4 and P21. High V_T ventilation has been reported to cause injury in newborn rat lung (13, 14) and was included as positive control. We hypothesized that continuous cyclic (over)stretching of the primitive airsacs would adversely affect cytokine/chemokine production and the adverse effect would be stretch-amplitude dependent. Secondly, we assessed the effect of low V_T ventilation with controlled oxygen superimposed on a systemic inflammation on cytokine/chemokine production. To induce a mild systemic inflammation we pre-treated the newborn rats with lipopolysaccharide (LPS) (7). We hypothesized that low V_T ventilation with 50% oxygen superimposed on a relatively mild systemic inflammation would enhance the adverse inflammatory mediator production by low tidal volume alone.

METHODS

Animals: In two series of experiments newborn (postnatal day 8) Sprague-Dawley rats (average weight 16.7 ± 1.0 g) were ventilated for 8 hours using rodent ventilators (FlexiVent Scireq, Montreal, PQ). After rats were anesthetized by i.p. injection of 30 mg kg⁻¹ pentobarbital, a tracheotomy was performed. The trachea was cannulated with a 1 cm 22G cannula. Dynamic compliance was estimated from data obtained during a single-frequency forced oscillation manoeuvre, using a mathematical model-fitting technique according to the specifications of Scireq Inc. (Montreal, PQ). To determine ventilator settings we started with the normal breathing frequency of a 8-day old rat (~160 minute⁻¹ (15)) and adjusted V_T and PEEP to achieve normal blood gases. The V_T and PEEP values for this frequency were ~12.5 ml.kg⁻¹ and 2 cm H₂O, respectively. Next we choose a lower and higher V_T and adjusted the ventilator frequency accordingly. Increasing the positive end expiratory pressure (PEEP) in the low V_T group led to increase of CO₂ and early death, most likely due to inadvertent PEEP. Animals were monitored by ECG. Rectal temperature was maintained around 37°C by using a thermal blanket, lamp and plastic wrap. To prevent spontaneous respiratory efforts 5 mg.kg⁻¹ pancuronium was administered i.p. Every two hours 0.1 ml saline was administered to prevent dehydration. At the end of the ventilation period a blood sample from the carotid artery was taken for blood gas analysis prior to euthanasia. Lung tissues were processed for histology or fresh frozen for molecular/protein analyses. The study was conducted according to the guidelines of the Canadian Council for Animal Care and with approval of the Animal Care Review Committee of the Hospital for Sick Children.

Series I: Different Tidal Volumes. Animals were randomly assigned to one of the following four groups: 1) Non-ventilated (NV) controls; 2) Low V_T ($V_T \sim 3.5$ ml.kg⁻¹, freq. 600 minute⁻¹, PEEP 0 cm H₂O); 3) Moderate V_T ($V_T \sim 12.5$ ml.kg⁻¹, freq. 160 minute⁻¹, PEEP 2 cm H₂O); 4) High V_T ($V_T \sim 25$ ml.kg⁻¹, freq. 20 minute⁻¹, PEEP 2 cm H₂O).

Series II: Pre-exposure to LPS and Low V_T Ventilation with Oxygen. Rats were randomly assigned to injection (i.p.) of either 3 mg.kg⁻¹ body weight of LPS from *E. coli* serotype 026:B6 or the same volume of 0.9% NaCl (7). Twenty four hours after treatment animals were randomly assigned to one of the following six groups: 1) Non-ventilated after NaCl injection (NV); 2) NV after LPS injection (NV+LPS); 3) Low V_T ($V_T \sim 3.5$ ml.kg⁻¹, freq. 600 minute⁻¹, PEEP 0 cm H₂O) with room air after NaCl injection (LV_T); 4) Low V_T with room air after LPS injection (LV_T + LPS); 5) Low V_T with 50% oxygen after NaCl injection (LV_T+O₂); 6) Low V_T with 50% oxygen after LPS injection (LV_T+LPS/O₂).

Immunohistochemistry: After flushing lungs were infused in situ with 4% (v/v) paraformaldehyde (PFA) in phosphate-buffered saline (PBS) with a constant pressure of 20 cm H₂O to equalize filling pressure over the entire lung. Under these constant pressure conditions the cannula was removed and the trachea immediately ligated. The excised lung tissue was immersed in 4% (v/v) PFA in PBS overnight and then dehydrated in an ethanol/xylene series and embedded in paraffin. Five micron sections were deparaffinized, rehydrated in a graded series of ethanol. Following antigen retrieval by heating in 10 mM sodium citrate pH 6.0, endogenous peroxidase quenching and blocking with NGS/BSA, sections were stained with 1:200 diluted mouse anti-CD68 (Serotec, Raleigh, USA) and 1:100 diluted rabbit anti-myeloperoxidase (MPO) antibodies (Lab Vision Corporation, Fremont, Canada), using the avidin-biotin (ABC) immunoperoxidase method. Biotinylated rabbit anti-mouse IgG or goat anti-rabbit IgG were used as secondary antibodies, respectively. All sections were counterstained with hematoxylin.

Quantitative RT-PCR: Total RNA was extracted from lung tissues and reverse transcribed. Complementary DNA was amplified for our target genes (IL-1 β , IL-6, IL-10, CXCL-2 (GRO2/MIP-2: macrophage inflammatory protein-2, a functional rodent homolog of human IL-8) and 18S as previously described (7, 10). For relative quantification, polymerase chain reaction signals were compared between groups after normalization using 18S as an internal reference. Fold change was calculated.

Cytokine protein measurement in BALF: Lungs were infused with 0.5 ml of saline, followed by withdrawal and re-infusion two more times (7). Total protein was determined and IL-1 β , IL-6 and CXCL-1 (also known as GRO1/KC) were measured in BALF using multiplex immunoassays for Luminex technology (7). CXCL-1 was measured because of lack of CXCL-2 detection kit for the Luminex system.

Statistical analysis: Stated otherwise all data are presented as mean \pm SD. Data were analyzed, using SPSS software version 15 (SPSS Inc, Chicago, IL). Depending on the distribution and the homogeneity of variation within the groups, statistical significance ($p < 0.05$) was determined by using either one-way ANOVA, or Kruskal-Wallis test. *Post hoc* analysis was performed using Duncan's multiple-range test (data presented as mean \pm SD) or Mann-Whitney test (data presented as median and interquartile range). Since data of NV and LV_T groups of series I and II were similar, they were combined in the analysis.

RESULTS

Series I: Different Tidal Volumes

Physiologic data: Blood gases were in the normal range after eight hours of ventilation with different tidal volumes (Table 1). Ventilator set tidal volumes differed from inspired tidal volumes, namely 6, 16 and 40 ml.kg⁻¹ for low, moderate and high V_T, respectively.

Table 1. Blood gas analysis after 8 hours of mechanical ventilation

	Tidal Volume		
	LV _T (n=4)	MV _T (n=4)	HV _T (n=4)
pH	7.39 ± 0.08	7.38 ± 0.02	7.37 ± 0.04
PaCO ₂ (mmHg)	45.0 ± 4.4	44 ± 6.4	42 ± 1.1
PaO ₂ (mmHg)	72.0 ± 13.8	83 ± 8.1	91.0 ± 9.8
Saturation (%)	95.0 ± 3.2	96.0 ± 0.8	97.0 ± 1.2

LV_T = low tidal volume;

MV_T = moderate tidal volume

HV_T = high tidal volume

Dynamic compliance of the respiratory system is shown in Figure 1. Dynamic compliance of animals ventilated with high V_T significantly increased within minutes of ventilation and then remained stable for the rest of the experiment, indicative of larger airspaces and loss of tissue recoil due to hyperinflation (Fig. 2). Overall mortality during ventilation was 16.4% with no differences between V_T groups. No autopsy was performed and electrolytes were not measured.

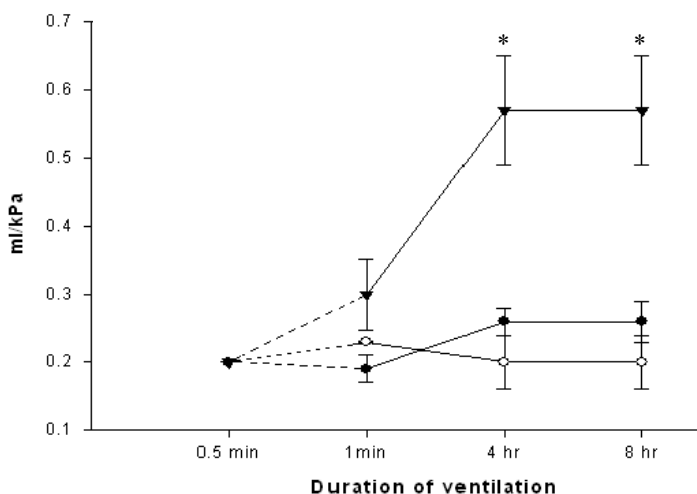


Figure 1. Dynamic compliance of 8-day newborn rats (n=8) ventilated with room air and low V_T (○), moderate V_T (●) and high V_T (▼). Dash lines are extrapolated. *p < 0.05 vs. ventilation for 1 minute.

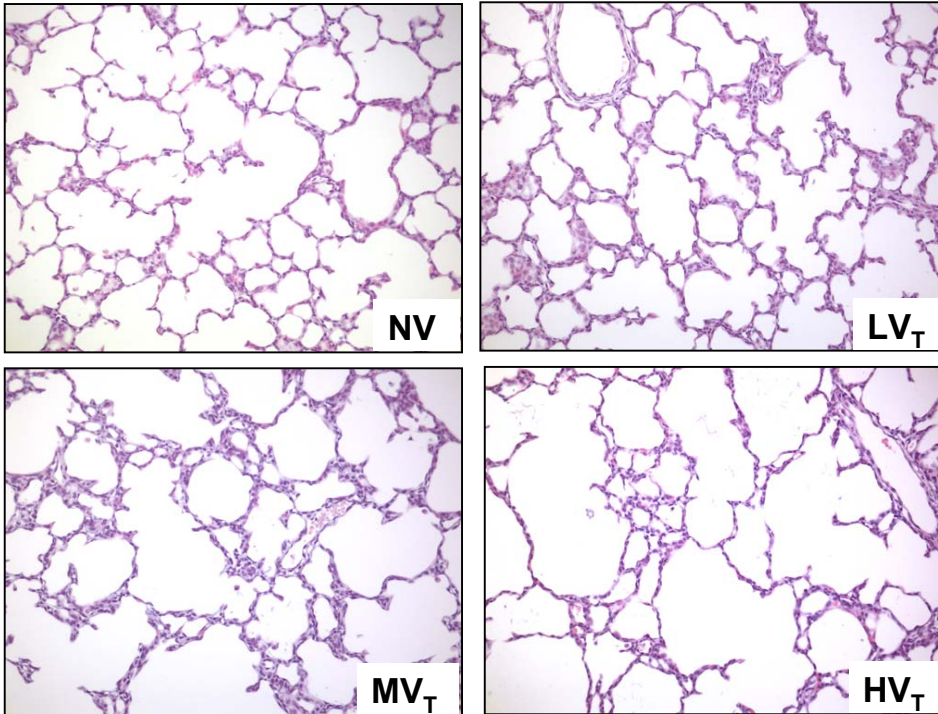


Figure 2. Representative sections of ventilated lungs stained with haematoxylin and eosin. Low, moderate and high V_T ventilation of 8-day neonatal rats do not show any areas of atelectasis, indicating no differences in lung recruitment. Air spaces are larger after high V_T ventilation due to hyperinflation.

Inflammatory cells in lung: High V_T ventilation was associated with a significant increase of MPO-positive neutrophils in comparison with NV, LV_T and MV_T . To a lesser extent, moderate V_T ventilation also increased the number of MPO-positive neutrophils (mainly in the alveolar space) in comparison with LV_T (Table 2). HV_T increased the number of neutrophils in both lung parenchyma and alveolar space. The number of macrophages (CD-68 antigen) did not alter among the ventilation groups (Table 2).

Cytokine mRNA expression: The effect of ventilation with different V_T on IL-1 β , IL-6, CXCL-2, and IL-10 mRNA expression is shown in Figure 3. Low V_T ventilation increased CXCL-2 and IL-6 mRNA levels vs. non-ventilated animals ($p < 0.05$), while those of IL-1 β and IL-10 were not altered. Moderate V_T ventilation appeared to further increase the expression of CXCL-2 vs. non-ventilated animals and that of IL-6 versus non-ventilated and low V_T rat pups, but the differences were not significant. Message levels of IL-1 β and IL-10 were also not altered by MV_T . High V_T ventilation significantly increased mRNA expression of IL-1 β , CXCL-2 and IL-6, but not IL-10, versus all other groups ($p < 0.05$).

Table 2. Effect of low, moderate and high V_T ventilation on number of myeloperoxidase- and CD68-positive inflammatory cells in lungs of 8-day newborn rats

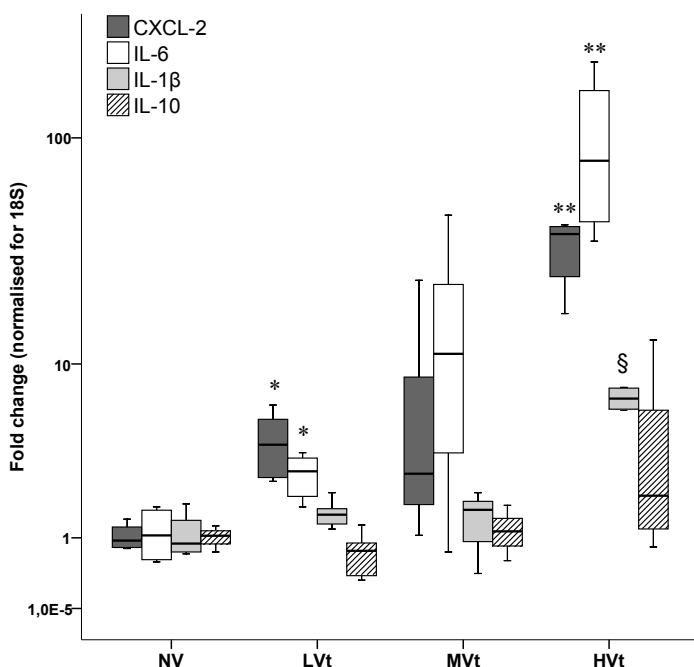
	Tidal Volume			
	NV	LV _T	MV _T	HV _T
MPO total	19.6 ± 8.4	14.0 ± 5.6	28.0 ± 3.0**	58.3 ± 4.2 [§]
MPO tissue	9.7 ± 3.5	10.8 ± 6.3	8.3 ± 0.3	32.3 ± 13.1 [§]
MPO air	10.0 ± 5.6	3.3 ± 2.8	19.7 ± 3.3*	26.0 ± 11.1*
CD 68	4.9 ± 0.9	6.1 ± 0.88	3.7 ± 0.4	4.4 ± 1.0

Number of immunopositive cells per unit area (40X high power field); n = 5 fields per slide, 3 slides per animal and 4 animals per group.

*p < 0.05 vs. NV and LV_T

**p < 0.05 vs. LV_T and HV_T

[§]p < 0.05 vs. other groups.

**Figure 3.** Effect of low, moderate and high V_T ventilation on pro-inflammatory gene expression in lungs of 8-day newborn rats. NV and LV_T; n = 8; MV_T; n = 6; HV_T; n = 4.

*p < 0.05 vs. non-ventilated group

**p < 0.05 vs. NV and LV_T group

[§]p < 0.05 vs. other groups.

Cytokines in BALF: Table 3 shows the amount of IL-1 β , IL-6 and CXCL-1 in BALF after eight hours of ventilation. The volume of lavaged material recovered from each animal (0.29 ± 0.08 ml) and BALF total protein content (0.22 ± 0.08 μ g/ μ l) did not differ

Table 3. Effect of low, moderate and high V_T ventilation on BALF cytokine protein content of 8-day newborn rats

Cytokine	NV (n=8)	LV_T (n=4)	MV_T (n=4)	HV_T (n=4)
IL-6	0.7 (0.6-1.1)	2.2 (1.1-4.9)	8.0 (3.3-11.7)	5.2 (1.7-21.0)
IL-1 β	0.13 (0.1-0.2)	0.2 (0.17-0.4)	0.33 (0.24-0.43)	1.0 (0.94-1.5)*
GRO/KC	10.3 (7.5-12.3)	9 (8.5-9.8)	7.9 (3.7-12.0)	14.9 (12.3-20.7)**

Concentrations are expressed as pg cytokine/100 pg of total BALF protein.

* $p < 0.05$ vs. NV, LV_T and MV_T

** $p < 0.05$ vs. LV_T and MV_T .

significantly between treatment groups. There was a trend towards an increase of IL-1 β and IL-6 with increasing tidal volume. However, only HV_T ventilation significantly increased IL-1 β and CXCL-1 levels.

Series II: Pre-exposure to LPS and Low Tidal Volume Ventilation with Oxygen

Physiologic data: Ventilation for 8 hours with low V_T with room air after exposure to LPS (LV_T +LPS), ventilation with 50% oxygen (LV_T + O_2), and ventilation with oxygen after exposure to LPS (LV_T +LPS/ O_2) resulted in normal pH and PaCO₂ (Table 4). Low V_T ventilation with room air after exposure to LPS (LV_T +LPS) and ventilation with 50% oxygen (LV_T + O_2) significantly increased the PaO₂ when compared to ventilation with room air (LV_T). The combination of ventilation with oxygen and exposure to LPS (LV_T +LPS/ O_2) further increased PaO₂ vs. LV_T and LV_T +LPS groups ($p < 0.05$). Mean airway pressures and peak pressures remained stable during the ventilation period and were not different between groups.

Table 4. Blood gas analysis after 8 hours of mechanical ventilation of 8-day newborn rats after exposure to endotoxin and/or 50% oxygen

	Tidal Volume		
	LV_T + LPS	LV_T + O_2	LV_T + LPS/ O_2
pH	7.40 \pm 0.04	7.32 \pm 0.18	7.30 \pm 0.08
PaCO ₂ (mmHg)	36.0 \pm 3.7	58.3 \pm 14.4	54.3 \pm 16.6
PaO ₂ (mmHg)	87.7 \pm 6.6*	131.0 \pm 52.1*	167.0 \pm 40.4**
Saturation (%)	96.5 \pm 1.2	96.4 \pm 3.6	98.4 \pm 1.6

* $p < 0.05$ versus animals ventilated with LV_T

** $p < 0.05$ versus animals ventilated with LV_T and LV_T + LPS.

Dynamic compliance of the respiratory system is shown in Figure 4. Ventilation with room air after exposure to LPS (LV_T +LPS), ventilation with 50% oxygen (LV_T + O_2), and ventilation with oxygen after exposure to LPS (LV_T +LPS/ O_2) significantly decreased the dynamic compliance after 4 hours of ventilation. Loss of compliance can be explained by increase of stiffness of the lung as a result of lung injury. No further worsening of dynamic compliance was seen during the last 4 hours of ventilation. Overall mortality

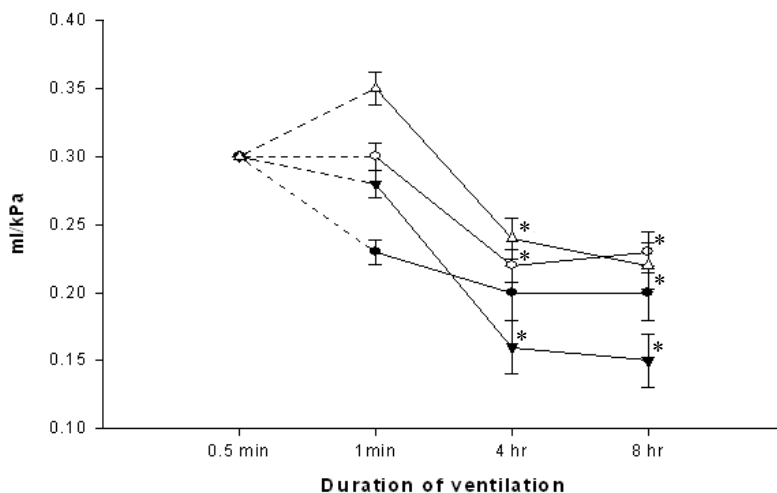


Figure 4. Dynamic compliance of 8-day newborn rats ventilated with low V_T and room air (●), low V_T and room air after exposure to LPS (○), low V_T and 50% oxygen (▼) and low V_T with 50% oxygen after exposure to LPS (▽). $n = 8$ animals per group. The dash lines are extrapolated. * $p < 0.05$ versus ventilation for 1 minute.

during ventilation was 8.1% with no differences between the 4 groups. No autopsy was performed.

Inflammatory cells in lung: Mean values and ranges for the number of macrophages (CD68- antigen) and MPO-positive neutrophils per unit area are shown in Table 5. Exposure to LPS, independent of ventilation with or without oxygen, was associated with a significant increase of MPO-positive neutrophils as well as CD-68 positive macrophages. The number of neutrophils was profoundly increased in the parenchyma and to a lesser extent in the alveolar space.

Cytokine mRNA expression: The effect of LPS, LV_T ventilation and LV_T ventilation after exposure to LPS (LV_T +LPS) on IL-1 β , IL-6, CXCL-2 and IL-10 mRNA expression is shown in Figure 5A. LPS significantly increased the expression of IL-6 and IL-1 β even 24 hours after administration when compared to saline treated animals ($LPS > NV$, $p < 0.05$). However, mRNA expression of CXCL-2 and IL-10 was not altered by the LPS pre-treatment. The combination of LV_T and LPS pretreatment did not increase the expression of IL-6 and CXCL-2 above that observed in animals ventilated with LV_T ($LV_T \approx LV_T$ +LPS $> NV$; $p < 0.05$) and decreased IL-6 mRNA levels compared to LPS treatment alone ($LPS > LV_T$ +LPS, $p < 0.05$). LV_T ventilation after exposure to LPS significantly decreased the expression of IL-10 mRNA ($p < 0.05$). Figure 5B shows the effect of ventilation with room air or 50% oxygen on IL-1 β , IL-6, CXCL-2 and IL-10 mRNA expression. As shown in Series I, low V_T ventilation with room air increased the expression of IL-6 and CXCL-2 vs. non-

Table 5. Combined effect of endotoxin, oxygen and low V_T ventilation on number of myeloperoxidase- and CD68-positive inflammatory cells in lungs of 8-day newborn rats

	Tidal Volume		
	NV	NV+LPS	LV_T
MPO total	19.6 ± 8.4	211.0 ± 74.0**	14.0 ± 5.6
MPO tissue	9.7 ± 3.5	187.3 ± 73.3**	10.8 ± 6.3
MPO air	10.0 ± 5.6	23.8 ± 2.2 [†]	3.3 ± 2.8
CD 68	4.9 ± 0.9	11.8 ± 0.33**	6.1 ± 0.88
	LV_T +LPS	LV_T +O ₂	LV_T +LPS/O ₂
MPO total	186.0 ± 117.5**	12.0 ± 1.4	188.7 ± 257.9 [§]
MPO tissue	169.3 ± 114.3**	8.3 ± 2.9	176.8 ± 259.3 [§]
MPO air	16.7 ± 9.7*	3.7 ± 3.5	11.8 ± 3.4**
CD 68	2.5 ± 2.8**	7.4 ± 0.6	13.4 ± 2.0**

Number of immunopositive cells per unit area (40X high power field). LV_T +O₂ group was not compared with NV+LPS and LV_T +LPS groups; n = 5 fields per slide, 3 slides per animal and 4 animals per group.

*p < 0.05 vs. LV_T

**p < 0.05 vs. NV and LV_T

[§]p < 0.05 vs. NV, LV_T and LV_T +O₂

[†]p < 0.05 vs. NV, LV_T and LV_T +LPS/O₂

ventilated controls. Ventilation with 50% oxygen further increased the message levels of both cytokines (LV_T +O₂> LV_T , p < 0.05). The expression of IL-1 β and IL-10 mRNA was not altered by ventilation with room air or oxygen. Ventilation with 50% O₂ after LPS pre-treatment (Fig. 5C) resulted in the greatest increase in IL-6 mRNA levels (LV_T +LPS/O₂>NV+LPS> LV_T +LPS \approx LV_T +O₂> LV_T >NV) (Fig. 5A-C). Interestingly, the combination of ventilation with oxygen and pre-exposure to LPS decreased the expression of CXCL-2 vs. ventilation with room air (LV_T +LPS/O₂ \approx NV< LV_T , p < 0.05) (Fig. 5C).

Cytokines in BALF: Table 6 shows the amount of IL-1 β , IL-6 and CXCL-1 protein in BALF after exposure to either LPS, ventilation with room air or oxygen, ventilation after exposure to LPS or the combination of ventilation with oxygen after exposure to LPS. The amount of BALF total protein was significantly increased after exposure to LPS and further increased after ventilation with room air or oxygen and after ventilation with oxygen of a LPS exposed lung (LV_T +LPS/O₂ \approx LV_T +O₂ \approx LV_T +LPS \approx LV_T >LPS>NV, p < 0.05), consistent with lung injury (Fig. 6). The volume of lavaged material recovered from each animal (0.31 ± 0.06 ml) did not differ significantly between the groups. IL-6 content of BALF increased after ventilation with oxygen (LV_T +O₂>NV, p < 0.05). An increase was also noted after ventilation of a LPS-exposed lung (LV_T +LPS>NV \approx NV+LPS, p < 0.05). Ventilation of a LPS exposed lung with oxygen did not further increase the BALF IL-6

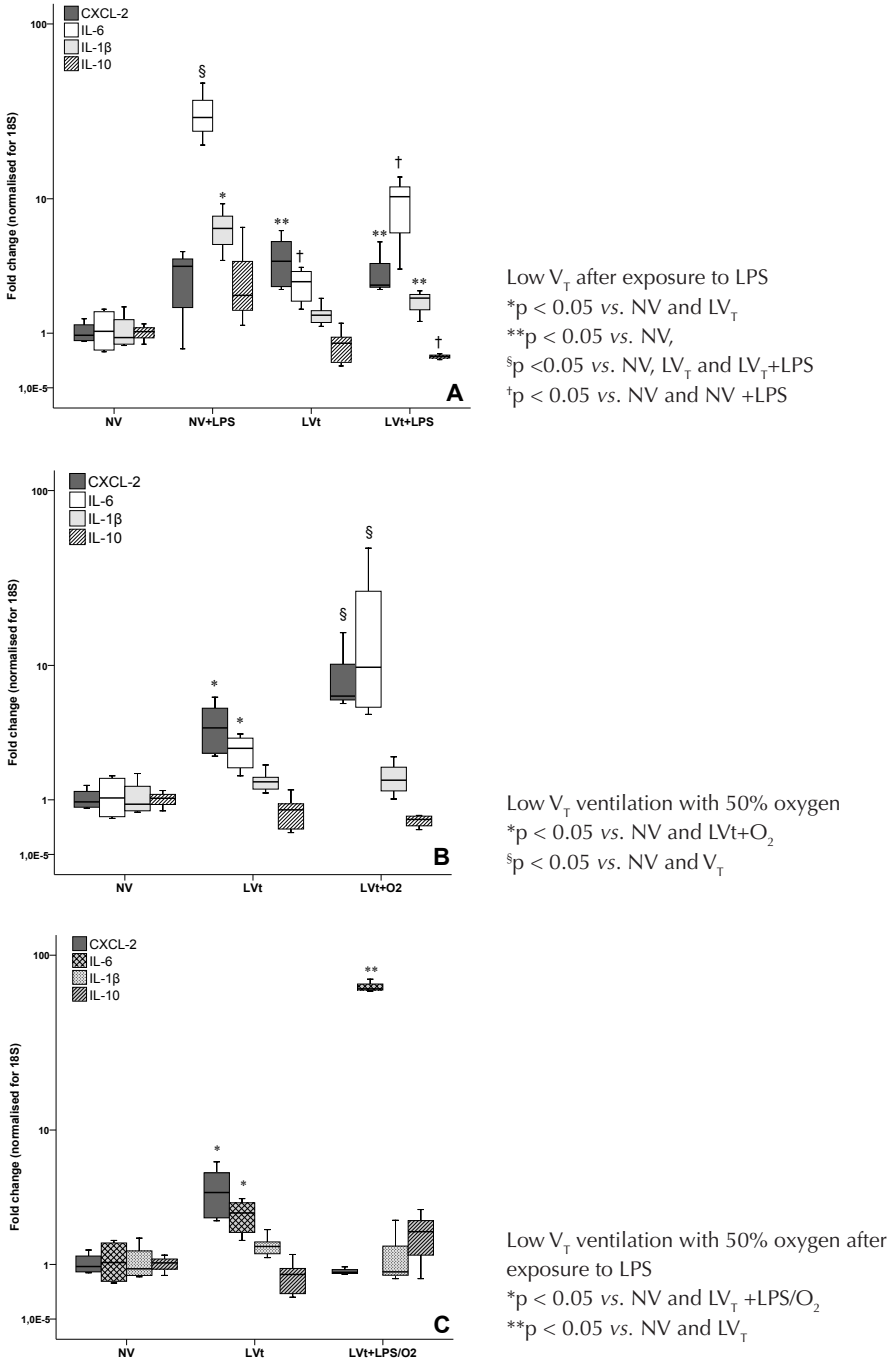


Figure 5. Effect of endotoxin and oxygen on low V_T ventilation triggered pro-inflammatory gene expression in lungs of 8-day newborn rats. NV and LV_T ; n = 8; NV+LPS, LV_T + O_2 and LV_T +LPS: n = 5; LV_T +LPS/ O_2 : n = 6.

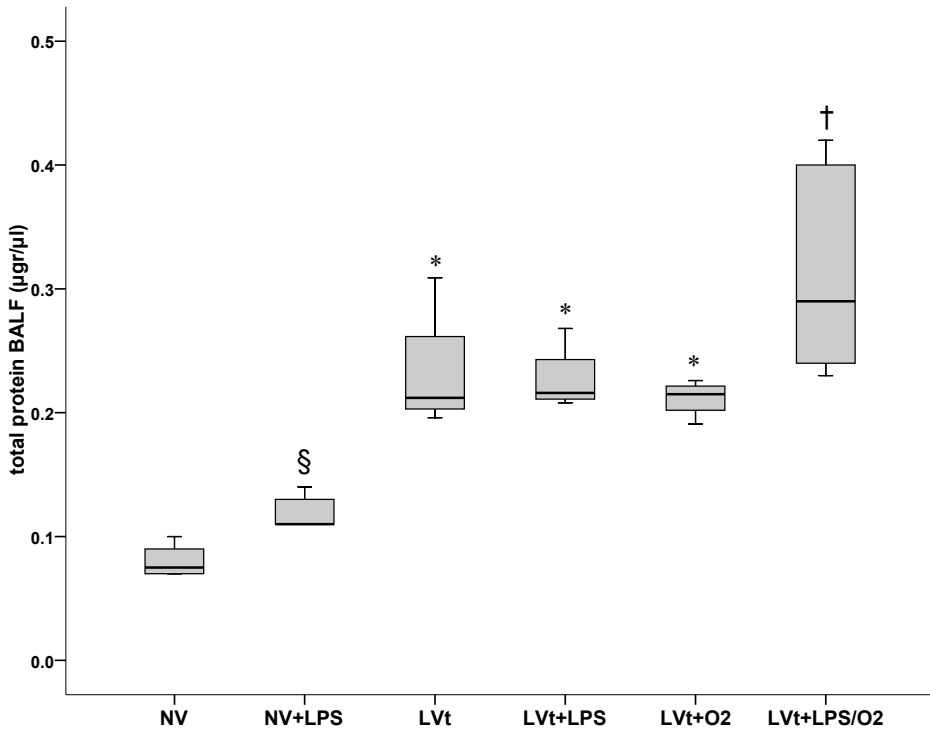


Figure 6. Effects of low V_T ventilation, endotoxin and oxygen on BALF total protein of 8-day newborn rats. NV and LV_T : n = 8; NV+LPS: n = 5; LV_T +LPS and LV_T + O_2 : n = 4; LV_T +LPS/ O_2 : n = 6. LV_T + O_2 group was not compared with NV+LPS and LV_T +LPS groups.

* $p < 0.05$ vs. NV

§ $p < 0.05$ vs. LV_T , LV_T +LPS and LV_T +LPS/ O_2

† $p < 0.05$ vs. LV_T +LPS and LV_T + O_2 .

content (LV_T +LPS/ $O_2 \approx LV_T$ + $O_2 \approx LV_T$ +LPS, $p > 0.05$). The concentration of IL-1 β in BALF only increased after ventilation with oxygen and after ventilation with oxygen after LPS exposure (LV_T +LPS/ $O_2 \approx LV_T$ + $O_2 > LV_T$ +LPS $\approx LV_T \approx NV$, $p < 0.05$). Independent of ventilation and oxygen, the concentration of CXCL-1 was increased after exposure to LPS (NV+LPS $\approx LV_T$ +LPS $> LV_T \approx LV_T$ + $O_2 \approx LV_T$ +LPS/ O_2 , $p > 0.05$).

Table 6. Combined effect of endotoxin, oxygen and low V_T ventilation on BALF cytokine protein content of 8-day newborn rats

Cytokine	NV	NV+LPS	LV_T
IL-6	0.7 (0.6-1.1)	0.7 (0.5-1.1)	2.2 (1.1-4.9)
IL-1 β	0.13 (0.1-0.2)	0.07 (0.06-0.08) [§]	0.2 (0.17-0.4)
GRO/KC	10.3 (7.5-12.3)	22.2 (18.3-23.4) [†]	9 (8.5-9.8)
	LV_T +LPS	LV_T +O ₂	LV_T +LPS/O ₂
IL-6	3.5 (2.6-3.8)**	2.1 (1.3-2.9)*	3.0 \pm 0.68**
IL-1 β	0.22 (0.18-0.23)	0.45 (0.38-0.55)*	0.29 (0.25-0.45) [†]
GRO/KC	15.7 (10-21.4) [†]	7.6 (7.0-9.2)	9.6 (7.4-12.2)

Concentrations are expressed as pg cytokine/100 pg of total BALF protein. n=8 for groups NV and LV_T , n = 4 animals for other groups. LV_T +O₂ group was not compared with NV+LPS and LV_T +LPS groups.

[§]p < 0.05 vs. NV, LV_T , LV_T +LPS and LV_T +LPS/O₂

[†]p < 0.05 vs. NV, LV_T ;

[‡]p < 0.05 vs. NV, LV_T and LV_T +LPS/O₂

[†]p < 0.05 vs. NV, NV+LPS and LV_T +LPS

*p < 0.05 vs. NV

**p < 0.05 vs. NV and NV+LPS

DISCUSSION

Mechanical ventilation, (intrauterine) infection and oxygen are well-recognized risk factors for BPD and known to trigger a pro-inflammatory response. In the present study we demonstrate that low V_T ventilation -presumed to be a less injurious form of ventilation- triggers a pro-inflammatory cytokine/chemokine response in neonatal rats, which is amplified by ventilation with oxygen, but not endotoxin pre-treatment. The combination of ventilation with oxygen and endotoxin pre-treatment either intensifies or abrogates the low V_T response, depending on individual cytokine or chemokine.

In the first series of experiments we assessed the effect of low, moderate, and high V_T ventilation on pro- and anti-inflammatory cytokine/chemokine production. Clinical data and experimental studies using premature animal models have compared high frequency oscillatory ventilation (HFOV: low V_T) and conventional ventilation (CMV: high V_T) with respect to pro-inflammatory cytokine/chemokine production and release into the alveolar and/or vascular compartment. Some studies reported findings favouring low V_T ventilation (4, 16) whereas others did not find any significant differences in cytokine/chemokine production/release between CMV and HFOV ventilation (17, 18). In the present study, we found a significant increase of mRNA expression of CXCL-2 and IL-6 after 8 hours of low V_T ventilation. Further increases were noted with increasing V_T as reported previously (14). Although there was a tendency to higher concentrations of IL-6 and IL-1 β protein in the BAL fluid, we did not find significant increases in total

number of inflammatory cells after low V_T ventilation. In contrast, moderate and to a higher extent high V_T ventilation increased the inflammatory response as shown by increases in IL-6, IL-1 β and CXCL-1 content in BALF and number of inflammatory cells in the lung. In addition, high V_T altered the dynamic compliance due to hyperinflation as shown previously (7). The difference between set and inspired V_T suggests tube leakage and/or expansion of tubing of the ventilator circuit which may influence the compliance measurement. No clinical signs for pneumothorax were observed. Thus, low V_T ventilation with room air for 8 hours results in a mild inflammatory response in the neonatal lung that is not overtly injurious (no change in dynamic compliance but an increase in the amount of protein in BALF). However, it is plausible that longer durations of low V_T ventilation increase the levels of pro-inflammatory cytokines sufficiently to cause lung injury. Although the low V_T ventilation strategy ($\sim 3.5 \text{ ml.kg}^{-1}$) cannot be compared with clinical applied HFOV ($V_T 0.5\text{-}2.0 \text{ ml.kg}^{-1}$), our finding of an inflammatory response may explain why randomised controlled trials did not show any beneficial effect of protective HFOV in preventing BPD in premature infants. This explanation is supported by several studies in which MV elevates pulmonary cytokines without cellular injury (19-21).

LPS triggers a network of inflammatory responses by activation of macrophages and recruitment of neutrophils, which was also observed in the present study. Activated macrophages release different pro-inflammatory cytokines and neutrophil activation causes the production of oxygen radicals and the release of granular enzymes, which are associated with injurious processes in the lung (10, 22, 23). Especially CXC chemokines and IL-8 activate and attract neutrophils into interstitial and alveolar spaces of the lung. Blocking neutrophils by blocking the CXCL-2 receptor led to increased alveolar formation and CXCL-2 null mice exhibited less ventilator-induced lung injury (10, 24). Several studies have shown that high V_T ventilation combined with another lung injury amplifies the inflammatory response in adult lungs (25, 26). A significant increase of CXCL-2 was measured in BALF of adult rats when high V_T ventilation (40 ml.kg^{-1}) was superimposed on a systemic inflammatory process (25). Ventilation of adult mice with smaller tidal volumes (6 ml.kg^{-1}) after induction of lung injury with hydrochloric acid showed a significant increase of IL-6 content in lung tissue versus ventilation alone (26). High V_T ventilation of neonatal rat lungs superimposed on a systemic inflammation induced by LPS (7) significantly increased IL-6 mRNA expression compared to high V_T ventilation alone. In the present study, we found an additive effect of 50% oxygen on LV $_T$ -induced expression of IL-6, in agreement with our previous study using 100% oxygen and high V_T (14). Whether the additive effect of oxygen on IL-6 expression is harmful remains a matter of speculation. IL-6 has long been considered a pro-inflammatory cytokine but adult transgenic mice that over-express IL-6 are more resistant to oxidative injury (27), while newborn IL-6 transgenic mice demonstrated more cell death after 100% oxygen

exposure (28). These data suggest that high levels of IL-6 in the lung may actually be beneficial in adult mice, but harmful in newborn mice. Surprisingly, we found that pre-exposure to LPS did not further increase IL-6 message levels when compared to LV_T ventilation alone. In contrast, the combination of low V_T ventilation with 50% oxygen after exposure to LPS further amplified the mRNA expression of IL-6. The increased expression of IL-6 mRNA after LV_T ventilation with and without oxygen, which was reflected in increased concentrations of this cytokine in BALF. LPS pre-exposure did not further increase BALF levels of IL-6. LV_T ventilation with 50% oxygen also increased the mRNA expression of CXCL-2 versus ventilation alone. Pre-treatment with LPS had no significant additive effect on CXCL-2 expression, in contrast to our previous findings with high V_T ventilation (7). No correlation between CXCL-2 mRNA expression and CXCL-1 protein content in BALF was observed, suggesting that CXCL-2 and CXCL-1 are not interchangeable. Together, these findings suggest that low V_T ventilation avoids the synergistic effect of ventilation and systemic inflammation on cytokine/chemokine expression seen with high V_T (7, 10). In contrast, oxygen has an additive effect on ventilation-induced cytokine/chemokine expression, which is tidal volume independent. Interestingly, upregulated CXCL-2 message was reduced to non-ventilated control levels when LPS-treated newborn rats were ventilated with 50% oxygen. This complex immunomodulatory regulation of CXCL-2 and IL-6 resembles that seen in LPS tolerant mice (29). Pulmonary IL-6 levels were significantly increased in the tolerant mice upon further LPS challenge, while CXCL-2 levels were significantly reduced. However, it is unlikely that a single exposure to LPS induced tolerance in our model.

We conclude that even low tidal volume ventilation can mount an inflammatory response in the newborn rat, which is amplified by a clinically relevant concentration of inspired oxygen.

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Chapter 4

Apoptotic Cell Death in Bronchopulmonary Dysplasia

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ABSTRACT

Apoptosis plays an important role in normal lung development as well as repair after lung injury and contributes to the pathophysiology of many lung diseases. Bronchopulmonary dysplasia (BPD) is a major cause of neonatal pulmonary and non-pulmonary morbidity and is characterized by an arrest in alveolar development. Currently there is no specific treatment for BPD. Mechanical ventilation, exposure to high concentrations of oxygen and inflammation are important risk factors for the development of BPD. Emerging evidence links the pathophysiology of BPD to an imbalance between anti-apoptotic and pro-apoptotic signaling pathways. Different apoptotic signaling pathways have been implicated, including Fas/FasL, caspase-dependent and -independent pathways, pro-survival Akt, transforming growth factor- β and p53. To what extent these pathways are involved in the pathogenesis of BPD is a hot topic of research. The aim of this review is to describe the timing and apoptotic events during lung development and the pathogenesis of BPD with particular focus upon apoptotic pathways activated by mechanical ventilation, hyperoxia and inflammation. An appreciation of these apoptotic pathways is essential for understanding the aetiology of and the development of treatments for pulmonary diseases such as BPD.

INTRODUCTION

Apoptosis is important for the right balance between the formation of new cells by mitosis, and the orderly destruction and removal of cells that are no longer needed by the organism. It is critical for normal lung development and function (1-2). In addition, apoptosis plays a vital role in remodelling of lung tissue after injury by clearance of excess epithelial and mesenchymal cells (3-4). Dysfunctional apoptotic mechanisms appear to be important in the pathophysiology of many lung diseases, including interstitial pulmonary fibrosis, chronic obstructive pulmonary disease and acute respiratory distress syndrome (5-11). Bronchopulmonary dysplasia, most commonly defined as an ongoing requirement for supplemental oxygen and/or respiratory support at 36 weeks post-menstrual age (12), is a chronic respiratory disease that develops as a consequence of neonatal lung injury. BPD is mainly seen in very low birth weight infants and is characterized by an arrest in alveolar and vascular development (13-14). Mechanical ventilation, intra-uterine infections and oxidative stress are well-recognized risk factors for development of BPD (15-17). Patients with BPD remain oxygen dependent for many months and in the first two years they frequently require hospital readmission and use more medication than patients without BPD (18-19). The most severely affected may remain symptomatic and have evidence of airway obstruction as adults (20).

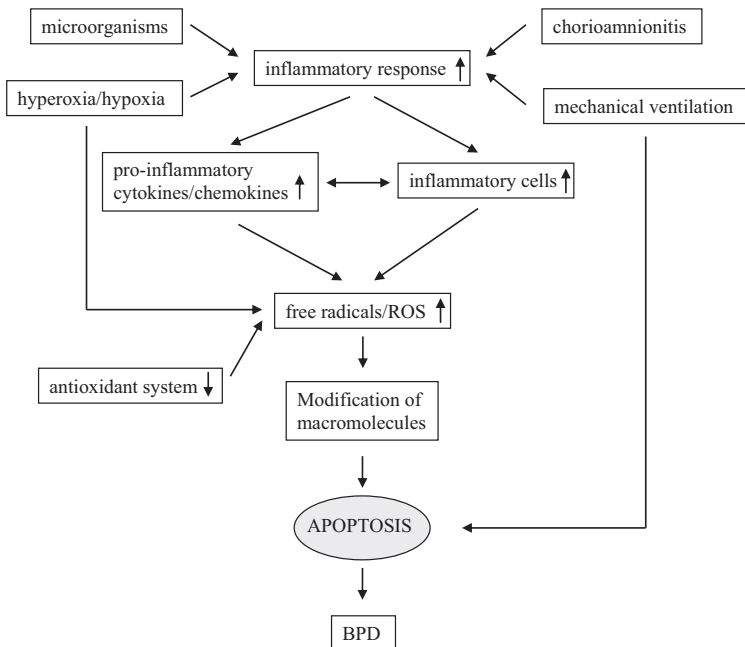


Figure 1. Possible pathogenetic sequence of events in the development of bronchopulmonary dysplasia.

The clinical treatment of preterm infants is complex. For example, mechanical ventilation is combined with oxygen treatment, making it hard to study the influence of each risk factor separately. Here we will discuss the importance of apoptosis for normal lung development and how that process can be altered by cell stretch (mechanical ventilation), hyperoxia and inflammation - the prime risk factors for BPD (Fig. 1).

Apoptotic pathways

Apoptosis is only one of several types of programmed cell death (21). Apoptotic cells are characterized by cytoplasmic and nuclear shrinkage, and the formation of apoptotic bodies. The cellular membrane of apoptotic cells remains intact (22). The result is the complete elimination of cellular debris without induction of an inflammatory response (23-24). Apoptosis is genetically determined during the course of development, but can also be triggered by external stimuli, such as inflammation and intrinsic stimuli, resulting from alteration of cellular function and metabolism (25). During apoptosis various proteases including caspases are activated. The human genome encodes 13 distinct caspases of which seven have been suggested to participate in apoptosis as initiator (caspase-2, -8, -9 and 10) or effector (caspase-3, -6 and 7) caspases (25-26). Caspases can be activated by either an extrinsic or intrinsic pathway (27-28). The extrinsic pathway is activated by binding of members of the tumor necrosis family (TNF) such as Fas Ligand (FasL) and TNF- α to specific cell surface death receptors including Fas receptor (CD95), TNF receptors I and II, DR4 and DR5 (29-31). Ligand binding is translated into intracellular signals that eventually leads to activation of effector caspases 3 and 7 via upstream initiator caspases 8 and 10 (27). Activated caspases 3 and 7 set off enzymes responsible for apoptosis resulting in nuclear condensation and fragmentation (26, 29). Studies have found increased concentrations of soluble Fas and FasL in the bronchoalveolar lavage (BAL) of patients with acute lung injury and acute respiratory distress syndrome (10). Excessive apoptosis of epithelial cells and infiltrating lymphocytes expressing FasL mRNA has been observed in fibrotic lung tissue of bleomycin-treated mice, which was abolished by the administration of a soluble form of Fas antigen or anti-FasL antibody (32). These findings suggest involvement of the Fas/FasL pathway in mediating apoptosis during lung injury. The intrinsic pathway is activated by physical and chemical stress signals, leading to changes in mitochondrial outer membrane integrity (26). The integrity of the mitochondrial outer membrane is determined by the balance between anti-apoptotic (e.g., Bcl-2, Bcl-_{XL}) and pro-apoptotic (e.g., Bax, Bad, Bak) B-cell lymphoma (Bcl-2) family proteins (29, 33). Permeabilization of the mitochondrial outer membrane will lead to release of pro-apoptotic factors such as cytochrome c. The release of these factors leads to the activation of caspase-9 and ultimately results in the activation of effector caspases including caspase-3. In certain conditions the extrinsic and intrinsic pathway are interlinked by caspase-8 (29). Besides

caspsases as executioners of programmed cell death, there is increasing evidence of the existence of caspase-independent pathways leading to apoptosis (34-35). Cathepsin B, a proteolytic lysosomal enzyme, seems to be the main executor of this pathway (36). In addition, lung cells can die via oncosis and autophagy (37-41).

Apoptosis and lung development

Development of the fetal lung can be divided into six overlapping stages (42). Apoptotic activity has been observed throughout each stage (43). During the embryonic stage (weeks 4-5 in humans) a diverticulum from the foregut endoderm invades into the surrounding splanchnopleuric mesoderm. The lung anlage gives rise to lung sacs on either side of the foregut tube that begins to separate at the same time into a ventral trachea and a dorsal oesophagus. Apoptosis occurs in the peripheral mesenchyme surrounding these lung sacs and underlying airway branch points (44). The pseudoglandular stage (weeks 5-17 in humans) is characterized by branching morphogenesis, establishing the prospective pre-acinar airways (i.e. those proximal to the respiratory bronchioles) (45). Apoptosis is found in the interstitial tissue, leading to mesenchymal involution (2, 46). The subsequent transition to the canalicular stage (weeks 16-26 in humans) is marked by completion of airway branching. The terminal bud epithelium is reduced from a pseudostratified form to simple cuboidal, marking the appearance of the future acini. The epithelium begins to differentiate into alveolar type II cells. The mesenchyme thins out by apoptosis -first peak of apoptosis during lung development- and developing capillaries begin to "canalize" the lung parenchymal interstitium of the primitive respiratory units (2, 42, 46). Apoptosis at this stage of lung development maybe mediated via the Fas/FasL pathway (47). During the saccular period (weeks 24-38 in humans) distal airways expand and obtain a saccular shape. There is further thinning of the mesenchyme by apoptosis and flattening of the cuboidal alveolar type II epithelial cells into squamous alveolar type I cells, leading to a close link between the capillary network and airway epithelium (2, 46, 48-49). The alveolar period (weeks 36-8 years in humans) is characterized by the formation and subsequent extension of secondary septa, thereby subdividing the immature saccules into smaller units. The capillary network forms a double layer of capillaries in each saccular wall. During this period an excess of alveolar type II cells are produced (50-51). These cells serve as putative stem cells for alveolar type I cells and produce surfactant. An excess of alveolar type II cells guarantees that enough alveolar type II cells can be formed when the alveolar surface area increases during the alveolarization period. Moreover, an excess of alveolar type II cells ensures enough pulmonary surfactant around birth (51). After birth excess of alveolar type II cells and fibroblasts are removed by apoptosis; second peak of apoptosis during lung development (2, 46, 50-51). The expression of p53 is up-regulated during this period in a time-dependent manner, indicating that a

p53-dependent pathway may be involved in regulating apoptosis at this time (52). Cell cycle regulator p53 is a transcriptional factor that can either arrest the cell cycle for repair of damaged DNA or initiate apoptotic cell death (53-54). Both pro-apoptotic proteins Bcl-2 and Bax are transcriptionally controlled by p53 (55). The third peak of apoptosis takes place during the period of microvascularization, the final stage of lung development (2, 46, 50-51). The air-blood interface thins further; the double layer of capillaries is reduced to a single layer and the interstitial tissue decreases (48). All peaks of apoptosis during lung development are preceded by peaks in proliferation, indicating the importance of apoptosis for the removal of excess lung cells (46, 50-51). Thus, apoptosis is vital for proper lung development but the signaling cascades leading to cell death remain largely unknown.

Apoptosis and stretch

Premature born infants with respiratory insufficiency often require mechanical ventilation. Mechanical ventilation causes cellular stretch, which may trigger an inflammatory response and apoptosis (56-57). The mechanism by which mechanical stretch triggers apoptosis is unknown.

Stretch-induced apoptosis during lung development was first demonstrated in a fetal rabbits after tracheal occlusion (TO). Ligation of the trachea caused a depletion of alveolar type II cells due to increased apoptosis (49). A mechanical stretch simulating fetal breathing movements stimulated apoptosis of day 19 (canalicular stage) rat lung fibroblasts, but not epithelial type II cells, compared to control, unstretched cells (58). This observation and the finding of increased mesenchymal apoptosis during the canalicular stage *in vivo* (2, 42, 46) suggests a role for physiological stretch induced-apoptosis in lung remodelling during development.

The mechanisms by which excessive stretch of fetal lung cells contributes to the development of BPD are not well defined. A continuous cyclic stretch of 20% elongation for 24 hours, mimicking injurious ventilation, increased apoptosis and cell death of day 19 fetal rat type II cells and fibroblasts (58-59). Mechanical ventilation for 24 hours of newborn mice with air led to a 5-fold increase in the number of apoptotic lung cells (60). Unfortunately, the apoptotic lung cell type was not identified. *In utero* ventilation of fetal sheep for 6 hours with 21% oxygen increased the number of caspase-3 positive cells, which were mainly interstitial cells. (61). In both studies the ventilation-induced increase in apoptosis coincided with a significant reduction in alveolarization (60-61). So far only one study has investigated the effect of ventilation on apoptosis in infants with BPD. In lung sections from ventilated preterm infants delivered at 22-36 weeks the number of apoptotic epithelial cells increased significantly compared to stillborn fetuses (62). Besides ventilation, however, these infants were also treated with oxygen.

That apoptosis of fetal lung cells is triggered by stretch is now widely recognized, but the signalling pathways that mediate apoptosis remain to be elucidated. Stretch induces the activation of caspases (58, 60). It has been suggested that activation of the Fas/FasL system is pivotal in activating the caspases (43). FasL has a temporal pattern of expression in the developing lung (63-64) maximal during perinatal transition. The time-specific upregulation of FasL gene and protein expression during development suggests a tightly upregulated transcriptional (*or posttranscriptional*) regulation of the FasL gene. Fas is continuously expressed in the fetal rabbit lung (64), while in the murine lung its expression increases at birth (63-64). Both Fas and FasL immunolocalize to alveolar type II cells (63-64). The high FasL expression in distal epithelial cells during perinatal transition corresponds to increasing type II cell apoptosis and maximal airway distension (63-64) implicating Fas/FasL as a mediator of late-gestational apoptosis which may be mediated by stretch. Indeed, sustained lung expansion in fetal rabbits due to TO resulted in a time-specific increase of FasL in alveolar type II cells, which coincided with increased alveolar type II cell apoptosis (63). However, stretching of day 19 fetal rat fibroblasts induced apoptosis without detection of FasL (58). Hence, signalling pathways independent of FasL must be involved in stretch-induced apoptosis of fetal and neonatal lung fibroblasts.

Tumor necrosis factor (TNF)- α and its receptors represent another major extrinsic cell death pathway. Mechanical ventilation up-regulates TNF- α (65). Whether this pathway is important in stretch-induced apoptosis is unknown. Other pro-inflammatory cytokines/chemokines up-regulated by mechanical ventilation are interleukin (IL)-1 β , IL-6, IL-8, and transforming growth factor (TGF)- β (59, 66-67). Some of these inflammatory mediators have also pro-apoptotic properties. The role of TGF- β in the pathogenesis of BPD has extensively been studied. Prolonged ventilation of newborn mice elevated pulmonary levels of Smad2, a downstream signaling molecule of the TGF- β pathway (68), indicating increased TGF- β signaling (60). TGF- β induces apoptosis by enhancing the Fas/FasL system via caspase-3 activation, but also via down regulation of p21^{Cip1/Waf1} expression (43, 69). It has been shown that p21^{Cip1/Waf1} suppresses apoptosis (70-71).

Apoptosis and hyperoxia

Oxygen is often life saving for prematurely born infants suffering from respiratory distress syndrome (RDS). To maintain adequate blood oxygen levels they require supra-physiological concentrations of oxygen. However, hyperoxia (or oxidative stress in a broader sense) is also a major contributing factor to the development of BPD (72-74). Oxidative stress is the result of an imbalance between exposure to oxidants and antioxidants. Prematurely born infants have a less developed and efficient antioxidant system (75-76) and oxygen treatment likely shifts the balance towards oxidative stress. In particular, free radicals and reactive oxygen species contribute to the development

of lung injury. These compounds have the ability to modify cellular macromolecules (proteins, lipids, carbohydrates and DNA), thereby promoting cell death (77-78).

Animal studies have shown that apoptosis is significantly increased in lungs exposed to hyperoxia. Exposure of newborn mice to 92% oxygen increased pro-apoptotic Bax mRNA and number of apoptotic lung cells (79). The increase in apoptosis was proportional to the duration of hyperoxia exposure. An increase in TUNEL-positive lung cells, mainly epithelial cells, and Bax expression was also demonstrated in neonatal rats exposed to > 90% oxygen (80). The increase in apoptosis coincided with an increase in p53, but not caspase-3, mRNA expression (81). Exposure of adult mice to 100% oxygen also increased p53 and Bax expression without affecting that of caspase-3 (82). Premature baboons delivered at 125 and 140 days of gestation and ventilated with high oxygen exhibited increased lung cell apoptosis and p53 expression (53, 83). Together, these findings suggest that p53 plays an essential role in hyperoxia-induced lung cell death. However, p53-deficient mice do not manifest an increased resistance to hyperoxia-induced lung damage (82), suggesting that multiple apoptotic pathways are involved in cell death during hyperoxia.

Interestingly, newborn wild-type mice raised in 95% oxygen had increased expression of pulmonary Fas and pro-apoptotic Bax, Bad and Bak mRNA, compared to newborn Fas-deficient *lpr* mice (84). Although apoptotic activity was similar in both groups, alveolar disruption was more severe in Fas-deficient *lpr* mice, suggesting a pro-proliferative rather than pro-apoptotic role of the Fas/FasL system (84). This contrasts to *in vitro* findings (85) and observations in adult *lpr* mice exposed to hyperoxia (82). These differences in pulmonary response to hyperoxia suggest that the effect of hyperoxia on pulmonary apoptosis and apoptosis-related gene expression may be age and species specific, which makes extrapolation of these data to the neonate complicated.

The TGF- β signaling pathway is also activated by hyperoxia. Neonatal mice and rats exposed to hyperoxia exhibited an arrest in alveolarization together with changes in the expression and localization of key signaling components of the TGF- β pathway (86-87). Exposure of alveolar type II cells to hyperoxia increased the sensitivity of these cells to TGF β -induced apoptosis (86). Tracheal aspirates of extremely low birthweight neonates contain increased levels of bioactive TGF- β , which may be predictive of the need for oxygen therapy at home (67).

Besides activation of pro-apoptotic pathways, there is also evidence of decreased expression of anti-apoptotic pathways. Pulmonary pro-survival Akt expression was decreased in an experimental model of BPD induced by oxygen exposure of newborn rats (88). Akt over-expression preserved and restored normal alveolarization in this BPD model. This coincided with decreased protein expression of active caspase-3.

As mentioned earlier, clinically, mechanical ventilation is combined with oxygen treatment, making it hard to study the influence of each risk factor on cell death sepa-

rately. Lungs of ventilated and oxygen-treated preterm infants have increased apoptosis of alveolar epithelial cells (89). Caspase-3 appears activated but not caspase-8 and/or -9. The mechanism by which caspase-3 was activated in human neonates remains to be elucidated.

Apoptosis and inflammation

Inflammation plays a critical role in the pathogenesis of BPD (66, 90-92). Preterm infants developing BPD have increased numbers of inflammatory cells in their airways and elevated serum concentrations of interleukin (IL)-6, IL-8, IL-1 β and IL-10 (93-95). An inflammatory response within the lungs of premature infants may result from prenatal (chorioamnionitis) and/or postnatal infections (bacteremia and sepsis), but can also be secondary to hyperoxia, hypoxia and mechanical ventilation or a combination of these factors. Each of these injurious stimuli triggers a different inflammatory response by activating different molecular pathways. In general, each stimulus increases the concentration of neutrophils, macrophages, proinflammatory cytokines and other inflammatory mediators.

Experimental animal models, mimicking chorioamnionitis in humans, have been used to study the effect of inflammation on lung development. In these models the effect of ventilation and oxygen is eliminated. Chorioamnionitis is an intra-uterine infection that starts early in pregnancy but remains undetectable until (preterm) delivery. It is associated with increased levels of pro-inflammatory cytokines in the amniotic fluid, including IL-6, IL-8, IL-1 β , TNF- α and granulocyte colony stimulating factor (G-CSF) (91, 96-98). Infants of mothers with increased levels of pro-inflammatory cytokines in the amniotic fluid have a higher risk of developing BPD (91, 98). The incidence of chorioamnionitis in prematurely born infants varies between 33% and 71% (99). Antenatal infection induced by intra-amniotic injection of endotoxin to pregnant rats, rabbits or sheep results in a BPD-like disorder, characterized by pulmonary inflammation, reduced alveolar formation and vascularisation (100-103). Twenty four hours after being delivered via Caesarean section, lungs of premature lambs exposed to intra-amniotic endotoxin showed a transient increase in the number of apoptotic alveolar type II cells (104). Similar observations have been made in lung tissue of still born human fetuses exposed to chorioamnionitis. Exposure triggered an pulmonary inflammatory response and apoptosis of epithelial cells (89). The increase in apoptotic epithelial cells coincided with an increase in active caspase-8 (89). Fetuses that were exposed to chorioamnionitis and a pneumonia exhibited an even greater apoptotic index, suggesting a further increase of mediators inducing apoptosis or the release of other pro-apoptotic mediator such as fibronectin and elastase (89). BPD patients have increased concentrations of fibronectin in their tracheal lavage (105) and fibronectin has been implicated in regulating apoptosis during lung development (106). Elastase is

released by neutrophils and degrades elastin. Elastin is essential for alveolar development (107-108). In a newborn mouse model dysregulation of elastin was associated with increased lung cell apoptosis and impaired alveolar formation (107). TUNEL staining demonstrated significant more apoptotic cells, located in the walls of distal air spaces, and increased levels of active caspase-3. These mice were ventilated with 40% oxygen. The effect on apoptosis has also been demonstrated in a neonatal mouse model with a postnatal inflammation (109). Systemic lipopolysaccharide induced lung inflammation and a 2-fold increase in apoptotic alveolar epithelial cells in neonatal mice when compared to non-infectious controls (109).

Diminished apoptosis of inflammatory cells may also play a role in the development of BPD. It has been suggested that activated neutrophils in the lungs have an prolonged half-life due to delayed apoptosis by the macrophages (110). The delayed apoptotic response provides the neutrophils with a longer life span of 8 hours, resulting in accumulation of activated neutrophils at the local site of injury and inflammation. This may induce a persistent inflammatory state and delay repair processes. A potential mechanism for the diminished neutrophil apoptosis involves NF κ B activation, which results in diminished caspase-3 and -9 activation (111-112) and decreased expression of pro-apoptotic proteins Bax, Bad and Bak (113). Inhibition of neutrophils influx

Table 1. Bronchopulmonary dysplasia and apoptotic molecules

Event	apoptotic molecules		Reference
	pro-apoptotic	anti-apoptotic	
Mechanical ventilation/ stretch	caspase-3		56, 58, 59
	Fas/FasL		61
	TNF- α		63
	TGF- β		58, 65, 66
	IL-8, IL-6, IL-1 β		57, 64
Hyperoxia		IL-10	57
	p53		53, 79, 81
	Bax		78, 82
	Bad, Bak	Fas/FasL	82
	TGF- β		84, 85
Inflammation		Akt	86
	caspase-3		107
	caspase-8		87
	IL-6, IL-8, IL-1 β		
	TNF- α , G-CSF		89, 96-98
	Fas/FasL fibronectine, elastase		87

in newborn rat lung with a selective CXC chemokine receptor-antagonist enhanced postnatal alveolar formation (114). Indeed, apoptosis of neutrophils in tracheal fluid of infants developing BPD is decreased (115-116). Impaired neutrophil apoptosis has been associated with a number of respiratory diseases (117), but its exact role in the pathogenesis of BPD is not known.

CONCLUSION

Inflammation, ventilation and oxygen are the most important risk factors for BPD and all three trigger apoptosis (Table 1). Abnormal apoptosis may contribute to the development of BPD. Apoptosis plays also an important role during normal growth and development of the lung as well as during repair after injury. Therefore, treatment strategies based on inhibiting apoptosis do carry the potential harm of inhibiting physiological apoptosis and inducing proliferation in cells other than targeted cells such as alveolar epithelial cells, fibroblasts and neutrophils. This can result in enhanced fibrosis and inefficient alveolar repair and recovery. A better understanding of the role of apoptosis in the pathogenesis of BPD and the signaling cascades leading to apoptosis is necessary before therapeutic strategies can be developed for the treatment of BPD.

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Chapter 5

Mechanical Ventilation-Induced Apoptosis in Newborn Rat Lung is mediated via FasL/Fas Pathway

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Submitted



ABSTRACT

Rationale: Mechanical ventilation induces pulmonary apoptosis and inhibits alveolar development in preterm infants, but the molecular basis for this apoptotic injury is unknown. *Objective:* To determine the signaling mechanism(s) of ventilation(stretch)-induced apoptosis in newborn rat lung. *Methods:* Seven-day old rats were ventilated with room air for 24 h using moderate tidal volumes (8.5 mL.kg⁻¹). Isolated fetal rat lung epithelial and fibroblast cells were subjected to continuous cyclic stretch (5, 10 or 17% elongation) for up to 12 h. *Measurements and Main Results:* Prolonged ventilation increased significantly the number of apoptotic alveolar type II cells (i.e. TUNEL-labelling, anti-cleaved caspase-3 immunocytochemistry) and was associated with increased expression of the apoptotic mediator FasL. Fetal lung epithelial cells, but not fibroblasts, subjected to maximal (i.e. 17%, but not lesser elongation) cyclic stretch exhibited increased apoptosis (i.e. nuclear fragmentation; DNA laddering) which appeared to be mediated *via* the extrinsic pathway (increased expression of FasL and cleaved caspase-3, -7 and -8). The intrinsic pathway appeared not to be involved (minimal mitochondrial membrane depolarization (i.e. JC-1 flow analysis); no activation of caspase-9). Universal caspases inhibition and neutralization of FasL abrogated the stretch-induced apoptosis. *Conclusion:* Prolonged mechanical ventilation induces apoptosis of alveolar type II cells in newborn rats and the mechanism appears to involve activation of the extrinsic death pathway *via* the FasL/Fas system.

INTRODUCTION

Mechanical ventilation is frequently required in preterm infants. Although contemporary 'protective' ventilation is designed to minimize volutrauma (1-2), this has not reduced the incidence of bronchopulmonary dysplasia (BPD) (3), a disease characterized by arrest of alveolar development. How mechanical ventilation adversely affects alveolar development is unknown, but a potentially important mechanism is apoptosis. For example, apoptotic cell (particularly alveolar epithelial cells) numbers are increased in ventilated pre-term infants (4-6). Apoptosis is central to -and occurs in all stages of lung development (6-9), and abnormal apoptotic activity may reduce alveolar number. Indeed, prolonged mechanical ventilation of neonatal mice increased lung epithelial cell apoptosis which was paralleled by inhibition of alveolar development (10).

Apoptosis can occur through the extrinsic or intrinsic (mitochondrial) pathways, but the pathway related to ventilator-induced apoptosis in the preterm lung is not known. Emerging evidence suggests a role for the extrinsic (Fas Ligand (FasL)/Fas) system in lung development (11). Fas is continuously expressed in fetal rabbit lung (12), in murine lung its expression increases at birth (9), and both Fas and FasL immunolocalize to bronchial epithelial cells and alveolar type II cells (9, 12). The temporal expression of FasL throughout development suggests transcriptional (or posttranscriptional) regulation of the FasL gene, and its expression in distal epithelial cells during perinatal transition corresponds to maximal airway distension and increasing apoptosis (i.e. in type II epithelial cells) (9, 12); thus, FasL-Fas may mediate stretch-induced apoptosis of late gestation. Indeed, this is consistent with the finding that tracheal occlusion (a potent cause of lung stretch) increases type II cell apoptosis as well as FasL protein in the fetal lung (13). Finally, induction of lung-specific FasL (using inducible transgenic mice) causes increased lung epithelial apoptosis during postcanalicular remodeling and impaired alveolar development (14). However, the role of the Fas/FasL system in ventilation-induced apoptosis in preterm infants remains unknown. Mechanical ventilation (stretch) may also activate the mitochondrial apoptotic pathway, leading to activation of initiator caspase-9. This pathway has received little attention in preterm infants. Such infants, when ventilated, have increased levels of cleaved-caspase 3, but not caspases-8 or -9 (5). Thus, it is unclear by which pathway apoptosis is activated during mechanical ventilation of immature lungs.

In this study we demonstrated *in vivo* (mechanical ventilation) and *in vitro* (cell stretch) stretch-induced apoptosis in pre-term and newborn lung models, and that apoptosis develops in type II epithelial cells (but not fibroblasts), and that the mechanism is through activation of the extrinsic (not intrinsic) pathway.

METHODS

Animals: Female timed-pregnant Wistar rats were obtained from Charles River (St. Quebec, Canada) and were housed in the Hospital for Sick Children animal facilities until used. All animal procedures were in accordance with Canadian Council of Animal Care guidelines and were approved by the Animal Care Review Committee of the Hospital for Sick Children.

Mechanical ventilation: Seven-day old rat pups were anesthetized by *i.p.* injection of pentobarbital (30 mg.kg⁻¹) and a tracheotomy was performed. Isoflurane was used as general anesthesia during the ventilation period and 0.9% saline (100 ml.kg⁻¹) was administered subcutaneously to prevent dehydration. Rat pups were ventilated (FlexiVent Scireq, Montreal, PQ) with room air and moderate V_T (7-9 ml.kg⁻¹, RR 150 min⁻¹, PEEP 2 cm H₂O) for 24 h as previously described (15) Rectal temperature was maintained at 37°C using a thermal blanket, lamp and plastic wrap. At the end of ventilation lung tissues were processed for histology or fresh frozen for molecular/protein analyses.

Immunohistochemistry: After flushing whole lungs were infused in situ with 4% (wt/vol) paraformaldehyde (PFA) in PBS with a constant pressure of 20 cm H₂O over 5 minutes to have equalized filling pressure over the entire lung. Under these constant pressure conditions the cannula was removed and the trachea immediately ligated. The lungs were excised and immersed in 4% PFA in PBS overnight, dehydrated in a ethanol/xylene series and embedded in paraffin. Following sectioning and antigen retrieval by heating in 10 mM sodium citrate pH 6.0, sections were washed in PBS and endogenous peroxidase was blocked in 3% (v/v) H₂O₂ in methanol. Blocking was done with 5% (w/v) normal goat serum (NGS) and 1% (w/v) bovine serum albumin (BSA) in PBS. Sections were then incubated overnight at 4°C with either rabbit anti-cleaved caspase 3 (Asp 175) antibody (1:200 dilution; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-Egr1 (1:50 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-FasL (1:500 dilution; Thermo Scientific Inc., Ottawa, ON, CA). Biotinylated goat anti-rabbit IgG was used as secondary antibody at a 1:300 dilution. Color detection was performed according to instruction in the Vectastain ABC and DAB kit (Vector Laboratories, Burlingame, CA, USA). Sections were counterstained with hematoxylin.

Immunofluorescence double staining: Following blocking sections were first incubated with rabbit anti-FasL (1:500 dilution; Thermo Scientific Inc., Ottawa, ON, CA) and then with biotinylated goat anti-rabbit IgG (dilution 1:300) and fluorescein-streptavidin (Invitrogen Corporation, Carlsbad, California, USA). Subsequently, sections were blocked again and then incubated with rabbit anti-prosurfactant protein C (pro-SPC)(1:1000

dilution, Abcam, Cambridge, USA), biotinylated goat anti-rabbit IgG (dilution 1:300) and CYTM3-streptavidin (Invitrogen).

TUNEL Assay: Sections were treated with proteinase K, washed in PBS and the terminal transferase dUTP end-labeling (TUNEL) assay was conducted according to manufacturer's instructions (in situ cell death detection-POD Kit; Roche, Montreal, Quebec, Canada). Following incubation with anti-fluorescein antibody staining was visualised using DAB. Sections were counterstained with hematoxylin. For quantitative analysis, digital images were captured at 20× magnification with random sampling of all tissue in an unbiased fashion. Images were captured randomly from 15 non-overlapping fields/slide with 3 slides/animal and 4 animals/group.

Cell stretch: Timed pregnant rats and their fetuses were sacrificed at day 19 of gestation (term=22 days). Rat fetal lung fibroblast (FLF) and epithelial (FLDE) cells were isolated as described previously (16). Within 24 hours of isolation, fibroblast and epithelial cells were separately inoculated at a density of 10⁶ cells/well on Bioflex multiwell plates precoated with collagen I and maintained in MEM + 10% FBS. Six hours prior to stretch medium was changed to MEM + 0.5% FBS. Cells received fresh MEM + 0.5% FBS and were then mounted in a Flexercell FX-4000 Strain Unit (Flexercell Int., Hillsborough, NC). Cyclic continuous radial elongations of 5, 10 or 20% were applied at intervals of 30 cycles per min for 2, 4, 6 and 12 h. In some cases, cyclic intermittent radial elongation of 5% at an interval of 30 cycles per min, 15 min/hour, was applied. Control cells were grown on the Bioflex collagen I plates, treated in the same manner as stretched cells, but were subjected to no stretch. Cells treated for 3 h with staurosporine, which induced apoptosis, served as positive control.

Analysis of DNA fragmentation: Following exposure to stretch, cells were stained with DAPI to visualize nuclear fragmentation. Apoptosis was confirmed by analysis of oligonucleosomal DNA cleavage. For DNA extraction, cells were incubated overnight at 55°C in lysis buffer (10mM Tris HCl, pH7.5, 10mM EDTA, 0.1% SDS, 0.2% Triton X-100, and 0.1 mg/ml proteinase K). After overnight lysis at 50°C, the samples were extracted with phenol-chloroform-isoamyl alcohol (25:24:1). The DNA was then precipitated with ethanol and sodium acetate and resuspended in Tris-EDTA, pH 7.5. The ApoAlert LM-PCR Ladder Assay Kit (Clontech Laboratories, Palo Alto, CA) was used for the detection of nucleosomal ladders in apoptotic cells, according to the manufacturer's instructions. Amplified products were visualized by electrophoresis in 1.5% agarose ethidium-bromide gels.

Analysis of PARP activity: After stretching, cells were trypsinized and resuspended in MEM (1.5×10^5 cells/ml). The cell suspension was centrifuged at $1,200 \times g$ for 3 min and the supernatant was discarded. The pellet was resuspended in 1 ml of 56 mM Hepes, 28 mM KCl, 28 mM NaCl, 2 mM $MgCl_2$ and vortexed. A 500 μ l aliquot of cell suspension was added to pre-mixed Eppendorf tubes containing 25 μ l digitonin, 25 μ l Nicotinamide Adenine Dinucleotide (NAD) and 3 μ l [3H]-NAD (ICN Biomedicals, St. Laurent, PQ) and incubated at $37^\circ C$ for 5 minutes. After the incubation 200 μ l of ice-cold 50% (vol/vol) TCA was added to the tube and left on ice for 10 minutes. The tubes were then centrifuged at $10,000 \times g$ for 10 min. The supernatant was discarded and the pellet was washed twice with ice-cold 20% (vol/vol) TCA. The final pellet was solubilized by the addition of 100 μ l of 4% (vol/vol) SDS and 100 μ l of 0.2M NaOH. The tubes were incubated overnight at $37^\circ C$. The next day the tubes were gently vortexed and 200 μ l of the solubilized samples were placed in scintillation tubes. Four ml of cytoscint (Ready-Safe, Millipore, Billerica, MA) and 30 μ l 100% acetic acid were also added to the scintillation tubes. The tubes were gently vortexed to ensure that everything was mixed and liquid scintillation counting was performed. PARP activity in counts per minute (cpm/ 10^6 cells), was expressed as fold change of PARP activity versus control samples.

Analysis of membrane permeability: After stretching, cells were harvested and washed twice with cold PBS. The cell density was adjusted to 1×10^6 cells per ml in PBS. One μ l of 100 μ M YO-PRO-1 and 1.5 mM Propidium Iodine (Molecular Probes, Eugene, OR) were added to one ml of cell suspension. The cells were then incubated on ice for 30 minutes in the dark and analyzed by flow cytometry (FACS Calibur using CELLQuest software; Becton Dickinson, Mountain View, CA), measuring the fluorescence emission at 530 nm and >575 nm.

Analysis of mitochondrial membrane potential: After stretching, cells were harvested, adjusted to a density of 0.5×10^6 per ml and stained with 2 μ g/ml JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide; Molecular Probes, Eugene, OR) dye for 30 min at $37^\circ C$. Cells were then washed twice with cold PBS containing 1% BSA and 7 mM sodium azide. Cells were resuspended in PBS and analyzed by flow cytometry. Mitochondrial membrane depolarization was monitored as a result of a shift in emission spectra after 480 nm excitation from orange-red (590 nm, for JC-1 aggregates) to green (530 nm, for JC-1 monomers). The green and red fluorescence signals were resolved by detection in the conventional FL1 and FL2 channels, respectively.

Caspase activity measurements: Activation of caspases was determined by fluorometry. After stretch, cells were harvested and protein lysates were extracted. Lysates were

incubated with and without either caspase 3 (DEVD-fmk), caspase 8 (IETD-fmk) or caspase 9 (LEHD-fmk) substrates (Biomol, Plymouth Meeting, PA) for 2 hours at 37°C in 96-well bottom black coated plates (Sigma, St. Louis, MO). Activity was measured on microplate reader set a wavelength of 400 nm. The relative increase in caspase activity in stretched cells versus static control cells was calculated after subtracting the background obtained from lysates containing no substrate.

Western blot Analysis: Total protein was extracted from cells and protein content was measured according to Bradford (17). Samples (40 g protein) were subjected to 10% SDS-PAGE and transferred to PVDF membranes. After blocking with 5% skim milk in TBST (20mM Tris, 137 mM NaCl, 0.1% Tween 20) membranes were incubated with appropriate primary antibody overnight in 4°C. Primary antibodies were rabbit anti-cleaved caspase 8, (dilution of 1:500), rabbit anti-cleaved caspase 7 (dilution of 1:1000), rabbit anti-cleaved caspase 3 (dilution 1:500), rabbit anti-cleaved caspase 9 (dilution 1:500) and rabbit anti-FasL (dilution of 1:1000). The next day the membranes were washed TBST and incubated with either horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (dilution of 1:1000; Cell Signaling Technology, Danvers, USA). After several washes with TBST, protein bands were visualized using an enhanced chemiluminescence detection kit (Amersham, Piscataway, NJ, USA). Equal protein loading was confirmed by β -actin immunoblotting of same membrane.

Inhibition of Caspase Activation: Two broad spectrum caspase inhibitors benzylo-carbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) (R&D, Minneapolis, MN) and IDN6556 ((Idun Pharmaceuticals; donated by Dr. Mingyao Liu (University Hospital Network, Toronto, ON)) were dissolved in DMSO. Cells were pretreated with either medium containing DMSO or inhibitor (1-10 μ M) for the duration of the stretch regiment. After the stretch, the cells were analyzed for DNA-laddering and caspase-3 activity.

Neutralizing FasL: Cells were stretched in the presence and absence of 3 μ g/ml of either purified hamster anti-(mouse and rat) CD178 (Fas Ligand) antibody or nonimmune hamster IgG (BD Biosciences, Pharmingen, San Jose, CA) followed by assessment of cl-caspase-3 and -8 expression, caspase 8 activation and DNA fragmentation.

Quantitative RT-PCR: Total RNA was extracted from either lung tissues or cells and reverse transcribed. Complementary DNA was amplified for target genes Egr1, Fas and FasL as previously described (18-19). For relative quantification, polymerase chain reaction signals were compared between groups after normalization using 18S as internal reference. Fold change was calculated (20).

Statistical Analysis: All values of animal experiments are presented as mean \pm SEM of at least 4 separate animals. All values of cell experiments are shown as means \pm SD of at least 3 separate experiments carried out in triplicate. Statistical analysis was by Student's *t* test or, for comparison of more than two groups, by one-way analysis of variance followed by Duncan's multiple range comparison test, with significance defined as $p < 0.05$.

RESULTS

In Vivo Stretch-induced Apoptosis

Prolonged (24 h) mechanical ventilation with moderate V_T significantly increased the number of TUNEL-positive cells in the lungs of 7-day old rat pups vs. non-ventilated 8-day rat pups (Fig. 1). The increase in lung cell apoptosis by prolonged ventilation was confirmed by anti-cleaved caspase-3 immunohistochemistry (Fig. 1A:d-f). In adult rat lung, high V_T rapidly induces early growth response (Egr)-1, a transcription factor implicated in regulating FasL expression (21-22). Therefore, we investigated the effect of prolonged ventilation with moderate V_T on Egr-1 and FasL expression in newborn rat lung. Both Egr-1 and FasL mRNA and protein levels were increased by 24 h ventilation (Fig. 2A), and immunohistochemical localization indicated up-regulation primarily in alveolar epithelial type II cells (Fig. 2B), in agreement with previous findings for Egr-1 in adult lung (23). Co-localization of FasL and pro-SPC (type II cell marker) immunofluorescence confirmed the presence of FasL in type II epithelial cells (Fig. 2C).

We then utilized rat fetal lung distal epithelial (FLDE) cells in order to investigate the mechanism of ventilation (stretch)-induced apoptosis.

In Vitro Stretch-induced Apoptosis

Nuclear fragmentation and DNA laddering were detected in FLDE cells subjected to continuous 17% cyclic stretch (mimics moderate/high V_T ventilation) of 6 h when compared to static cultured control cells (Fig. 3A). This cell stretch regimen also increased PARP activity in FLDE cells but did not trigger DNA fragmentation or changes in PARP activity in fetal lung fibroblast (FLF) cells (Fig. 3B). Additionally, FLDE membrane permeability (i.e. fluorescent dye YO-PRO-1 absorbance) was increased compared to control cells (Fig. 3C). After 6 hours of stretch, membrane transport became transiently defective and the rate of uptake of the YO-PRO-1 was significantly increased (1.5-fold increase vs. static control; $p < 0.001$), and was further increased (1.7-fold vs. control; $p < 0.001$) after 12 hours of stretch. This stretch-induced permeability increase over time correlated with increased DNA fragmentation (Fig. 4A). Together, these data suggest

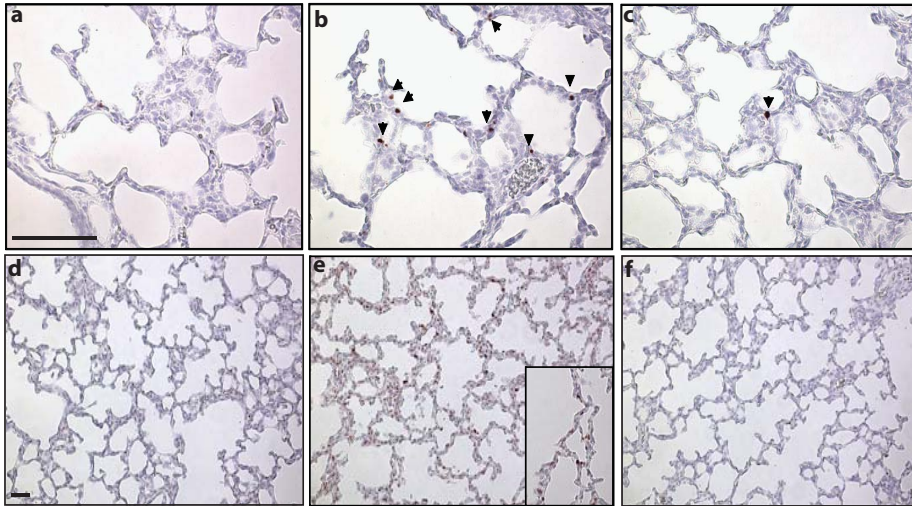
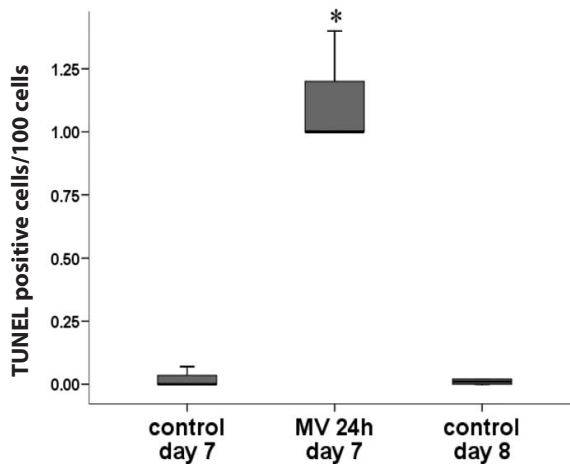
A**B**

Figure 1. Prolonged ventilation of newborn rat with moderate V_T triggers pulmonary apoptosis. TUNEL analysis (A, a-c) and cleaved caspase-3 immunohistochemistry (A, d-f) revealed increased numbers of apoptotic (brownish) cells (arrows) in 7-day newborn rat lung ventilated for 24 h with V_T of 7-9 ml.kg⁻¹ (A-b, A-e) when compared to non-ventilated 7- (A-1, A-d) and 8-day (A-c, A-f) newborn rat lungs. (B) Quantification of number of TUNEL-positive cells in ventilated and non-ventilated control rat lungs.

that a continuous 17% cyclic stretch regimen rapidly induces apoptosis in FLDE cells. To determine if lower levels of stretch would also initiate an apoptotic response in FLDE cells, cells were subjected to lesser stretch regimens. Neither intermittent stretch (5%; mimics fetal breathing movements) or continuous cyclic stretch (mimics regular

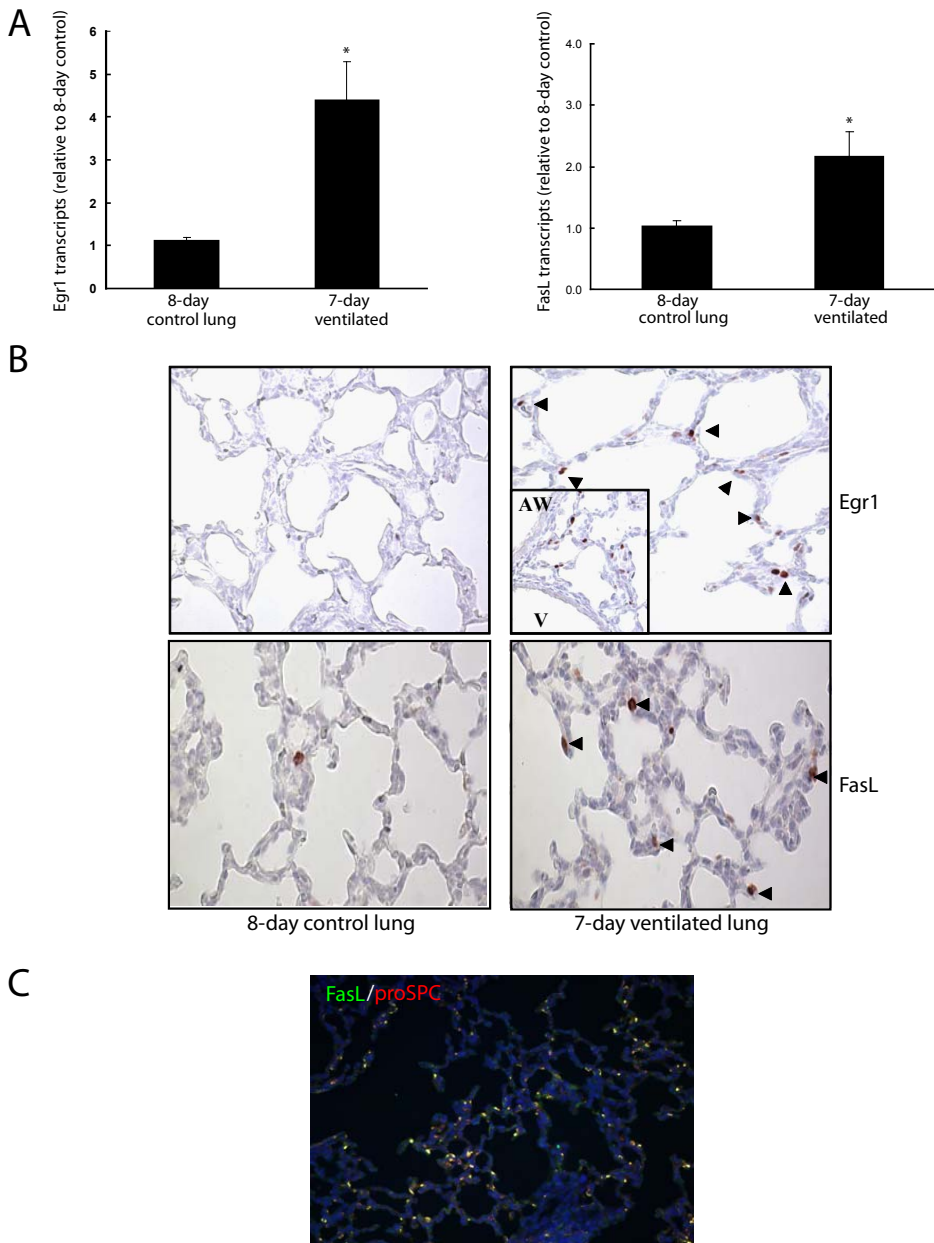


Figure 2. Prolonged ventilation of newborn rat with moderate V_T induces pulmonary Egr1 and FasL expression. A 24-h ventilation of 7-day rat with V_T of 7-9 ml.kg⁻¹ increased number of Egr1 and FasL transcripts (A) and immunopositive (brownish) cells (B) in whole lung compared to 8-day non-ventilated rat lung. (C) FasL (green) immunolocalizes with pro-SPC (alveolar type II cell marker: red) in the ventilated newborn rat lung (merged yellow indicates co-localization).

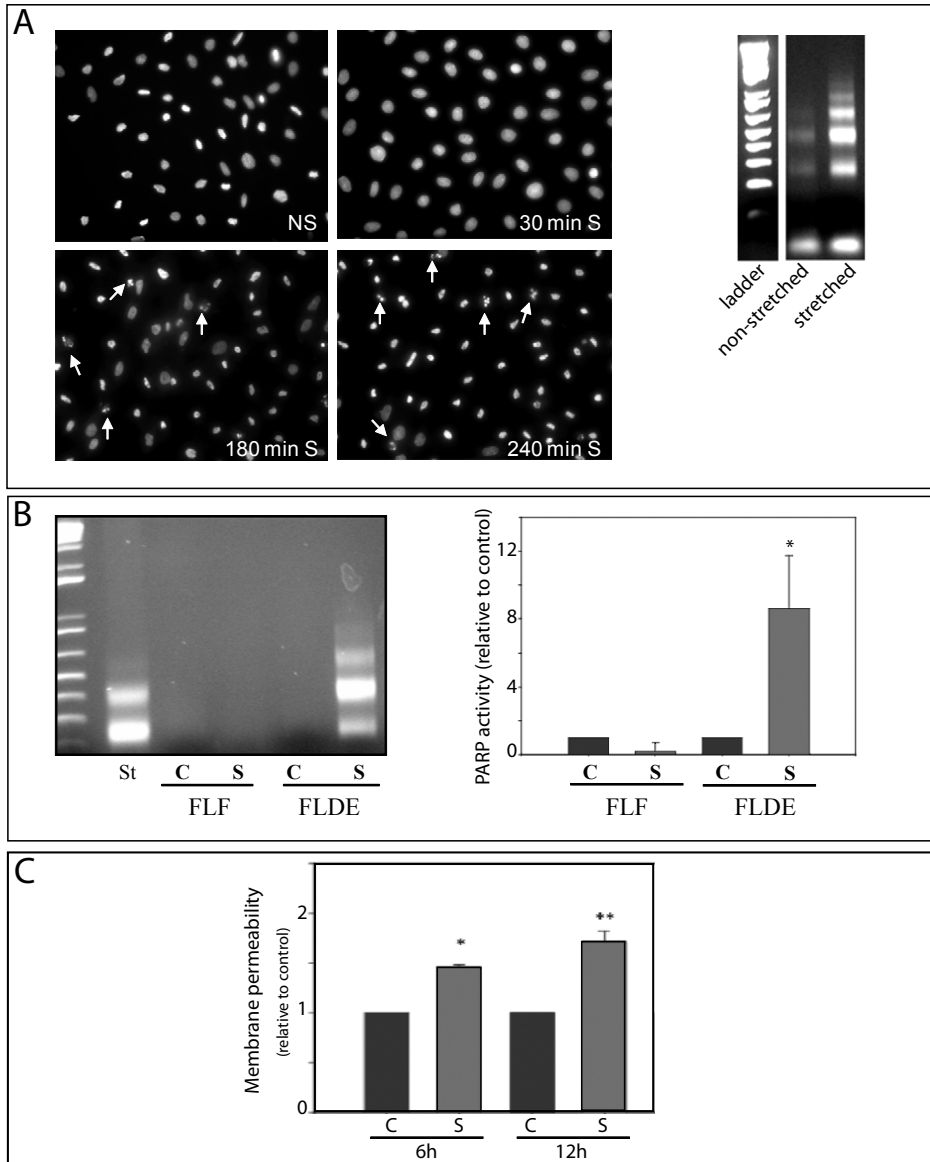


Figure 3. A continuous 17% cyclic stretch induces apoptosis in fetal lung epithelial cells. Increases in nuclear fragmentation (**A, left panel** - arrows), DNA laddering (**A-right panel** and **B-left panel**), PARP activity (**B-right panel**) and membrane permeability (**C**) were noted fetal lung distal epithelial (FLDE) cells, but not fetal lung fibroblasts (**B**), after 6 (**A, B, C**) and 12 (**C**) hours of stretch. C = static control; S = stretch; FLF: fetal lung fibroblasts. * or ** $p < 0.05$ vs. 6 or 12 h controls

breathing) did not induce DNA fragmentation, nor did the membrane permeability change in FLDE cells (Figure 4-B,C). Longer duration of stretch (17% cyclic stretch for 72 hours) also induced apoptosis in fetal lung fibroblasts (Fig. 4D).

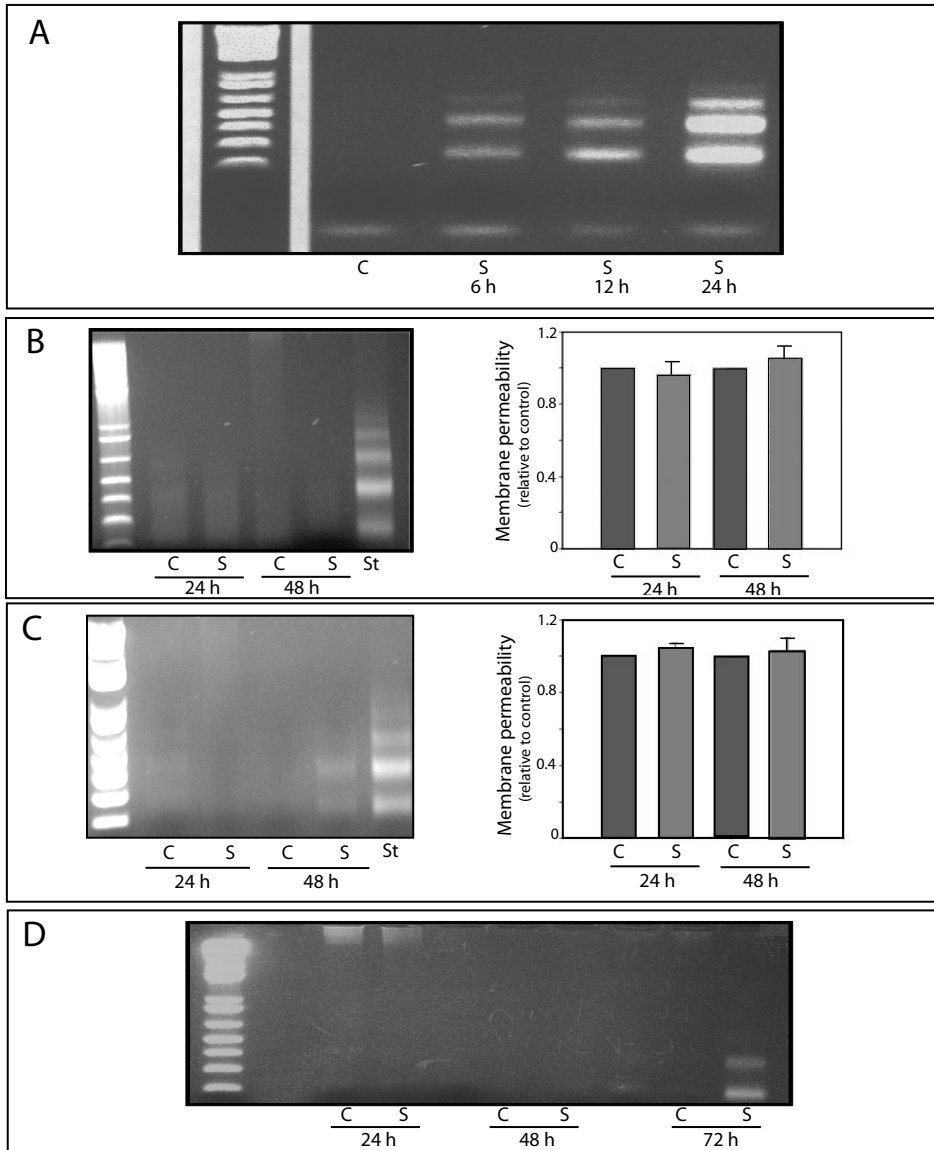


Figure 4. Stretch-induced apoptosis is time-, amplitude- and cell type-dependent. **(A)** Increased DNA fragmentation in rat fetal lung epithelial cells with duration of continuous 17% cyclic stretch. **(B and C)** Exposure of rat fetal lung epithelial cells to either an intermittent 5% cyclic stretch **(B)** or continuous 10% cyclic stretch **(C)** for 24-48 hours did not trigger DNA fragmentation (left panels) or alter the membrane permeability of the cells (right panels). **(D)** DNA fragmentation occurred in rat fetal lung fibroblast after 72 hours of continuous 17% cyclic stretch. C = static control; S = stretch.

Stretch-induced Apoptosis – Role of Caspases: Universal inhibitors of caspases (*ZVAD-fmk* and *IDN6556*) abrogated the stretch-induced DNA laddering in FLDE cells (Fig. 5A). Indeed, stretch increased the amount of cleaved-caspase-3, -7 and -8 (Fig. 5B)

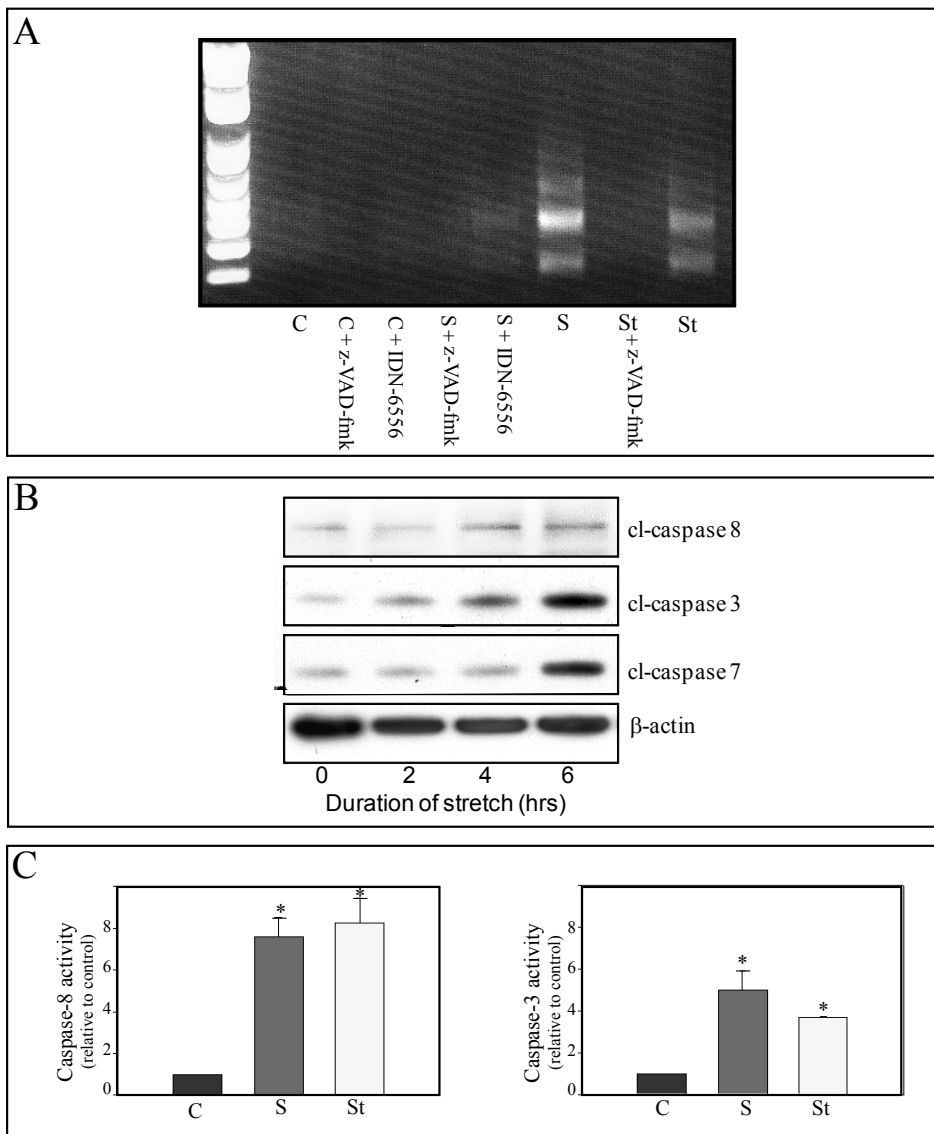


Figure 5. Stretch-induced apoptosis in fetal lung epithelial cells is caspase-dependent. (A) Universal caspase inhibitors, z-VAD-fmk and IDN-6556, abrogate DNA fragmentation in fetal lung epithelial cells subjected to a continuous 17% cyclic stretch of 6 hours. (B) Cleaved caspase-3, -7 and -8 content increases with duration of 17% stretch. (C) Increases of caspase-3 and -8 activities after 6 hours of 17% stretch. C = static control; S = stretch; St = staurosporine positive control; * $p < 0.05$.

as well as the activities of caspase-3 and -8 (Fig. 5C), implying activation of the extrinsic death pathway. Activation of the mitochondrial (intrinsic) death pathway was determined by measuring caspase-9 activation, as well as mitochondrial membrane depolarization. There was no stretch-induced increase in caspase-9 activation (Fig. 6A,

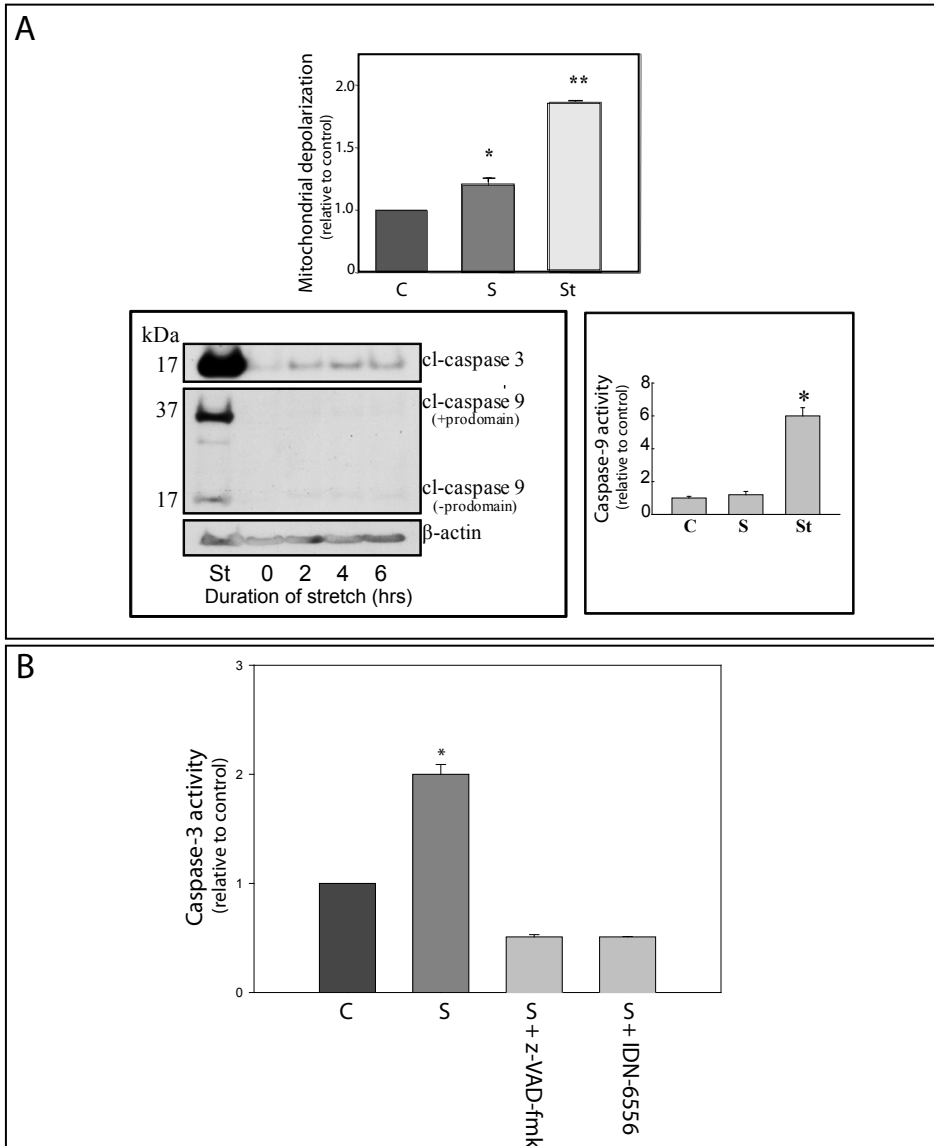


Figure 6. Stretch effect on mitochondrial membrane permeability, caspase -3 and -9 activity. (A) Mitochondrial membranes were depolarized but caspase-9 (in contrast to caspase-3) was not activated (bottom panel left: cleaved caspase-3 and -9 formation; bottom panel right: caspase-9 activity) in fetal lung epithelial cells subjected to continuous 17% cyclic stretch for 6 hours. (B) Universal caspase inhibitors, z-VAD-fmk and IDN-6556, abrogated 17% stretch-induced caspase-3 activation in rat fetal lung epithelial cells. C = static control; S = stretch; St = staurosporine positive control; * $p < 0.05$.

bottom panels), suggesting no or minimal activation of the mitochondrial (intrinsic) death pathway fluorescence (JC-1 monomers) when compared to static control cells, suggesting some by stretch. There was a small, shift in stretched FLDE cells from red

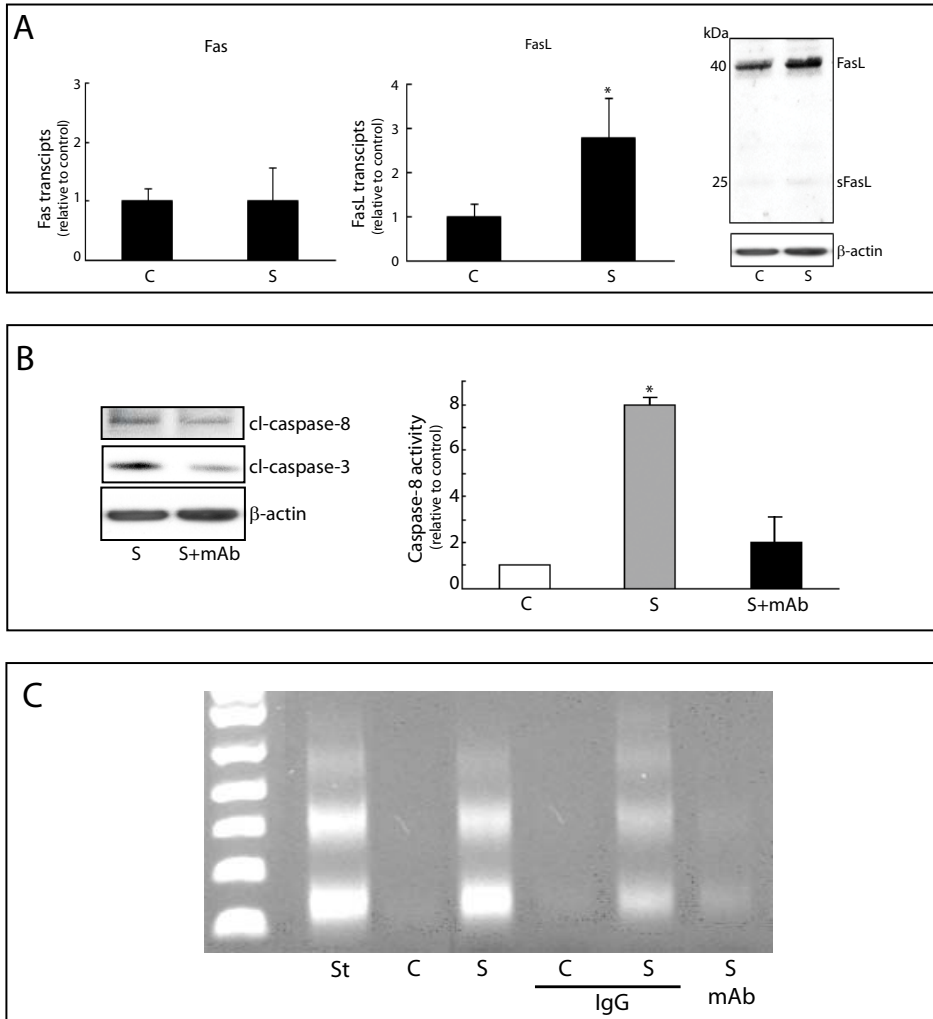


Figure 7. Stretch-induced apoptosis in fetal lung epithelial cells is mediated via FasL. (A) FasL, but not Fas, expression is increased in fetal lung epithelial cells subjected to 6 hours of 17% stretch. Neutralizing FasL monoclonal antibodies decrease cleaved caspase-3 and -8 content and caspase-8 activity (B) as well as DNA fragmentation (C) in fetal lung epithelial cells subjected to 17% stretch. C = static control; S = stretch; St = staurosporine positive control; IgG = antibody control, mAb = monoclonal antibody; *p < 0.05.

fluorescence (JC-1 aggregates) to green mitochondrial membrane depolarization in cells subjected to stretch (Figure 6A, top panel), but insufficient to activate caspase-9. Both universal caspase inhibitors ((ZVAD-fmk and IDN6556) abrogated stretch-induced caspase-3 activation (Fig. 6B).

Stretch-induced Apoptosis – Role of Fas/FasL: FasL mRNA levels were increased in FLDE cells subjected to stretch (Fig. 7A-middle panel) while Fas mRNA expression was

not changed (Fig. 7A-left panel). Western blotting revealed a small increase in FasL protein in stretched FLDE cells (Fig. 7A, right panel), and a neutralizing anti-rat FasL monoclonal antibody attenuated the stretch-induced levels of cleaved caspases-3 and -8 (Figure 7B-left panel) as well as stretch-induced caspase 8 activity (Fig. 7B-right panel) and stretch-induced DNA laddering (Fig. 7C). An IgG control had no effect (not shown).

DISCUSSION

Apoptosis is critical for normal lung development and function (7, 24), and plays a vital role in remodelling of lung tissue by clearing excess epithelial and mesenchymal cells after injury (25-26). Dysfunctional apoptotic mechanisms likely contribute to the pathophysiology of many lung diseases, including BPD. Mechanical ventilation is a well-recognized risk factor for development of BPD (27-29) and, in the present study, we demonstrate that mechanical ventilation of a newborn rat for 24 h with moderate V_T and in the absence of supplemental O_2 increases apoptosis of alveolar type II cells *via* activation of the FasL/Fas (extrinsic apoptotic) pathway.

Several *in vitro* studies have shown that mechanical stress stimulates apoptosis in isolated lung cells (30-32). Stretch-induced apoptosis *in vivo* was first demonstrated in fetal rabbits after tracheal occlusion (a major lung stretch stimulus) (33), and a recent report demonstrated that mechanical ventilation of newborn mice for 24 h led to a 5-fold increase in the number of apoptotic lung cells (10), and in keeping with the current data, a concomitant increase in cleaved caspase-3 protein (10). The number of caspase-3 positive cells, mainly interstitial cells, was also increased after *in utero* ventilation of fetal sheep for 6 hours (34). In both studies, ventilation-induced apoptosis coincided with a significant reduction in alveolarization (10, 34), in agreement with our findings using the newborn rat model (15). The death pathway(s) by which mechanical ventilation reduces alveolarization were not investigated. In the present study, we demonstrated that the extrinsic pathway (caspase-8 activation) was predominantly activated by mechanical stretch (mimicking moderate/high V_T ventilation) in fetal lung epithelial cells, resulting in activation of effector caspase-3 and -7 and subsequent DNA fragmentation. In contrast, the intrinsic pathway (caspase-9 activation) appeared not to contribute to stretch-induced apoptosis. The 17% cyclic stretch regimen increased apoptotic activity of fetal lung epithelial cells and not in fetal lung fibroblasts. In contrast, mechanical stretch simulating fetal breathing movements (FBM: 5% elongation at intervals of 60 cycles per min, 15 min per h) stimulated apoptosis of rat lung fibroblasts day 19 (canalicular stage), but not epithelial cells, when compared to control (non-stretched) cells (31). Thinning of the mesenchyme is essential during

this stage of lung development to create a close link between the capillary network and airway epithelium (35) and mesenchymal apoptosis during the canalicular stage of lung development has been reported previously (6-7, 36). These observations and our present findings suggest that during normal development, physiological stretch initiates apoptosis in fibroblasts that contributes to structural alignment in the canalicular stage; in contrast, during mechanical ventilation, pathological stretch initiates apoptosis in epithelial cells that contributes to lung injury and abnormal development. In support of this is the observation that pathological (20% cyclic stretch) increased apoptosis and cell death of rat fetal (day 19) type II epithelial cells (31, 37).

In adults with ARDS epithelial cell apoptosis appears to be induced by activation of the FasL/Fas pathway (38-40). In the setting of an inflammatory response, secreted FasL leads to lung epithelial cell apoptosis by binding to and activating Fas antigen in humans (41) and in newborn mice (42). In the present study, ventilation-induced apoptosis was accompanied by an increase in FasL expression in epithelial cells and stretch-induced FasL/Fas pathway activation was confirmed in fetal distal lung epithelial cells. A 17% cyclic stretch of these cells increased the expression of FasL, but not Fas, and neutralizing anti-rat FasL monoclonal antibody reduced the stretch-increased levels of cleaved-caspases 3, 7 and 8. These findings suggest that mechanical ventilation and cell stretch activate the FasL/Fas pathway by increased FasL expression, leading to activation of upstream initiator caspase-8, which in turn activates effector caspase-3 and -7. Alternatively, it is possible that mechanical stretch may increase the proteolytic release of soluble FasL from the membrane, thereby promoting binding to Fas-bearing cells and inducing apoptosis.

Together with the up-regulation of FasL, we found an increase of Egr-1 expression in alveolar type II cells of newborn rats subjected to prolonged mechanical ventilation. Previous *in vivo* studies using adult rats (21) and mice (43) and *in vitro* studies using fetal rat lung epithelial cells (44) have demonstrated that Egr-1 expression is up-regulated in response to mechanical stress while its down regulation by carbon monoxide attenuates ventilator-induced lung injury (43). Thus Egr-1 is an important pro-inflammatory transcriptional regulator that coordinates pro-inflammatory responses (45-46), including transcription of genes involved in growth and apoptosis (46), including FasL (47). The increased expression of both Egr-1 and FasL in alveolar type II cells after ventilation suggest that Egr-1 may activate the extrinsic caspase pathway *via* upregulation of FasL. Another possible mediator is interleukin (IL)-8, and it is known that Egr-1 regulates IL-8 expression (48) and that elevated levels of IL-8 induce apoptosis *via* increased FasL expression (49). Preterm infants developing BPD do, indeed, have elevated serum concentrations of interleukins, including IL-8 (50-51).

Only a few studies have investigated the effect of ventilation on apoptosis in infants with BPD. In preterm infants (gestational wk 24-37) elevated apoptotic activity was

found in bronchiolar and epithelial cells, but not in cells of mesenchymal origin (52). In lung sections from ventilated preterm infants delivered at 22-36 weeks the number of apoptotic epithelial cells was significantly greater than in the lungs of stillborn foetuses of similar gestational age (53), but in contrast to our experiments, these infants were also treated with oxygen. While newborn rats have immature lungs at birth, they do not need mechanical ventilation to survive and do not lack surfactant. However, rat lungs at birth have a saccular appearance (as in preterm infants of 24-30 wks) and alveolarization is an exclusively postnatal (between P4 and P21) event, which makes the newborn rat a suitable model of the infant population developing BPD.

In summary, our findings suggest a central role for the FasL-dependent pathway in ventilation -induced apoptosis of alveolar type II cells which may have therapeutic potential for preventing or treatment of BPD.

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Chapter 6

Prolonged Mechanical Ventilation Induces Cell Cycle Arrest in Newborn Rat Lung

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ABSTRACT

Rationale: The molecular mechanism(s) by which mechanical ventilation disrupts alveolar development, a hallmark of bronchopulmonary dysplasia, is unknown. **Objective:** To determine the effect of 24 hours (s) of mechanical ventilation on lung cell cycle regulators, cell proliferation and alveolar formation in newborn rats. **Methods:** Seven-day old rats were ventilated with room air for 8, 12 and 24 h using relatively moderate tidal volumes (8.5 mL.kg⁻¹). **Measurement and Main Results:** Ventilation for 24 h decreased the number of elastin-positive secondary crests and increased the mean linear intercept, indicating arrest of alveolar development. Proliferation (assessed by BrdU incorporation) was halved after 12 h of ventilation and completely arrested after 24 h. Cyclin D1 and E1 mRNA and protein levels were decreased after 8-24 h of ventilation, while that of p27^{Kip1} was significantly increased. Mechanical ventilation for 24 h also increased levels of p57^{Kip2}, decreased that of p16^{INK4a}, while the levels of p21^{Waf/Cip1} and p15^{INK4b} were unchanged. Increased p27^{Kip1} expression coincided with reduced phosphorylation of p27^{Kip1} at Thr¹⁵⁷, Thr¹⁸⁷ and Thr¹⁹⁸ (p<0.05), thereby promoting its nuclear localization. Similar -but more rapid- changes in cell cycle regulators were noted when 7-day rats were ventilated with high tidal volume (40 mL.kg⁻¹) and when fetal lung epithelial cells were subjected to a continuous (20% elongation) cyclic stretch. **Conclusion:** This is the first demonstration that prolonged (24 h) of mechanical ventilation causes cell cycle arrest in newborn rat lungs; the arrest occurs in G₁ and is caused by increased expression and nuclear localization of Cdk inhibitor proteins (p27^{Kip1}, p57^{Kip2}) from the Kip family.

INTRODUCTION

Introduction of more gentle ventilation strategies -together with surfactant replacement and antenatal corticosteroids- has improved the survival rate of very premature infants. In parallel, the number of infants with 'new' bronchopulmonary dysplasia (BPD) (1) has also increased. Currently, infants born at ≤ 26 weeks of gestation are at the greatest risk of developing such 'new' BPD (2), a syndrome of arrested lung development with fewer and larger alveoli and dysmorphic vasculature (3). BPD can no longer be considered only a pediatric disease because the substantial lung-function abnormalities -and significant symptoms- persist into adulthood (4-6). The pathogenesis of BPD is incompletely understood and its treatment is empirical, but mechanical ventilation remains a major risk factor.

Lung development between 24-32 weeks of gestation is characterized by extensive vasculogenesis within the developing terminal sacculles, followed by secondary crest formation as well as interstitial extracellular matrix loss and remodeling (7). This tissue remodeling requires well-coordinated regulation of cell proliferation and apoptosis. Recent studies have shown that prolonged mechanical ventilation increases apoptosis and impairs alveolar septation in newborn mice (8), however the effect of mechanical ventilation on lung cell growth is mostly unknown. *In vitro* studies have demonstrated that mechanical stretch (5% elongation, 60 cycles per min, 15 min/h for 24 h) and oxygen (95%) can inhibit lung cell proliferation (9-10), but molecular mechanisms are yet to be determined. Cell proliferation is a precisely coordinated set of events regulated by interaction of gene products that activate or suppress cell cycle progression. A series of cyclins and cyclin-dependent kinases (Cdk) act in concert to drive the cycle forward through the G_1 , S and G_2/M phases (11). In mammalian cells, G_1/S transition is an important checkpoint in the cell cycle. Entry into the cell cycle is initiated by mitogen-stimulated expression of D-type cyclins which activate Cdk4/6. Shortly thereafter, cyclin E expression is increased and cyclin E-Cdk2 complexes are formed, promoting entry into the S phase (12). While cyclin-Cdk complexes positively drive progression of the cell cycle, Cdk inhibitors (CKI) negatively regulate progression by binding to and inactivating cyclin-Cdks (13). There are two distinct CKI families in mammalian cells: INK4 proteins, which block the progression of the cell cycle by binding to either Cdk4 or Cdk6 and inhibiting the action of cyclin D; and, Cip/Kip proteins that inhibit a broader spectrum of cyclin-Cdk complexes (14-16).

In this study we determined the effect of prolonged (24 h) mechanical ventilation on lung cell cycle regulators, proliferation and alveolar formation in a newborn rat model (17). We hypothesized that continuous cyclic (over)stretching of the primitive airsacs would adversely affect proliferation of lung cells by influencing cell cycle regulators.

METHODS

Ethics statement: The study was conducted according to the guidelines of the Canadian Council for Animal Care and with approval of the Animal Care Review Committee of the Hospital for Sick Children (protocol #7217).

Animal preparation: Timed pregnant Wistar rats (Charles River, Oakville, Quebec, Canada) were allowed to deliver and immediately afterwards litters were reduced to 10 pups. Newborn rat pups were anesthetized by *i.p.* injection of 30 mg kg⁻¹ pentobarbital and a tracheotomy was performed. The trachea was cannulated with a 1 cm 19G cannula and connected to a rodent ventilator (FlexiVent Scireq, Montreal, PQ). Spontaneously breathing, non-ventilated, littermates served as sham controls. One pup per litter was ventilated and a littermate was used as non-ventilated control. Isoflurane was used as general anesthesia during the ventilation period and 0.9% saline (100 ml.kg⁻¹/24 h) was administered subcutaneously by continuous infusion with a 27G needle to prevent dehydration. First rat pups at postnatal days 6, 7, 8, 9, 10 and 14 were ventilated to assess lung cell proliferation. For all subsequent experiments 7-day old rat pups were used. Preliminary experiments were performed to determine ventilator settings (18). Starting from a normal respiratory rate for newborn rats (150 bpm), tidal volume was adjusted to achieve normal blood gas values after the ventilation period. Animals were monitored by ECG. Rectal temperature was maintained at 37°C using a thermal blanket, lamp and plastic wrap. At the end of the ventilation period a blood sample from the carotid artery was taken for blood gas analysis prior to euthanasia.

Mechanical ventilation: Rat pups were ventilated with room air and moderate-V_T (8.5 mL.kg⁻¹, RR 150 min⁻¹, PEEP 2 cm H₂O) for 8, 12 and 24 h. In a few cases, pups were ventilated for 4 h with high-V_T (40 mL.kg⁻¹, RR 30 min⁻¹, PEEP 2 cm H₂O). The 7-day old pups weighed 15.5-18.6 g. Dynamic compliance was estimated every 4 h from data obtained during a single-frequency forced oscillation manoeuvre, using a mathematical model-fitting technique according to the specifications of Scireq Inc. (Montreal, PQ). Two h before completion of ventilation pups were injected *ip* with 50 mg/kg 5-bromo-2-deoxyuridine (BrdU). At completion of ventilation a blood sample was taken from the carotid artery for blood gas analysis and the animals killed by exsanguination. Lung tissues were processed for histology or fresh frozen for molecular/protein analyses.

Histology: After flushing whole lungs were infused in situ with 4% (w/v) paraformaldehyde (PFA) in PBS with a constant pressure of 20 cm H₂O over 5 minutes to have equalized filling pressure over the entire lung. Under these constant pressure conditions the cannula was removed and the trachea immediately ligated. The lungs were excised

and immersed in 4% PFA in PBS overnight, dehydrated in a ethanol/xylene series and embedded in paraffin. Sections of 5 μm were stained with hematoxylin and eosin or stained for elastin using accustain artrazine solution (Sigma, St. Louis MO, USA).

Immunohistochemistry: Following sectioning and antigen retrieval by heating in 10 mM sodium citrate pH 6.0, sections were washed in PBS and endogenous peroxidase was blocked in 3% (v/v) H_2O_2 in methanol. Blocking was done with 5% (w/v) normal goat serum (NGS) and 1% (w/v) bovine serum albumin (BSA) in PBS. Sections were then incubated overnight at 4°C with either 1:50 diluted mouse anti-BrdU (Boehringer Mannheim, Germany) or 1:400 diluted rabbit anti-phospho-histone H3 (Millipore, Billerica, MA, USA) antibodies (Lab Vision Corporation, Fremont, Canada). Biotinylated rabbit anti-mouse IgG or goat anti-rabbit IgG were used as secondary antibodies, respectively. Color detection was performed according to instruction in the Vectastain ABC and DAB kit (Vector Laboratories, Burlingame, CA, USA). All sections were counterstained with hematoxylin. For quantitative analysis, digital images were captured using a Leica digital imaging system at 20 \times magnification with random sampling of all tissue in an unbiased fashion. Images were captured randomly from 15 non-overlapping fields from each slide, with 3 slides per animal and 4 animals per group.

Morphometric analysis: Digital images were captured from either H&E- or elastin-stained slides with random sampling of all tissue in an unbiased fashion. Images were captured randomly from 15 non-overlapping fields/slide with 3 slides/animal and 4 animals/group. Tissue fraction was calculated from pixel counts of black/white images (19), mean linear intercepts (Lm) were measured and calculated (20) and the number of elastin-positive secondary septa determined.

Western blot analysis: Lung tissues were lysed, protein content measured (21) and aliquots (40 g protein) were subjected to 10% SDS-PAGE and transferred to PVDF membranes. After blocking with 5% (w/v) skim milk in TBST (20mM Tris, 137 mM NaCl, 0.1% Tween 20) membranes were incubated with appropriate primary antibody overnight in 4°C. Because of decreased BrdU incorporation and cyclin D1 and E1 expression, we focused on CKIs inhibiting Cdk-2, -4 and -6 (22). Primary antibodies were rabbit anti-p15^{INK4B} (dilution of 1:500), rabbit anti-p16^{INK4A} (dilution of 1:1000), mouse anti-p21^{Waf1/Cip1} (dilution 1:500), rabbit anti-p27^{Kip1} (dilution 1:500) and rabbit anti-p57^{Kip2} (dilution of 1:1000), rabbit anti-cyclin D1 (dilution of 1:1000) (all from Cell Signaling Technology, Danvers, USA) and rabbit anti-cyclin E1 (dilution of 1:1000) (Santa Cruz Biotechnology, Santa Cruz USA). Primary phosphorylated p27^{Kip1} antibodies were rabbit anti-p27^{Kip1} (pThr198) (dilution of 1:400) and rabbit anti-p27^{Kip1} (pSer10)-R (dilution of 1:2000; both from Santa Cruz Biotechnology, Santa Cruz, USA), rabbit

anti-p27^{Kip1} (pThr157) (dilution of 1:300; R&D Systems Inc, Burlington, Canada) and rabbit anti-p27^{Kip1} (pThr187) (dilution of 1:400; Novus Biologicals, Littleton, USA). The next day the membranes were washed TBST and incubated with either horseradish peroxidase–conjugated anti-rabbit or anti-mouse IgG (dilution of 1:1000; Cell Signaling Technology, Danvers, USA). After several washes with TBST, protein bands were visualized using an enhanced chemiluminescence detection kit (Amersham, Piscataway, NJ, USA). Band densities were quantified using Scion Image software (Version 1.6, National Institutes of Health, Bethesda, MD, USA). Equal protein loading was confirmed by immunoblotting for β -actin of same membrane.

Quantitative RT-PCR: Total RNA was extracted from lung tissues and reverse transcribed [14]. Complementary DNA was amplified for target genes cyclin D1, cyclin E1 and p27 as previously described (17, 19). For relative quantification, polymerase chain reaction signals were compared between groups after normalization using 18S as internal reference. Fold change was calculated (23).

Stretch of epithelial cells isolated from fetal rat lungs: Distal fetal lung epithelial cells (day 19 of gestation) were isolated as previously described (24). Cells were cultured on type-1 collagen-coated BioFlex plates and subjected various durations of cyclic continuous 20% stretch using a FX-4000 Flexercell Strain Unit (Flexercell Int., NC, USA) (25). Neither cell viability (trypan blue exclusion) nor cell attachment was affected by any of the applied stretch regimens. Cell lysates were processed for Western Blotting.

Statistical analysis: Stated otherwise all data are presented as mean \pm SD. Data was analyzed using SPSS software version 15 (SPSS Inc, Chicago, IL). Statistical significance ($p < 0.05$) was determined by using one-way ANOVA or Kruskal-Wallis test. *Post hoc* analysis was performed using Duncan's multiple-range test (data presented as mean \pm SD) or Mann-Whitney test (data presented as median and interquartile range).

RESULTS

Physiologic data: Blood gases were in the normal range after 8, 12 and 24 h of ventilation (Table 1). Mean airway pressures, peak pressures and delivered V_T remained constant up to 8 h of ventilation (18), but altered slightly after 12 h of ventilation compared to baseline (Table 1). Dynamic compliance of the respiratory system was constant up to 8 h of ventilation (18) decreased after 12 h and remained stable afterwards (Fig. 1). These results are indicative of the impact of 8 h of ventilation that did not subsequently worsen.

Table 1. Blood gas analysis and airway pressures after 8, 12 and 24 h of ventilation

	8 hrs	12 hrs	24 hrs
pH	7.39 ± 0.07	7.29 ± 0.05	7.30 ± 0.05
pCO ₂ (mmHg)	44.3 ± 6.4	35.8 ± 6.2	39.4 ± 4.5
pO ₂ (mmHg)	83 ± 8.4	87.4 ± 11.2	73.5 ± 11.7
BE (mmol/L)	-4.2 ± 2.3	-3.9 ± 1.6	-5.2 ± 2.1
Ppeak (cm H ₂ O)	10.9 ± 1.1	12.7 ± 1.1*	13.1 ± 1.3*
Pmean (cm H ₂ O)	6.3 ± 0.5	7.2 ± 0.5*	7.5 ± 0.6*
PEEP (cm H ₂ O)	2	2	2
Frequency (breaths/min)	150	150	150
Delivered V _T (ml/kg)	8.9 ± 0.2	8.5 ± 0.4*	8.4 ± 0.2*

Values represent means ± SD, n = 12 animals per group. *p < 0.05 versus values at 0 hrs. Ppeak, peak pressure; Pmean, mean pressure; PEEP, positive-end expiratory pressure.

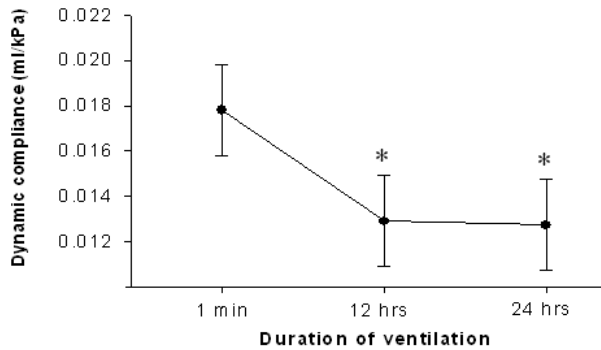


Figure 1. Dynamic compliance during 24 h of mechanical ventilation. Dynamic compliance decreased during first 12 h of ventilation with room air and low/moderate V_T but remained stable during the next 12 h. Data are mean ± SD, n = 12 rat pups per time group. *p < 0.05 prolonged versus 1-min ventilation.

Morphometric analyses: Seven-day old rat pups ventilated for 12 and 24 h had fewer and larger alveoli when compared to the lungs of non-ventilated 8 day-old pups (Fig. 2A). The tissue-to-air ratio corroborated these findings; it decreased after 12 h of ventilation and declined further during the next 12 h of ventilation (Fig. 2B). To quantify alveolar development, we calculated the number of elastin-positive secondary crests per unit area (Fig. 2D). The number of secondary crests -indicating alveolar formation-increased significantly between the 7th and 8th postnatal days in non-ventilated rat pups. The number of secondary crests increased after 12 h of ventilation when compared to day 7 controls. In contrast, the number of secondary crests was significantly lower in pups ventilated for 24 h vs. non-ventilated day 8 control pups, even when corrected for tissue fraction. To further evaluate alveolar development, we measured the mean linear intercept (Lm; Fig. 2C). Ventilation increased the Lm after 12 h, and more so after 24 h.

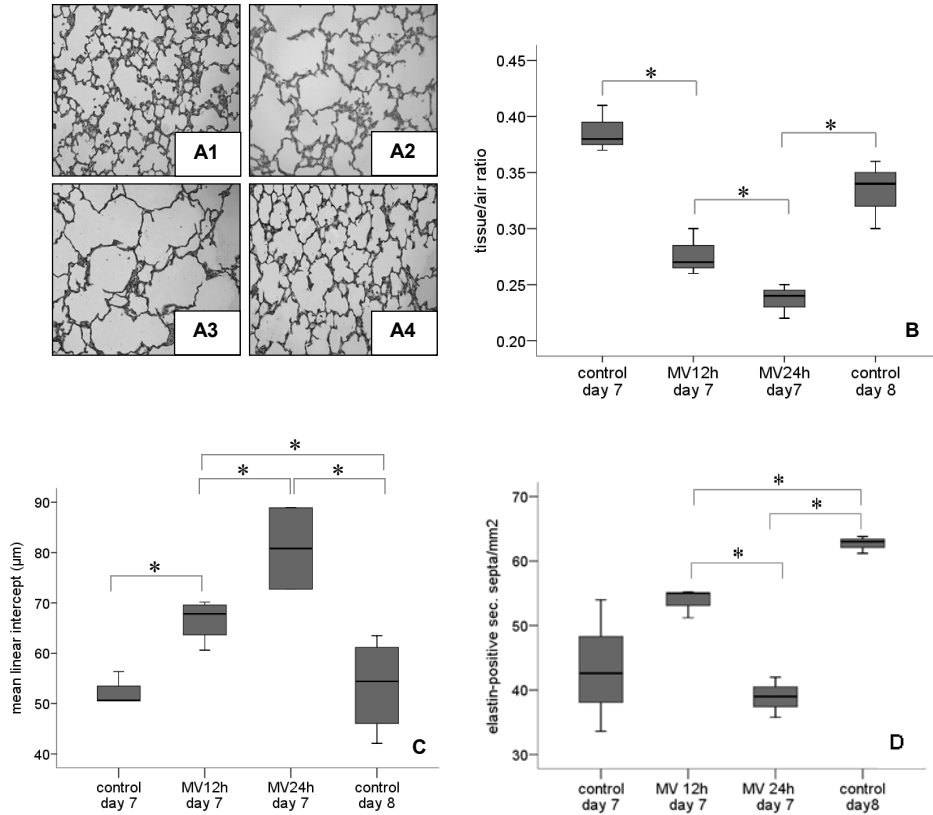


Figure 2. Ventilation inhibits alveolar growth. (A) Histology after mechanical ventilation: (A1) non-ventilated 7-day old rat (A2) 7-day old rat ventilated for 12 h, (A3) 7-day old rat ventilated for 24 h, (A4) non-ventilated 8-day old rat. (B) Mechanical ventilation for 12 and 24 h significantly increased alveolar airspace (reduction in tissue-to-air ratio (A) as well as increase in mean linear intercept (D)) but decreased number of elastin-positive secondary septa (C). Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles, n = 12 rat pups per time group. MV, mechanical ventilation. *p < 0.05.

Together the data suggest that during the first 12 h of ventilation alveolar space increases because of hyperinflation while a further increase of alveolar space during the next 12 h of ventilation is in part due to arrest in alveolar development as well as hyperinflation.

Lung cell proliferation: Lung cell proliferation was assessed in non-ventilated vs. ventilated rat pups at postnatal days 6, 7, 8, 9, 10 and 14. In non-ventilated rats, the number of proliferating lung cells was greatest at postnatal day 6 (BrdU labelling index: ~12%), which declined gradually to almost undetectable at day 15 (Fig. 3). Ventilation for 24 h clearly inhibited lung cell proliferation in pups of all studied ages (days 6-14). Next, 7-day old rat pups were ventilated for all subsequent experiments. Proliferation

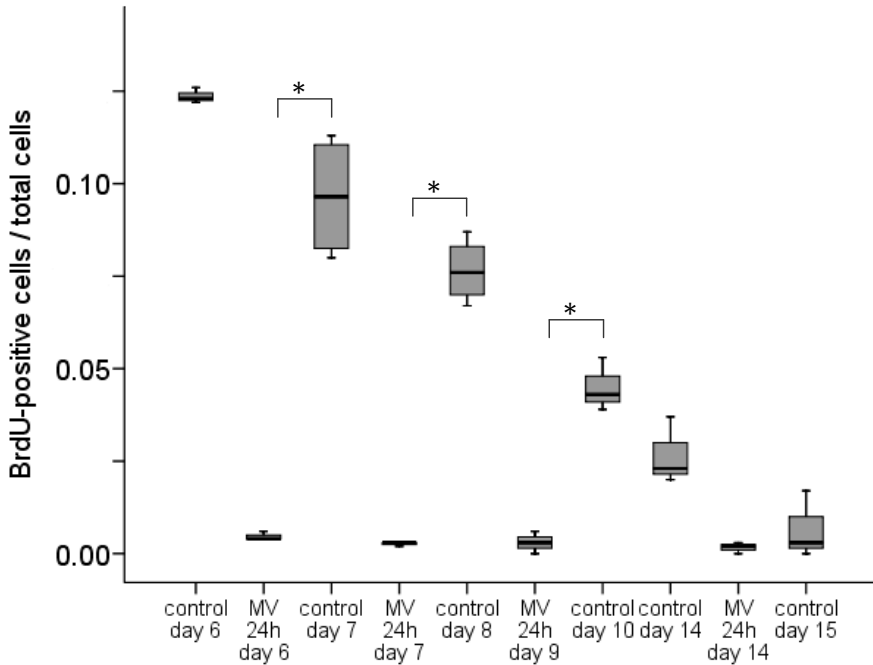


Figure 3. Ventilation inhibits BrdU uptake independent of postnatal age. Although the BrdU labeling index decreased gradually with advancing postnatal gestation, mechanical ventilation for 24 h inhibited cell proliferation at every postnatal age. Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles, n=4 rat pups per time group. *p < 0.05.

was not affected by 8 h of ventilation (data not shown) but longer durations of ventilation significantly decreased the number of proliferating cells (Fig. 4 A, B). The ratio of proliferating mesenchymal and epithelial cells did not significantly differ between non-ventilated pups and pups ventilated for 8 and 12 h, respectively (0.73 ± 0.05 vs. 0.65 ± 0.03 and 0.67 ± 0.1). Since a 12 h ventilation decreased the total number of proliferating cells (Fig. 4B) the unchanged ratio suggest that cell proliferation of both tissue layers was equally affected by mechanical ventilation. Hardly any proliferating cells were seen after 24 h of ventilation; in agreement with a reduction in cell proliferation in both tissue layers. The almost total arrest in lung cell proliferation by prolonged (24 h) ventilation was confirmed by anti-PH3 immunohistochemistry (PH3-positive cells decreased from 8 to 1% of total).

Cell cycle regulators: mRNA levels of lung cyclin D1 and E1 were significantly down-regulated after 8, 12 and 24 h of ventilation while that of p27^{Kip1} was increased (Fig. 5A). Immunoblot (i.e. protein) analysis of lungs ventilated for 24 h confirmed these mRNA changes of cyclin D1, E1 and p27^{Kip1} (Figs. 5B, 5C, 6A). The amount of p27^{Kip1} was 1.5-fold increased after 12 h of ventilation (not shown). Other members of the Cip/

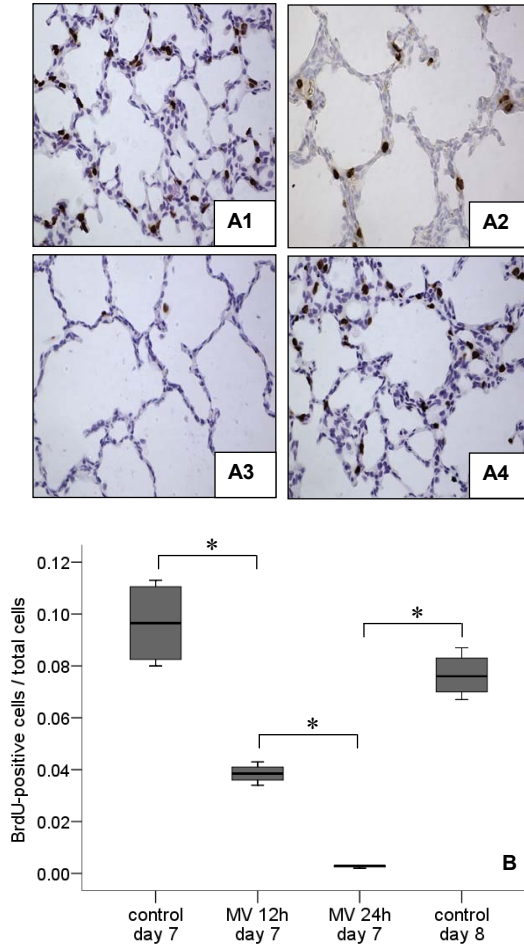


Figure 4. Ventilation inhibits lung cell growth. Immunohistochemistry ((A1) non-ventilated 7-day old rat, (A2) 7-day old rat ventilated for 12 h, (A3) 7-day old rat ventilated for 24 h, (A4) non-ventilated 8-day old rat) illustrates reduction in BrdU uptake (brown color) with duration of ventilation. (B) BrdU labeling index significantly decreased after 12 and 24 h of mechanical ventilation. Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles, n=12 rat pups per time group. MV, mechanical ventilation. *p < 0.05.

Kip family of CKIs were either increased (p57^{Kip2}, Fig. 6B) or unchanged (p21^{Waf1/Cip1}, not shown) by 24 h of ventilation. In contrast, CKIs belonging to the INK4 family were either reduced (p16^{INK4a}) or not affected (p15^{INK4b}) by 24 h of ventilation (Fig. 6C, D). p27^{Kip1} can be phosphorylated at different sites, which influences its localization and activity (26). A 12 h ventilation decreased phosphorylation of p27^{Kip1} at Thr¹⁵⁷ (Fig. 7A) but did not affect phosphorylation of Thr¹⁹⁸ (not shown). However, mechanical ventilation for 24 h decreased p27^{Kip1} phosphorylation at Thr¹⁵⁷, Thr¹⁸⁷ and Thr¹⁹⁸, thereby promot-

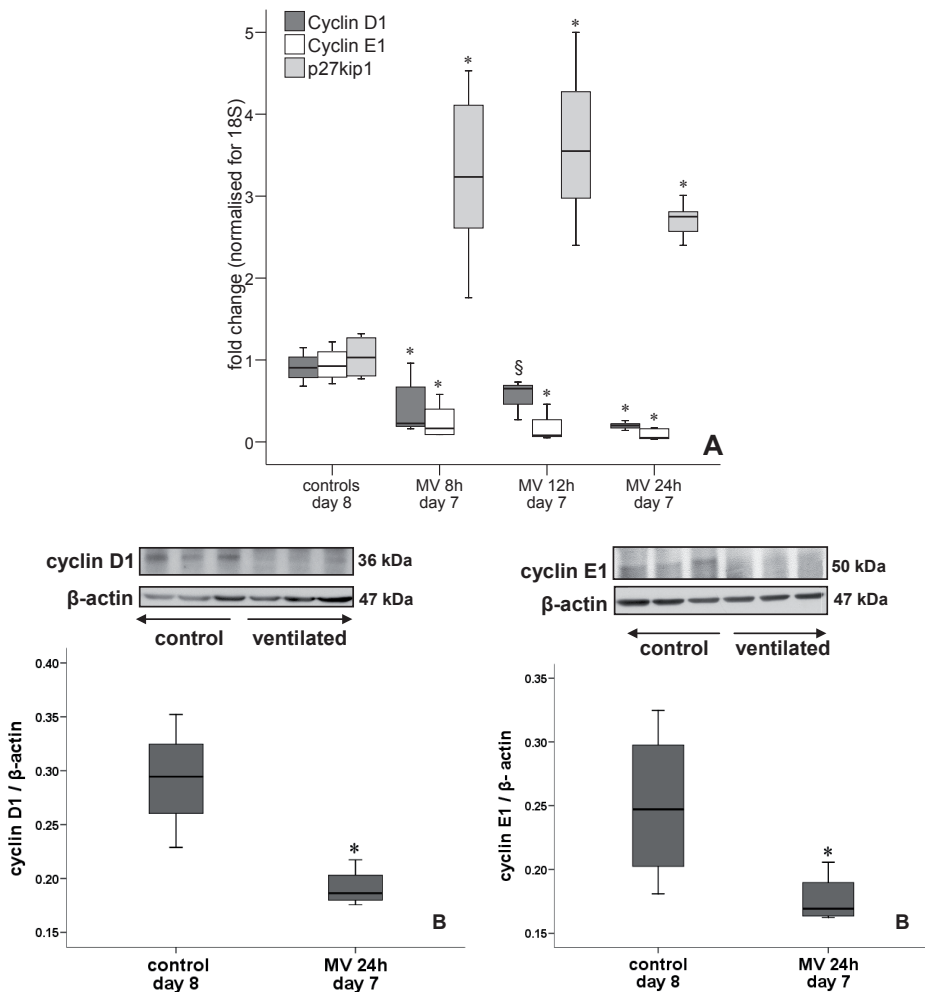


Figure 5. Impact on cyclin D, cyclin E and p27^{Kip1} expression. Mechanical ventilation for 24 h significantly decreased cyclin D1 and E1 mRNA (A) and protein (B and C) levels in lungs of 7-day old rats. In contrast, p27^{Kip1} mRNA increased (A). Inserts in (B) and (C) show cyclin D1 (B) and cyclin E1 (C) immunoblots of lung tissue of 7-day old rats ventilated for 24 h and non-ventilated 8-day old rats (controls). Blots were reprobated with β -actin for equal loading and transfer. Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles; qPCR, n=6 rat pups per time group; immunoblot, n=3 rat pups per time group. MV, mechanical ventilation. *p < 0.05 versus non-ventilated group, §p < 0.05 versus 24h ventilation.

ing stability and nuclear localization (Fig. 7B-D). Similar -but more rapid- changes in cell cycle regulators were noted when 7-day newborn rats were ventilated with high V_T . Although β -actin can be responsive to stretch, no significant differences in β -actin expression was noted between ventilated animals and controls (not shown). High V_T reduced the amount of D1 and D2 cyclins within 1 hour, while that of Cdk inhibitors

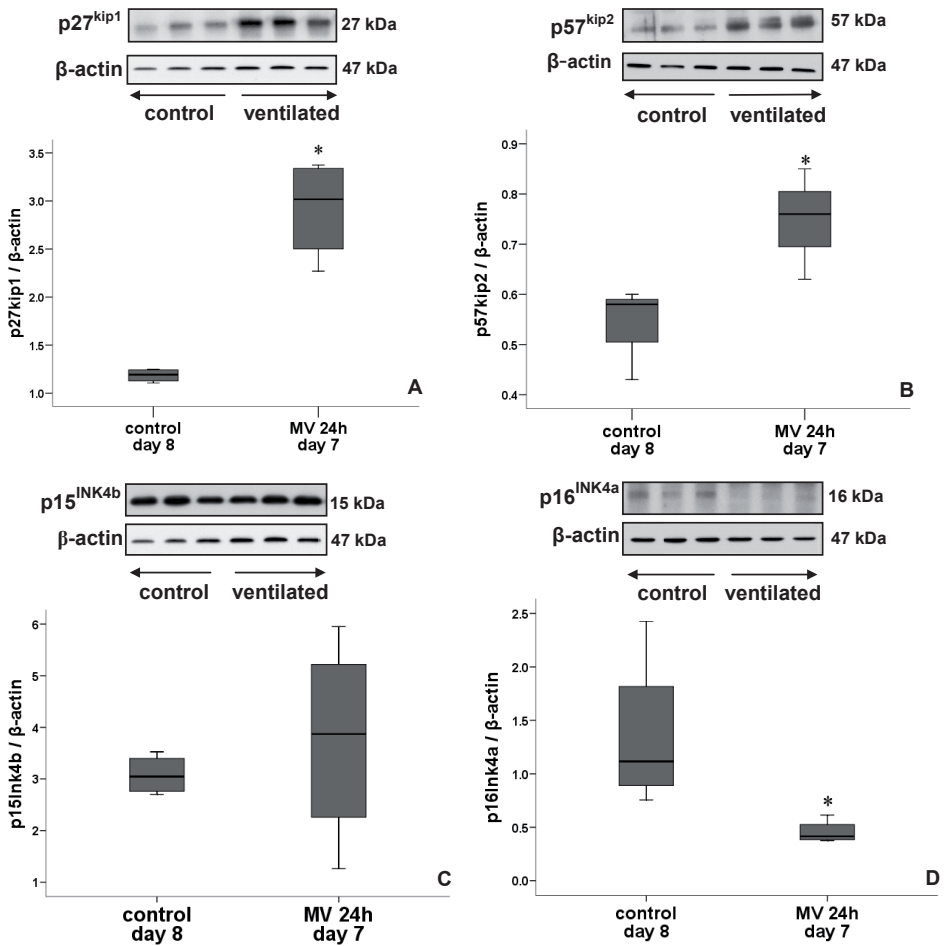


Figure 6. Different effects on Kip and INK proteins. Mechanical ventilation for 24 h significantly increased p27^{Kip1} (A) and p57^{Kip2} (B) protein levels. In contrast, p16^{INK4a} protein (D) was decreased by ventilation while p15^{INK4b} (C) was unchanged. Inserts show immunoblots of lung tissue of 7-day old rats ventilated for 24 h and non-ventilated 8-day old rats (controls). Blots were reprobed with β -actin for equal loading and transfer. Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles; $n=3$ rat pups per time group. * $p < 0.05$ versus non-ventilated group.

p27^{Kip1} and p57^{Kip2} increased (Fig. 8A); in contrast, p16^{INK4a} content was decreased by high- V_T ventilation.

We do not know in which particular tissue layer (epithelium, mesenchyme) these changes occurred *in vivo*, but they at least occur in epithelial cells as subjecting *ex vivo* fetal lung epithelial cells to cyclic continuous 20% stretch resulted in similar patterns of alteration in cell cycle regulators (Fig. 8B).

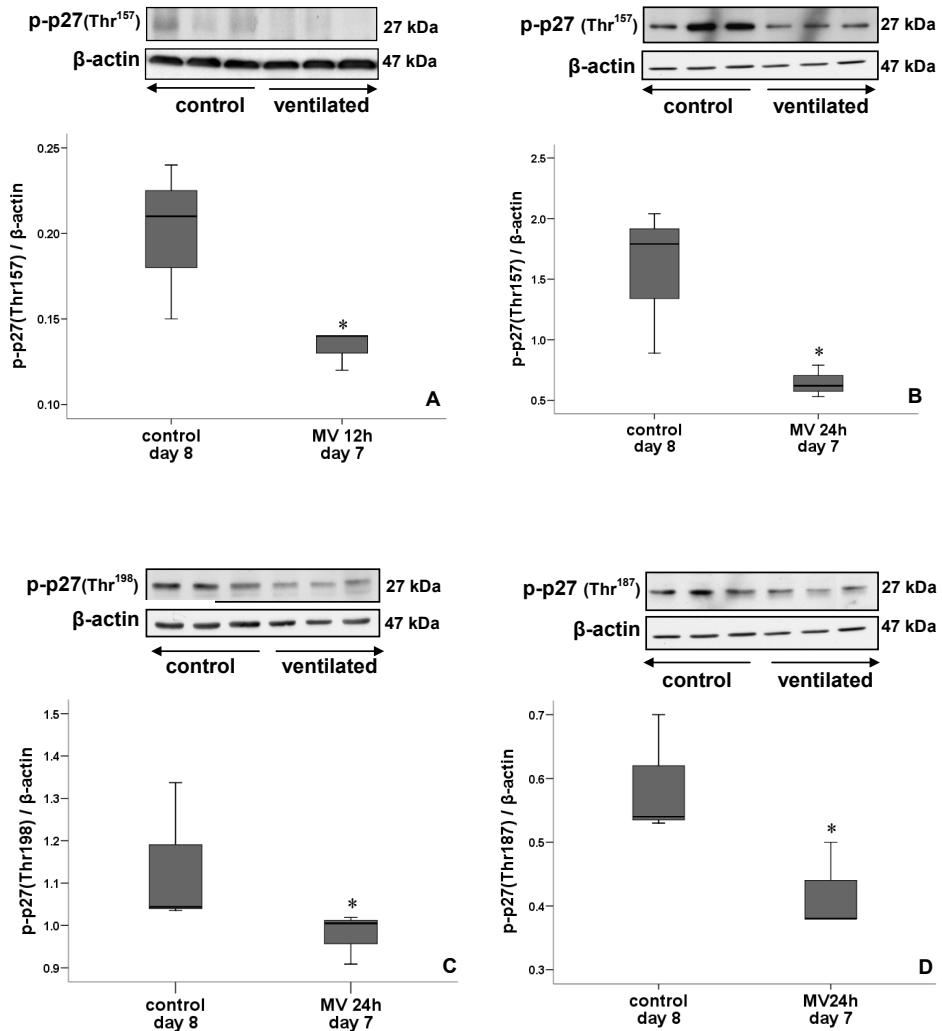


Figure 7. Effect on p27^{kip1} phosphorylation. A 24h-ventilation significantly decreased Thr¹⁵⁷-phosphorylated p27^{kip1} (A), Thr¹⁸⁷-phosphorylated p27^{kip1} (B) and Thr¹⁹⁸-phosphorylated p27^{kip1} (C). Phosphorylation of threonine 157 was already reduced for 12 h of ventilation (A). Inserts show immunoblots of lung tissue of 7-day old rats ventilated for 24 h and non-ventilated 8-day old rats (controls). Blots were reprobbed with β-actin for equal loading and transfer. Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles; n=3 rat pups per time group. *p < 0.05 versus non-ventilated group.

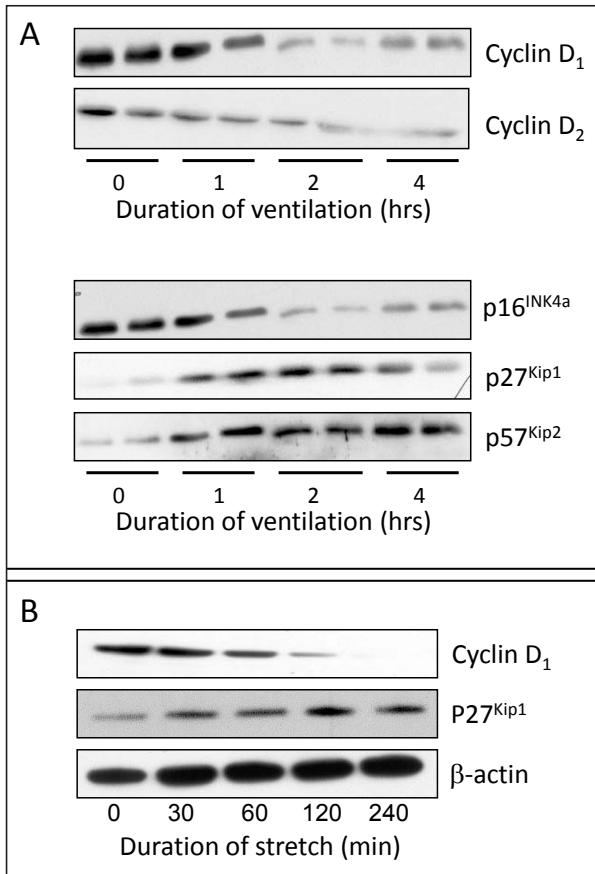


Figure 8. Effect of high tidal volume ventilation and in vitro stretch. High V_T ventilation of 7-day old rat lungs (A) and a continuous cyclic 20% stretch of fetal lung epithelial cells (B) rapidly decreased type-D cyclins and p^{16INK4a} while increasing Kip proteins, in particular p27^{Kip1}. Blots were reprobbed with β -actin for equal loading and transfer. Representative blots of 2 experiments carried out in duplicate (A) or 3 experiments (B).

DISCUSSION

The hallmark of ‘new’ BPD is arrested alveolarization (1), but the molecular and cellular basis of the alveolar arrest remains mostly unknown. Alveolarization occurs as the immature saccules, which form the lung parenchyma at birth, are subdivided into smaller units by the formation and extension of secondary septa; new tissue ridges are lifted off the existing primary septa and grow in a centripetal direction into the airspaces. This process, called septation, is mainly postnatal (human: 36 weeks-infancy; rat: Pnd5–Pnd21) (7, 27). Before septation of the air spaces starts, the lung expands for a short period of time, and the cells of the inter-airway walls actively proliferate, peaking at day

5 in rats and steadily declining thereafter (28). This active cell proliferation takes place just at the beginning of the septation of the distal airways. With the use of a newborn rat model (18) we demonstrate here that mechanical ventilation for 24 h with room air and moderate V_T results in cell cycle arrest, and reduced alveolar septation. This ventilation strategy (room air and moderate VT) was chosen to avoid/minimize lung injury.

In rats, lungs at birth have a saccular appearance and alveolarization is an exclusively postnatal (between P4 and P21) event, which makes this model relevant to the infant population developing BPD. However, major differences exist between mechanically ventilated newborn rats and premature born infants. Newborn rats have an immature lung architecture at birth, but they do not need mechanical ventilation to survive (likely due to differences in airway structure, with large airways extending almost to the lung periphery and quickly reducing in diameter to the alveoli) and they do not lack surfactant. Infants with BPD demonstrate interstitial thickening that may partly be due to fibroproliferation while in rat pups mechanical ventilation of 24 h caused cell arrest in both mesenchymal and epithelial cell layers. Despite these differences, our results suggest that the observed cell cycle arrest is due to increased expression of two CKIs (i.e. p27^{Kip1} and p57^{Kip2}) that are members of the Cip/Kip family; the other member, p21^{waf1/Cip1}, was not affected by 24 h of mechanical ventilation.

Knock-in mouse models have shown that p27^{Kip1} and p57^{Kip2} are interchangeable *in vivo* (29), suggesting similar mechanisms of regulation. Mechanical strain has been recognized as playing an important role in the regulation of fetal lung cell proliferation. Indeed the stimulatory effect of mechanical stretch (i.e. increased intratracheal pressure) on fetal lung growth has been extensively studied in tracheal occlusion (TO) models (30-31), where the number of proliferating alveolar type II cells significantly increased. Fetal sheep, exposed to TO during the alveolar stage of lung development, showed an increase in alveolar type II cells between days 2-4 after TO (31). This proliferative response of fetal lung cells to strain has also been demonstrated *in vitro*. Intermittent cyclic 5% stretching (simulating normal fetal breathing movements) of distal fetal rat lung cells (epithelial cells and fibroblasts) increased cell proliferation (32). However, a continuous cyclic 20% stretch (simulating mechanical ventilation (33)) for 24 h inhibited fetal lung cell proliferation (unpublished results), in agreement with our *in vivo* findings of a proliferative arrest after 24 h of mechanical ventilation. In the present study, continuous cyclic 20% stretch of fetal lung epithelial cells caused similar alterations in cell cycle regulators as observed in mechanically ventilated newborn lungs *in vivo*, namely increased levels of p27^{Kip1} and a decrease in the amount of cyclin D1. CKI members of the Cip/Kip family (p21^{waf1/Cip1}, p27^{Kip1} and p57^{Kip2}) preferentially inhibit cyclin-Cdk2 complexes (16). How mechanical stretch influences CKIs is unknown. In many cancers, the ras/raf/mitogen activated protein kinase (MAPK) pathway increases p27^{Kip1} proteolysis while downstream effectors of the PI-3K pathway such as protein

kinase B (also known as Akt) predominantly regulate p27^{Kip1} subcellular localization (26). Although the MAPK pathway is activated by ventilation/stretch (34-35) we found nuclear p27^{Kip1} accumulation instead of degradation. Thus, MAPK may regulate p27^{Kip1} differently in normal compared to cancer cells. The PI3K-Akt pathway during ventilation/stretch remains to be investigated. Mechanical ventilation of newborn rats triggers an inflammatory response(17-18) and various inflammatory mediators including tumor necrosis factor- α (TNF α), interleukin-6 and transforming growth factor- β (TGF- β) have been shown to induce p21^{WAF1/Cip1} expression (22, 36-37). Also p15^{Ink4b} is induced by TGF- β (38). In the current study, TGF β_1 mRNA expression was not changed after 24 h of ventilation (data not shown) and, indeed, neither p21^{WAF1/Cip1} nor p15^{Ink4b} expression was affected by mechanical ventilation.

The amount of p27^{Kip1} is regulated at the level of its synthesis (transcription, translation), degradation and localization (39). During the G₀ phase, it accumulates in the nucleus and inhibits cyclin-Cdk complexes. In response to growth stimuli, p27^{Kip1} translocates from the nucleus to the cytoplasm during G₁ phase and is degraded by the proteasome after ubiquitination (39), permitting the cell cycle to progress to S phase. Several signaling pathways that alter p27^{Kip1} phosphorylation influence its subcellular localization and function. For example, phosphorylation of the following essential sites regulate important functions: Thr¹⁵⁷ prevents nuclear import; Ser¹⁰ mediates nuclear export; Thr¹⁹⁸ promotes cytoplasmic translocation and increases p27-dependent motility, which may be important to prepare cells for shape changes in later phases of the cell cycle; and, Thr¹⁸⁷ results in proteolysis (26, 39). In the present study, mechanical ventilation of 24 h increased the transcription of p27^{Kip1} and altered its phosphorylation: less phosphorylation of Thr¹⁵⁷ (increasing nuclear import), Thr¹⁹⁸ (decreasing nuclear export) and Thr¹⁸⁷ (reduced proteolysis). No significant changes in Ser¹⁰ phosphorylation were noted (not shown). Together, these alterations in p27^{Kip1} phosphorylation favour its nuclear localization and stability. In addition, the reduced phosphorylation of p27^{Kip1} at Thr¹⁵⁷ and Thr¹⁹⁸ negatively affects the assembly function of p27^{Kip1} for cyclinD1-Cdk4, thereby negatively affecting cell cycle progression (26).

The second family of CKIs consists of INK4 proteins (p16^{INK4a}, p15^{INK4b}, p18^{INK4c} and p19^{INK4d}); they inhibit cyclin D-dependent kinases Cdk-4 and -6 (14-15) and, are thus specific for early G₁ phase. In the present study, we found a significant reduction in p16^{INK4a} protein after ventilation with low, moderate or high V_T. In addition to Cdk inhibition and G₁ growth arrest, p16^{INK4a} plays a role in regulating apoptosis. It has been shown that p16^{INK4a}-deficiency increases apoptosis in osteosarcoma U2OS and mouse embryonic fibroblast (MEF) cells exposed to ultraviolet (UV) light (40), because of down-regulation of the anti-apoptotic protein Bcl-2. In contrast, the pro-apoptotic protein Bax was down-regulated in p16^{INK4a} expressing cells (40). Thus, p16^{INK4a} appears to control apoptosis through the intrinsic mitochondrial death pathway. Prolonged

mechanical ventilation has been shown to significantly increase lung cell apoptosis in newborn mice lungs (8). Although p16^{INK4a} levels were decreased in the present study, it remains yet to be determined whether it plays a role in ventilator-induced apoptosis.

Another risk factor for BPD is oxygen (1). Hyperoxia has been shown to interfere with cell-cycle progression *in vitro* (36, 41-42) and hyperoxia-induced G₁ arrest appears to be mediated by p21^{Waf1/Cip1} (43-44). The hyperoxic induction of p21^{Waf1/Cip1} is p53-dependent (44) and its increase promotes survival of cells exposed to continuous oxidative stress by maintaining anti-apoptotic Bcl-2X(L) expression (45). Hyperoxic-ventilated premature baboons delivered at 125 and 140 days of gestation have increased p53 and p21^{Waf1/Cip1} expression (46-47). In the present study, we did not assess p53 but the absence of p21^{Waf1/Cip1} induction by 24 h of mechanical ventilation with room air suggests that p53 is likely not involved in ventilation-induced cell cycle arrest in these studies.

The increase of p27^{Kip1} and p57^{Kip2} by mechanical stretch *in vitro* and *in vivo* coincided with a reduced expression of cyclins D1 and E1, both of which are essential for cell cycle progression through G₁ and entry in S phase. D-type cyclins are induced by mitogenic stimuli in quiescent cells. After association with Cdk4/6 and activation by Cdk activating kinase, they promote entry into the G₁ phase, thereby triggering cyclin E expression. Cyclin E binds to Cdk2 and facilitates transition from G₁ to S phase (22). Both p27^{Kip1} and p57^{Kip2} are potent inhibitors of cyclin E-dependent kinase Cdk2, but at high concentrations they also block Cdk4/6. In addition, it is plausible that cell cycle progression is inhibited due to the reduced phosphorylation of p27^{Kip1} at critical threonines (Thr157, Thr198) which negatively affects the assembly function of p27^{Kip1} for cyclin D1-Cdk4 complexes (48). The down-regulation of cyclin D1 and E1 expression suggests a G₁ cell cycle arrest, a conclusion that is supported by the absence of BrdU incorporation (S-phase event) and positive PH3 staining (M-phase marker). In the 125-day premature born baboon model of BPD, the animals received ventilator support and oxygen as needed to achieve normal blood-gas values (49), and such treatment increased pulmonary expression of cyclin D1 and E at day 6 while prolonged ventilation and oxygen exposure led to a decrease in cyclin E (46). It is possible that lung cells were initially undergoing repair by increasing proliferation, but that prolonged exposure impairs the expression of cyclins, resulting in failure of repair and inhibition of further development. Furthermore, increased levels of the Cdk inhibitor p21^{Waf1/Cip1} in the baboon BPD model (46) suggests that G₁ growth arrest may occur in infants with BPD. Unfortunately, the expression of p27^{Kip1} or p57^{Kip2} has not been investigated in the baboon BPD model.

In summary, we conclude that mechanical ventilation for 24 h using moderate V_T without supplemental O₂ causes G₁ cell cycle arrest of lung cells in newborn rats due to increased transcription and altered phosphorylation (in favour of nuclear localization)

of Kip CKIs, and down-regulation of cyclins D and E. This proliferative arrest may cause a reduction in alveolarization, resulting in alveolar simplification. Such identification of ventilation-induced CKIs may have therapeutic potential for the prevention -or treatment- of arrested alveolarization in ventilated premature infants

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Chapter 7

Alterations in Expression of Elastogenic and Angiogenic Genes by Mechanical Ventilation in Newborn Rat Lung

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Submitted



ABSTRACT

Mechanical ventilation, inflammation and hyperoxia are important risk factors for the development of bronchopulmonary dysplasia. Here we investigated the effects of different tidal volumes (V_T), inflammation, hyperoxia and duration of ventilation on the expression of genes involved in alveolarization (tropoelastin (TE), lysyl oxidase (LOX), fibulin5 (Fib5) and tenascin-C (TN-C) and angiogenesis (platelet derived growth factors (PDGF) and vascular endothelial growth factors (VEGF)) in newborn rats. First, eight-day old rats were ventilated for 8 hours (h) with low (LV_T : 3.5 ml.kg^{-1}), moderate (MV_T : 8.5 ml.kg^{-1}) or high (HV_T : 25 ml.kg^{-1}) tidal volumes. LV_T and MV_T ventilation for 8 h hardly influenced the expression of TE, LOX and Fib5, while TN-C expression was significantly decreased. HV_T ventilation increased gene expression of all three elastogenic genes and TN-C. PDGF- α receptor (PDGF-R α) mRNA, but not protein, was significantly increased in all ventilation groups. PDGF-A gene expression was not affected by ventilation, but PDGF-B mRNA levels were decreased after MV_T and HV_T ventilation. VEGF expression was only up-regulated after HV_T ventilation, while, independent of V_T ventilation up-regulated VEGF receptor 1 (VEGF-R1) expression. In contrast, VEGF-R2 expression was down-regulated by MV_T and HV_T ventilation. Next we studied the effect of pre-exposure to LPS together with LV_T ventilation with room air and 50% oxygen on expression on these genes. Pre-exposure to LPS decreased the expression of Fib5, while oxygen increased Fib5 mRNA expression, which was abolished by pre-exposure to LPS. Pre-exposure to LPS and LV_T -ventilation with oxygen did not influence the expression of the angiogenic genes. Finally, we evaluated the duration (0-24 hours) of MV_T ventilation. Although expression of all three elastogenic genes peaked at 12 hours of ventilation, only that of Fib5 was negatively affected at 24 hours. In contrast, TN-C expression decreased with duration of ventilation. Disturbed elastin fiber deposition was only noticeable after 24 hours of ventilation. Ventilation-induced changes in expression of PDGF and VEGF receptors were maximal at 8 hours of ventilation, but a morphometric decrease in small vessel density was only observed after 24 hours. Thus, an imbalance between Fib5 and TE expression may trigger dys-regulated elastin fiber deposition during the first 24 h of mechanical ventilation. This imbalance in expression is accelerated by a systemic inflammation. Furthermore, ventilation-induced alterations in PDGF and VEGF receptors expression are tidal volume dependent and affect pulmonary vessel formation. Taken together the data are compatible with mechanical ventilation disturbing normal elastogenesis and angiogenesis in newborn rat lungs.

INTRODUCTION

Bronchopulmonary dysplasia (BPD), a chronic lung disease of premature born infants, is a major cause of morbidity and mortality. In severely affected infants symptoms last into early adulthood (1, 2). BPD is a consequence of an inflammatory lung injury that leads to remodeling of the lung. Inflammation results from a variety of stimuli, of which mechanical ventilation, hyperoxia and infection are most prominent. The incidence of BPD increases with decreasing gestational age. Due to the improved survival of extreme premature infants, the pathological features of BPD have evolved since its first description in 1967 by Northway *et al.* (3). Nowadays the pathology of BPD is characterized by alveolar simplification and enlargement, and dysmorphic vasculature. It is not surprising that these findings are believed to be the result of an arrest in alveolar development. After all, lungs of infants born at 24-32 weeks are in the saccular stage of development and structurally and biochemically immature. During this period of development extensive vasculogenesis takes place within the developing terminal sacculi, followed by secondary crests formation with interstitial extracellular matrix loss and remodelling with epithelial morphogenesis and capillary growth (4). Among the diverse factors that contribute to normal lung development, elastin, platelet-derived growth factors (PDGF) and vascular endothelial growth factor (VEGF) play a prominent role (5). However, the effect of each BPD risk factor, alone or combined on these regulatory molecules of alveolar and vascular development is still not understood.

Lung pathology of children with BPD revealed irregularly distributed alveolar elastic fibers (6). Other studies have shown increases in hydroxyproline (reflecting collagen) and elastin in lung injuries after ventilation (7, 8). Chronically ventilated preterm lambs have abundant and disordered elastin fibers (9) and display increased expression of tropoelastin (TE) and lysyl oxidase-like-1 (LOXL1): molecules involved in the assembly of elastin fibers. A recent study showed that blocking lung elastase in newborn mice ventilated with 40% oxygen prevented elastin degradation and preserved lung growth (10), providing evidence that ventilatory (stretch)-induced changes in lung elastin homeostasis impairs alveolar development.

The PDGF signalling network consists of four isoforms PDGFA-D, and two receptors, PDGF-R α and PDGF-R β . PDGF-A is needed for the development of alveolar myofibroblasts, which produce elastin. PDGF-B has been linked to compensatory lung growth and pulmonary vascular remodeling (11, 12). Vascular endothelial growth factor (VEGF) is a specific endothelial cell mitogen that regulates endothelial cell differentiation, angiogenesis, and maintenance of existing vessels (13). Mechanical ventilation has been shown to decrease pulmonary expression for VEGF, PDGF-A, and their receptors (VEGF-R2, PDGF-R α) in preterm and term lambs with BPD (9), thereby likely impacting pulmonary vascular development.

The main objective of this study was to investigate the effects of mechanical ventilation on pulmonary expression of genes that regulate formation of alveoli and blood vessels in lungs of newborn rats, in which alveolarization and angiogenesis occur mainly after birth (14). We hypothesized that the expression of these genes would be stretch-amplitude and time dependent. Furthermore, oxygen and a systemic inflammation would further alter the expression of these genes. We observed that fibulin5 and PDGF-B are the first genes to be down-regulated by moderate tidal volume ventilation. Inflammation accelerates this negative effect of mechanical ventilation on fibulin5 expression, while low tidal volume ventilation delays this downregulatory effect of moderate mechanical ventilation on the expression of PDGF-B.

METHODS

Ethics statement: The study was conducted according to the guidelines of the Canadian Council for Animal Care and with approval of the Animal Care Review Committee of the Hospital for Sick Children (protocol #7217).

Animal preparation: Timed pregnant Wistar rats (Charles River, Oakville, Quebec, Canada) were allowed to deliver and immediately afterwards litters were reduced to 10 pups. In three series of experiments newborn rats were ventilated to assess the effect of different tidal volumes, inflammation, oxygen and duration of ventilation on alveolar development. They were anesthetized by *i.p.* injection of 30 mg kg⁻¹ pentobarbital and a tracheotomy was performed. The trachea was cannulated with a 1 cm 19G cannula and connected to a rodent ventilator (FlexiVent Scireq, Montreal, PQ). Isoflurane was used as general anesthesia and 0.9% saline (100 ml.kg⁻¹/24h) was administered subcutaneously by continuous infusion with a 27G needle to prevent dehydration. Preliminary experiments were performed to determine ventilator settings (15). Animals were monitored by ECG. Rectal temperature was maintained at 37°C using a thermal blanket, lamp and plastic wrap. At the end of the ventilation period a blood sample from the carotid artery was taken for blood gas analysis prior to euthanasia. Lung tissues were processed for histology or flash frozen for molecular/protein analyses.

Mechanical ventilation:

Series I: Different Tidal Volumes. Rat pups (postnatal day 8) were randomly assigned to one of the following four groups: 1) Non-ventilated (NV) controls; 2) Low V_T (V_T 3.5 ml.kg⁻¹, freq. 600 minute⁻¹, PEEP 0 cm H₂O; increasing PEEP to 2 cm H₂O led to an increase of pCO₂ and early death); 3) Moderate V_T (8.5 ml.kg⁻¹, RR 150 min⁻¹, PEEP 2

cm H₂O); and 4) High V_T (V_T 25 ml.kg⁻¹, freq. 20 minute⁻¹, PEEP 2 cm H₂O). Pups were ventilated for 8 hrs.

Series II: Pre-exposure to LPS and Low V_T Ventilation with Oxygen. Rat pups (postnatal day 8) were randomly assigned to injection (*ip*) of either 3 mg.kg⁻¹ body weight of LPS from *E. coli* serotype 026:B6 or the same volume of 0.9% NaCl (14). Twenty four hours after treatment animals were randomly assigned to one of the following six groups: 1) Non-ventilated after NaCl injection (NV); 2) NV after LPS injection (NV+LPS); 3) Low V_T (V_T 6 ml.kg⁻¹, freq. 600 minute⁻¹, PEEP 0 cm H₂O) with room air after NaCl injection (LV_T); 4) Low V_T with room air after LPS injection (LV_T + LPS); 5) Low V_T with 50% oxygen after NaCl injection (LV_T+O₂); 6) Low V_T with 50% oxygen after LPS injection (LV_T+LPS/O₂). Pups were ventilated for 8 hrs.

Series III: Duration of Ventilation. Rat pups (postnatal days 7) were ventilated with room air and moderate-V_T (8.5 mL.kg⁻¹, RR 150 min⁻¹, PEEP 2 cm H₂O) for 12 and 24 h.

Histology: After flushing whole lungs were infused *in situ* with 4% (w/v) paraformaldehyde (PFA) in PBS with a constant pressure of 20 cm H₂O over 5 minutes to have equalized filling pressure over the entire lung. Under these constant pressure conditions the cannula was removed and the trachea immediately ligated. The lungs were excised and immersed in 4% PFA in PBS overnight, dehydrated and embedded in paraffin. Sections of 5 μm were stained for elastin using accustain artrazine solution (Sigma, St. Louis MO, USA).

Immunohistochemistry: Following sectioning and antigen retrieval by heating in 10 mM sodium citrate pH 6.0, sections were washed in PBS and endogenous peroxidase was blocked in 3% (v/v) H₂O₂ in methanol. Blocking was done with 5% (w/v) normal goat serum (NGS) and 1% (w/v) bovine serum albumin (BSA) in PBS. Sections were then incubated overnight at 4°C with 1:500 diluted rabbit anti-Tenascin (Chemicon Inc, Huissen, Netherlands). Biotinylated goat anti-rabbit IgG (1:2000) was used as secondary antibody. Color detection was performed according to instruction in the Vectastain ABC and DAB kit (Vector Laboratories, Burlingame, CA, USA). All sections were counterstained with hematoxylin.

Quantitative RT-PCR: Lung tissues were homogenized in Trizol[®] (Invitrogen Canada Inc, Burlington, ON, Canada) and total RNA was extracted according to the manufacturer's protocol. Total RNA was treated with DNase I[®] (Invitrogen Canada Inc, Burlington, ON, Canada) at 37°C to remove residual genomic DNA. Total RNA was reverse transcribed using Superscript II reverse transcriptase[®] (Invitrogen Canada Inc,

Table 1. Primer sequences for SYBR Green real-time PCR

Gene	Primer	Sequence
VEGFR1	Forward	GAGTGTGAACGGCTGCCTATGATG
	Reverse	GATGGCCGATGTGGGTCAAG
PDGFA	Forward	CATTGGCTGGGAAGACGGA
	Reverse	CCCACAGCCAGCTTACTTATT
PDGFB	Forward	TCTCTGCTGCTACCTGCGTCTG
	Reverse	GAGCTTCCGACTCGACTCCA
PDGF-R α	Forward	GGTGACCTGCGCCGTCTTTA
	Reverse	GGCGGGCAGCACATTCATAATC
PDGF-R β	Forward	TGGCCTTGGTGGTCCTTACTGT
	Reverse	CTCCACCACCTGTCCGAAAGC

VEGFR: vascular endothelial growth factor receptor, PDGF: platelet-derived growth factor, PDGR-R: platelet-derived growth factor receptor.

Burlington, ON, Canada) and random hexamers (Applied Biosystems, Foster City, CA, USA). Complementary DNA was quantified in a 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA). Amplification was performed with AmpliTaq Gold polymerase (Applied Biosystems, Foster City, ON, Canada) using TaqMan primers and probes (Applied Biosystems, Foster City, ON, Canada) for the target genes T1- α , tropoelastin (TE) and TN-C (17). VEGF, VEGFR2, PECAM, lysyl oxidase (LOX), fibulin 5 and 18S were Assays on Demand® from Applied Biosystems (Foster City, ON, Canada). VEGFR1, PDGF-A, PDGF-B, PDGF-R α and PDGF-R β cDNA (see Table 1) were quantified using SYBR Green. For relative quantification, polymerase chain reaction signals were compared between groups after normalization using 18S as an internal reference. Fold change was calculated according to Livak and Schmittgen (16).

Vessel numbers: The number of peripheral vessels of <20 μ m and 20–65 μ m in diameter were counted in 30 random, non-overlapping fields per lung with all fields being within 435 μ m of the edge of the lung on tissue cross-section as previously described (17).

Statistical analysis: Stated otherwise all data are presented as mean \pm SD. Data was analyzed using SPSS software version 15 (SPSS Inc, Chicago, IL). Statistical significance ($p < 0.05$) was determined by using one-way ANOVA or Kruskal-Wallis test. *Post hoc* analysis was performed using Duncan's multiple-range test (data presented as mean \pm SD) or Mann-Whitney test (data presented as median and interquartile range).

RESULTS

Physiologic data: *Series 1:* Blood gases were in the normal range after eight hours of ventilation with different tidal volumes (Table 2A) (15). *Series 2:* Ventilation for 8 hours with low V_T with room air after exposure to LPS (LV_T +LPS), ventilation with 50% oxygen (LV_T +O₂), and ventilation with oxygen after exposure to LPS (LV_T +LPS/O₂) resulted also in normal pH and PaCO₂ (Table 2B). Low V_T ventilation with room air after exposure to

Table 2A. Airway pressures and blood gas analysis after 8 hours of mechanical ventilation with low, moderate and high V_T (Series 1).

	Groups		
	LV _T	MV _T	HV _T
pH	7.39 ± 0.07	7.39 ± 0.03	7.39 ± 0.04
pCO ₂ (mmHg)	45 ± 4.4	44 ± 6.4	42 ± 2.2
pO ₂ (mmHg)	72 ± 14.0	83 ± 8.4	91 ± 9.8
Ppeak (cm H ₂ O)	6.0 ± 0.5	10.1 ± 1.5*	19.9 ± 0.4**
Pmean (cm H ₂ O)	3.6 ± 0.26	6.4 ± 1.0*	10.8 ± 0.6**
PEEP (cm H ₂ O)	0	2	2
Delivered V _T (ml/kg)	~3.5	~8.5	~25
Frequency (breaths min ⁻¹)	600	160	20

Statistical analysis (ANOVA) was done for measured values only. Values represent means ± SD, n = 4 animals per group. *p < 0.05 vs. animals ventilated with LV_T and HV_T; **p < 0.05 vs. animals ventilated with LV_T and MV_T. LV_T, low tidal volume; MV_T, moderate tidal volume; HV_T, high tidal volume; Pmean, mean pressure; Ppeak, peak pressure; PEEP, positive-end expiratory pressure.

Table 2B. Airway pressures and blood gas analysis after 8 hours of low V_T mechanical ventilation with room air or 50% oxygen and after exposure to LPS (Series 2)

	Groups			
	LV _T	LV _T +LPS	LV _T +O ₂	LV _T +LPS/O ₂
pH	7.36 ± 0.16	7.40 ± 0.04	7.32 ± 0.2	7.30 ± 0.08
pCO ₂ (mmHg)	40.7 ± 6.6	36.0 ± 3.9	58.3 ± 28.8	54.3 ± 16.6
pO ₂ (mmHg)	67.2 ± 10.4	87.7 ± 6.7*	131 ± 42.5*	167 ± 40.4**
Pmean (cm H ₂ O)	4.2 ± 0.6	4.2 ± 0.4	4.6 ± 0.6	4.3 ± 0.8
Pmax (cm H ₂ O)	6.6 ± 0.6	6.7 ± 0.4	7.4 ± 0.6	6.8 ± 1.2
PEEP (cm H ₂ O)	0	0	0	0
Delivered V _T (ml/kg)	~3.5	~3.5	~3.5	~3.5
Frequency (breaths min ⁻¹)	600	600	600	600

Statistical analysis (ANOVA) was done for measured values only. Group LV_T+LPS and group LV_T+O₂ were not compared. Values represent mean ± SD, n = 4 animals per group. LV_T, ventilated with low tidal volume; LV_T+LPS, ventilated with LV_T after exposure to LPS; LV_T+O₂, ventilated with low LV_T and 50% oxygen; LV_T+LPS/O₂, ventilated with LV_T and 50% oxygen after exposure to LPS. LV_T+O₂ group was not compared with LV_T+LPS. *p < 0.05 vs. animals ventilated with LV_T; **p < 0.05 vs. animals ventilated with LV_T and LV_T+LPS.

Table 2C. Blood gas analysis and airway pressures after 8, 12 and 24 hours of mechanical ventilation with room air (Series 3).

	Groups		
	8 hours	12 hours	24 hours
pH	7.39 ± 0.07	7.29 ± 0.05	7.30 ± 0.05
pCO ₂ (mmHg)	44.3 ± 6.4	35.8 ± 6.2	39.4 ± 4.5
pO ₂ (mmHg)	83 ± 8.4	87.4 ± 11.2	73.5 ± 11.7
BE (mmol/L)	-4.2 ± 2.3	-3.9 ± 1.6	-5.2 ± 2.1
Ppeak (cm H ₂ O)	10.9 ± 1.1	12.7 ± 1.1*	13.1 ± 1.3*
Pmean (cm H ₂ O)	6.3 ± 0.5	7.2 ± 0.5*	7.5 ± 0.6*
PEEP (cm H ₂ O)	2	2	2
Delivered V _T (ml/kg)	8.9 ± 0.2	8.5 ± 0.4*	8.5 ± 0.2*
Frequency	150	150	150

Statistical analysis (ANOVA) was done for measured values only. Values represent mean ± SD, n = 12 animals per group. *p < 0.05 versus values at 0 hrs. Ppeak, peak pressure; Pmean, mean pressure; PEEP, positive-end expiratory pressure.

LPS (LV_T +LPS) and ventilation with 50% oxygen (LV_T+O₂) significantly increased the PaO₂ when compared to ventilation with room air (LV_T). The combination of ventilation with oxygen and exposure to LPS (LV_T+LPS/O₂) further increased PaO₂ vs. LV_T and LV_T+LPS groups (p < 0.05). Mean airway pressures and peak pressures remained stable during the ventilation period and were not different between groups in series 1 and 2 (Table 2A, 2B). Series 3: Again, blood gases were in the normal range after 8, 12 and 24 h of ventilation (Table 2C) (18). Mean airway pressures, peak pressures and delivered V_T remained constant up to 8 h of ventilation, but altered slightly after 12 h of ventilation compared to baseline (Table 2C).

Expression of matrix molecules implicated in alveolarization: Series 1: Eight hours of ventilation with ~3.5 ml. kg⁻¹ and ~8.5 ml.kg⁻¹ decreased the expression of tenascin-C (TN-C) versus non-ventilated control animals (Fig. 1A). In contrast, injurious ventilation with ~25 ml.kg⁻¹ increased the expression of TN-C. The expression of TN-C between LV_T and MV_T group was not significantly different. Immunohistochemical analysis showed that the TN-C distribution pattern was similar in non-ventilated and ventilated lungs with different tidal volumes (Fig. 2. Lower panels, HV_T vs. non-ventilated group). TN-C staining was mesenchymal and no immunoreactivity was found in the larger airways and blood vessels. Western blotting did not reveal any differences in TN-C protein expression (data not shown). Gene expression of lysyl oxidase (LOX) and tropoelastin (TE) was increased versus non-ventilated group after 8 h of ventilation with ~3.5 ml. kg⁻¹. Fibulin 5 (Fib5) was not affected by low and MV_T ventilation, while it was increased by injurious ventilation with ~25 ml.kg⁻¹ (Fig. 1A). TE, LOX and Fib5 regulate elastin synthesis and assembly, a process crucial for alveologenesi. Although gene expression

of these elastogenic genes was increased after injurious HV_T ventilation, a similar pattern of elastin deposition and arrangement was observed in lungs of all groups (Fig 2. Upper levels, HV_T vs. non-ventilated group). Expression of type I cell marker (T1- α) was only increased by injurious ventilation with $\sim 25\text{ml}\cdot\text{kg}^{-1}$ (Fig. 1A). *Series 2:* Exposure to LPS decreased the expression of TN-C *versus* non-ventilated control animals (Fig. 1B).

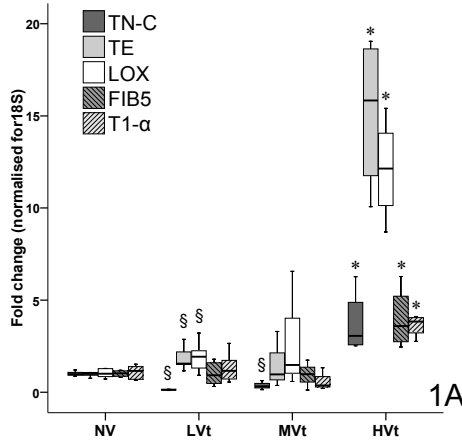


Figure 1A. Effect of low (LV_T), moderate (MV_T), and high (HV_T) ventilation for 8 hours with room air on the expression of matrix genes implicated in alveolarization in lungs of 7-day old rats. Median with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles. *p < 0.05 vs. other groups; §p < 0.05 vs. NV.

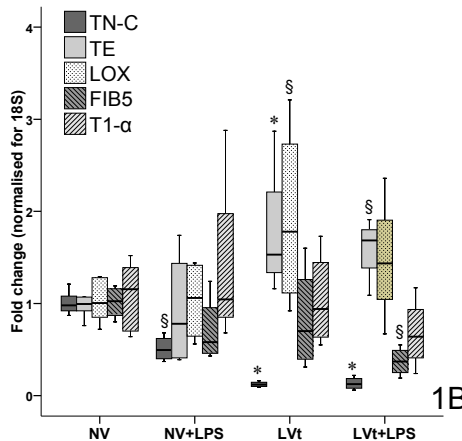


Figure 1B. Effect of exposure to LPS, low V_T ventilation (8 hours) with room air and low V_T ventilation (8 hours) after exposure to LPS on the expression of matrix genes implicated in alveolarization in lungs of 7-day old rats. Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles. NV: non ventilated (n = 8); NV+LPS: exposure to LPS, non ventilated (n = 5); LV_T: ventilated with low tidal volume (n = 6); LV_T+LPS: ventilated with LV_T after exposure to LPS (n = 5). §p < 0.05 vs. NV; *p < 0.05 vs. NV, NV+LPS.

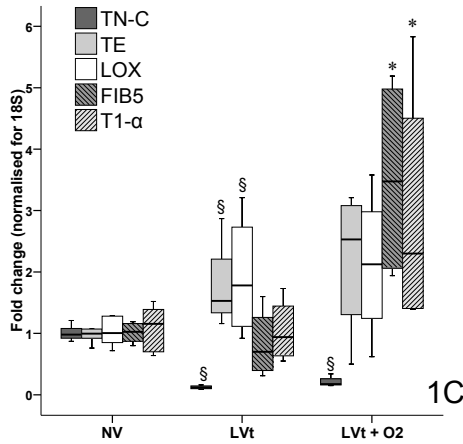


Figure 1C. Effect of low V_T ventilation (8 hours) with room air and low V_T ventilation (8 hours) with 50% oxygen on the expression of matrix genes implicated in alveolarization in lungs of 7-day old rats. Median with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles. NV: non ventilated (n=8); LV_T: ventilated with low tidal volume (n=6); LV_T+ O₂: ventilated with LV_T with 50% oxygen (n=5). §p < 0.05 vs. NV; *p < 0.05 vs. other groups.

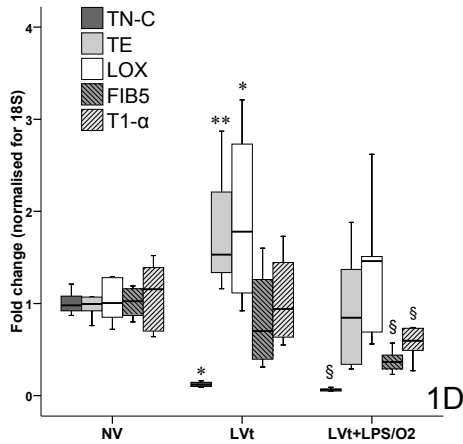


Figure 1D. Effect of exposure to low V_T ventilation (8 hours) with room air and low V_T ventilation (8 hours) with 50% oxygen after exposure to LPS on the expression of matrix genes implicated in alveolarization in lungs of 7-day old rats. Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles. NV: non ventilated (n = 8); LV_T: ventilated with low tidal volume (n=6); LV_T+LPS/O₂: ventilated with LV_T with 50% oxygen after exposure to LPS (n=6). *p < 0.05 vs. NV; **p < 0.05 vs. NV, LV_T+LPS/O₂; §p < 0.05 vs. other groups.

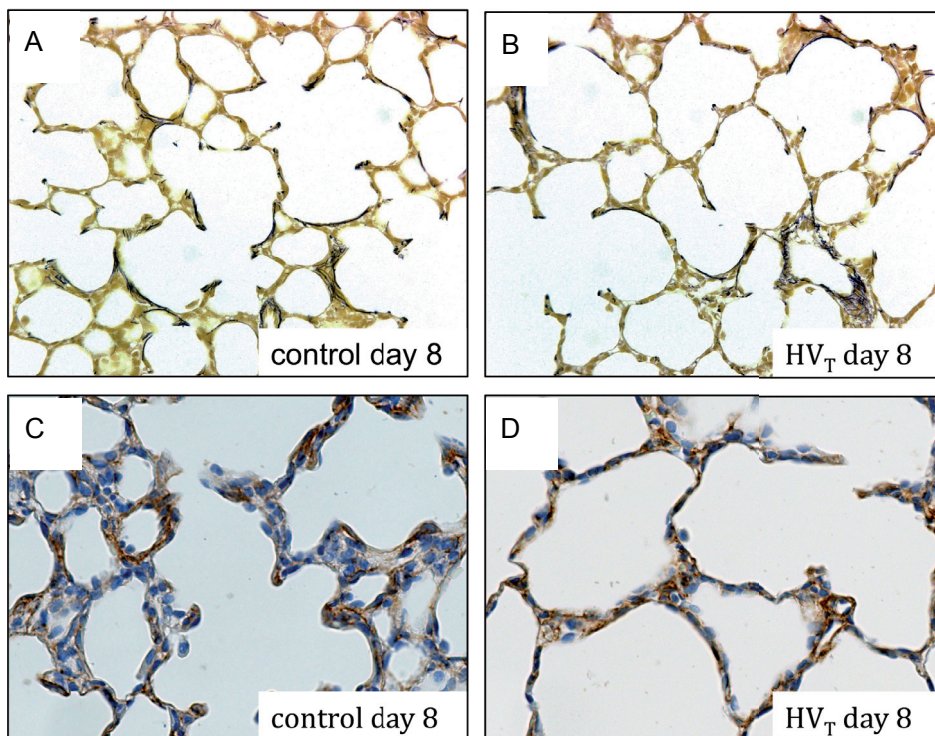


Figure 2. Immunostaining for elastin (Hart's staining) (upper panels) and immunostaining for TN-C (lower panels).

Ventilation with LV_T after exposure to LPS did further decrease the expression of TN-C *versus* non-ventilated control animals exposed to LPS but not *versus* LV_T ventilated animals. Also did LV_T ventilation after exposure to LPS decrease the gene expression of Fib5 and increase the gene expression of TE *versus* non-ventilated control animals (Fig. 1B). LV_T ventilation with 50% oxygen increased the gene expression of Fib5 and T1- α *versus* non-ventilated and LV_T ventilated animals (Fig. 1C). The combination of LV_T ventilation with 50% oxygen after exposure to LPS decreased the expression of TN-C, Fib5 and T1- α (Fig. 1D). Immunohistochemical analysis showed no differences in TN-C distribution pattern between different groups (data not shown). *Series 3:* A 24h-ventilation with moderate V_T further decreased the gene expression of TN-C *versus* 8 and 12 h of MV_T ventilation (Fig. 3A) without changing TN-C immunolocalization. Interestingly a peak of gene expression of LOX, TE and Fib5 was found after 12 h of MV_T ventilation (Figs. 3B, C, D). Although 24 h of MV_T ventilation did increase the expression of these genes *versus* non-ventilated animals, it was reduced compared to 12 h of MV_T ventilation (Figs. 3C, D). After 24 h of MV_T ventilation a different pattern of elastin deposition was observed. In non-ventilated animals and animals ventilated for 8 h, elastin was expressed mainly at the tips of the alveolar septa, whereas after 24 h of

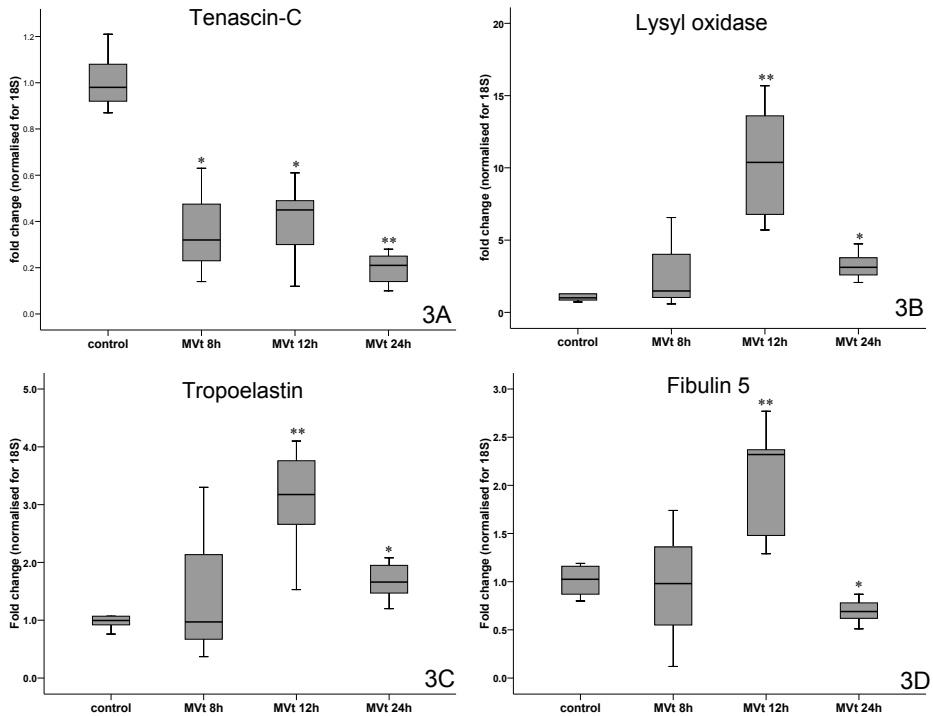


Figure 3. Effect of prolonged (8–24 hours) moderate (MV_T) ventilation with room air on the expression of matrix genes implicated in alveolarisation in lungs of 7-day old rats. Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles. Nonventilated controls (n=8), 8h MV_T (n=8), 12h MV_T (n=4) and 24 h MV_T (n=4). **A)** Tenascin C, *p < 0.05 vs. NV, **p < 0.05 vs. other groups; **B)** Lysyl oxidase, *p < 0.05 vs. NV, **p < 0.05 vs. NV, 24 hours; **C)** Tropoelastin, *p < 0.05 vs. NV, **p < 0.05 vs. NV, 24 hours; **D)** Fibulin 5, *p < 0.05 vs. NV, **p < 0.05 vs. NV, 24 hours.

MV_T ventilation elastic fibers were prominent throughout the walls of distal respiratory units in the lungs (Fig. 4). The gene expression of T1- α was not altered after 24 h of MV_T ventilation (Fig. 5A).

Increased lung MMP-9 activity is associated with the development of BPD in newborn infants (19, 20) as well as in animal models (21, 22). We observed an increase in gene expression of MMP-9 after 12 h of MV_T ventilation, but not after 24 h of MV_T ventilation (Fig. 5B).

Expression of angiogenic molecules implicated in alveolarization: Series 1: Transcript levels of PDGF-R α and PDGF-R β were significantly increased in all ventilation groups *versus* non-ventilated animals (Fig. 6). The increase in expression of PDGF-R α was much more pronounced. No changes in PDGF-R α immunoreactivity were detected (not shown). While PDGF-A expression was not altered, the number of PDGF-B transcripts was significantly decreased after MV_T and HV_T ventilation *versus* non- and LV_T

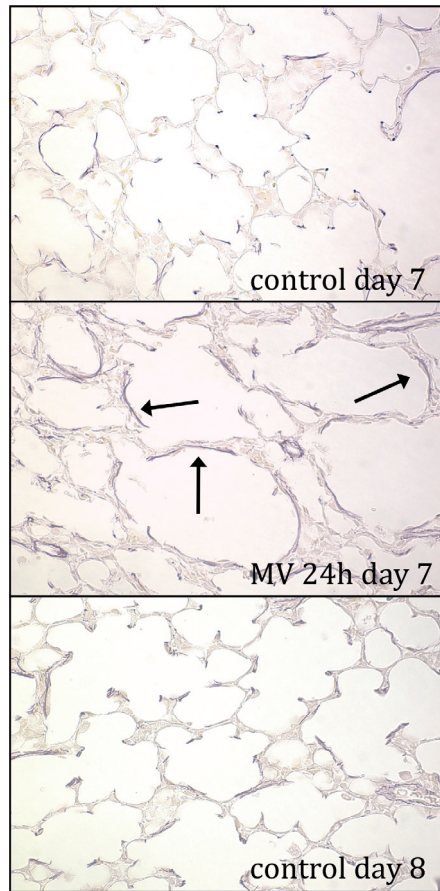


Figure 4. Immunostaining for elastin (Hart's staining): 7 day-old rat non-ventilated upper panel; 7 day-old rat ventilated for 24 h middle panel and 8 day-old rat non-ventilated (lower panel).

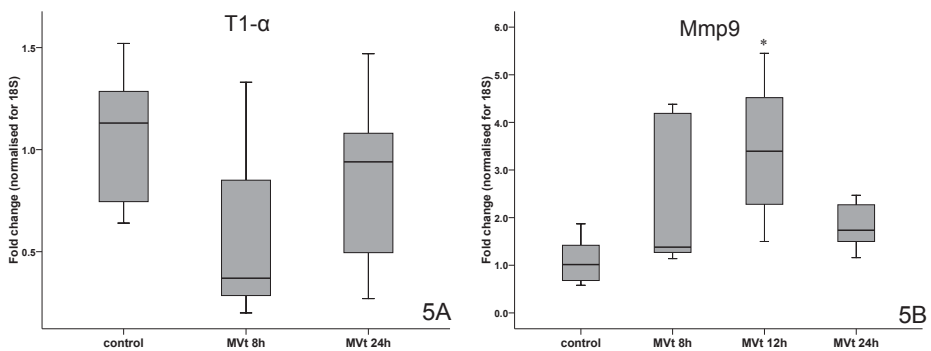


Figure 5. Effect of prolonged (8–24 hours) moderate (MV_T) ventilation with room air on the expression of T1- α (type I cell marker) and matrix metalloproteinase 9 (MMP9) in lungs of 7-day old rats. Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles. Nonventilated controls ($n=8$), 8h MV_T ($n=8$), and 24 h MV_T ($n=4$). **A)** T1- α ; **B)** MMP9, * $p < 0.05$ vs. NV, 24 hours.

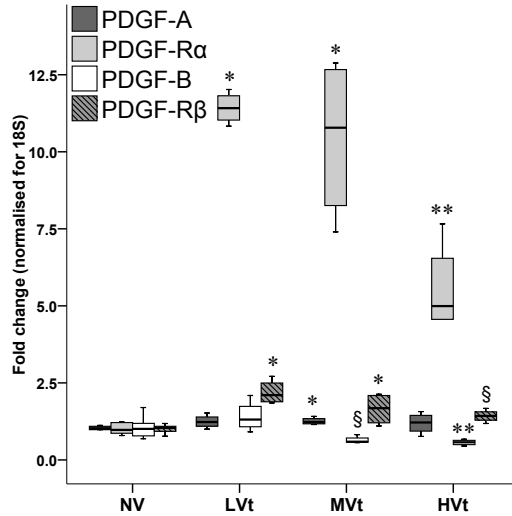


Figure 6. Effect of low (LV_T), moderate (MV_T) and high (HV_T) ventilation for 8 hours with room air on the expression of PDGF genes in lungs of 7-day old rats. Median with 25th and 75th quartiles are shown, bars are the 5th and 95th percentiles. *p < 0.05 vs. NV, §p < 0.05 vs. NV, LV_T, **p < 0.05 vs. other groups.

ventilation (Fig. 6). *Series 3:* Although 24 h MV_T ventilation also increased PDGF-Rα expression, this increase was less compared to 8 h MV_T ventilation (Fig 7A). The altered expression of PDGF-B and PDGF-Rβ after 8 h MV_T ventilation was normalized after 24 h MV_T ventilation (Fig 7B).

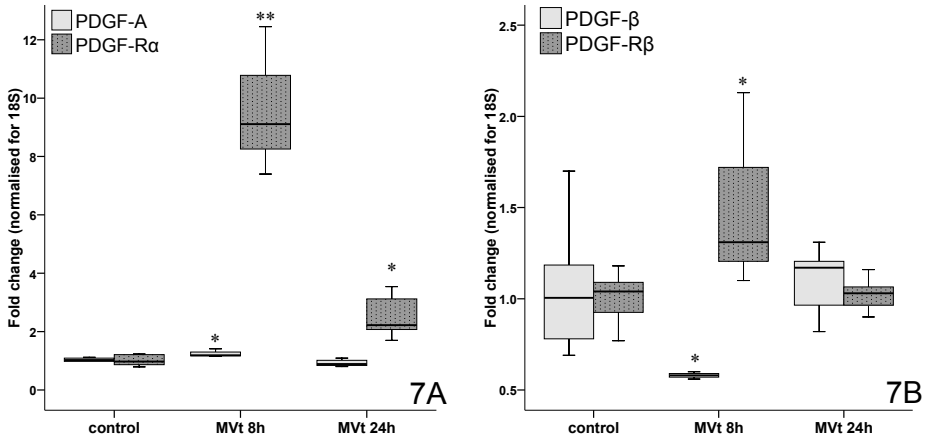


Figure 7. Effect of 8 and 24 hours of moderate (MV_T), ventilation with room air on the expression of PDGF genes in lungs of 7-day old rats. Median with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles. **A)** PDGF-A signalling, *P < 0.05 vs. NV, **p < 0.05 vs. NV, 24hours; **B)** PDGF-B signalling, *p < 0.05 vs. NV, 24 hours.

Various studies have shown the importance of vascular growth for lung development. VEGF is a potent inducer of endothelial cell growth that acts on vascular endothelial cells through two different receptors, VEGF-R1 and VEGF-R2. Inhibition of VEGF signaling has been shown to impair postnatal rat alveolar development (23). *Series 1*: VEGF-R1 expression was significantly up-regulated after LV_T , MV_T and HV_T ventilation, whereas VEGF-R2 expression was down-regulated by tidal volumes of ~ 8.5 ml.kg⁻¹ vs. other

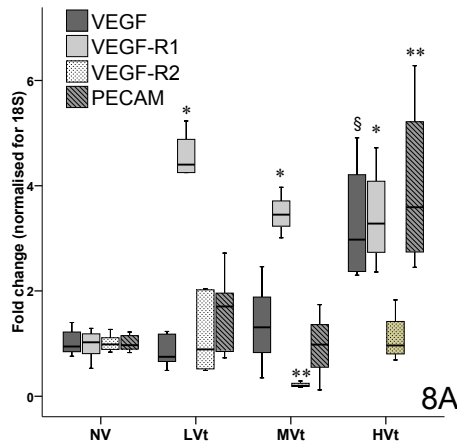


Figure 8A. Effect of low (LV_T), moderate (MV_T), and high (HV_T) ventilation for 8 hours with room air on the expression of matrix genes controlling vascularisation in lungs of 7-day old rats. Median with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles. * $p < 0.05$ vs. NV; § $p < 0.05$ vs. NV, LV_T ; ** $p < 0.05$ vs. other groups.

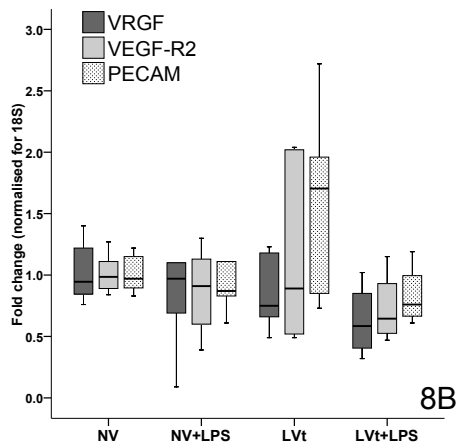


Figure 8B. Effect of exposure to LPS, low V_T ventilation (8 hours) with room air and low V_T ventilation (8 hours) after exposure to LPS on the expression of matrix genes controlling vascularisation in lungs of 7-day old rats. Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles. NV: non ventilated (n = 8); NV+LPS: exposure to LPS, non ventilated (n = 5); LV_T : ventilated with low tidal volume (n = 6); LV_T +LPS: ventilated with LV_T after exposure to LPS (n = 5).

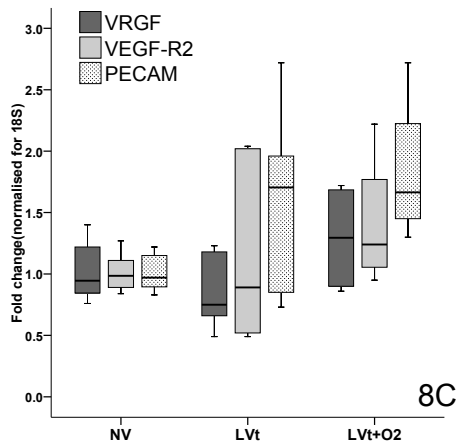


Figure 8C. Effect of low V_T ventilation (8 hours) with room air, low V_T ventilation (8 hours) with 50% oxygen on the expression of matrix genes controlling vascularisation in lungs of 7-day old rats. Median with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles. NV: non ventilated (n=8); LV_T: ventilated with low tidal volume (n=6); LV_T+O₂: ventilated with LV_T with 50% oxygen (n=5).

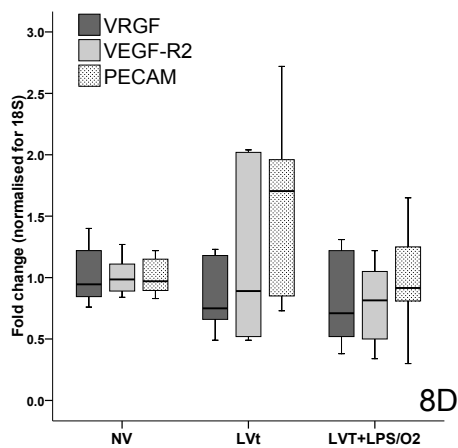


Figure 8D. Effect of exposure to low V_T ventilation (8 hours) with room air and low V_T ventilation (8 hours) with 50% oxygen after exposure to LPS on the expression of matrix genes controlling vascularisation in lungs of 7-day old rats. Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles.

groups (Fig. 8A). VEGF expression was not changed by ventilation with $\sim 3.5 \text{ ml.kg}^{-1}$ and $\sim 8.5 \text{ ml.kg}^{-1}$, but 8 h of ventilation with $\sim 25 \text{ ml.kg}^{-1}$ was associated with significant increased levels of VEGF mRNA (Fig. 8A). Endothelial cell (PECAM) marker expression was only increased by ventilation with $\sim 25 \text{ ml.kg}^{-1}$. Series 2: Interestingly, exposure to LPS and/or ventilation with 50% oxygen did not alter the expression of VEGF, VEGF-R2 and PECAM (Figs. 8B, C and D). Series 3: Prolonged (24 h) MV_T ventilation did increase

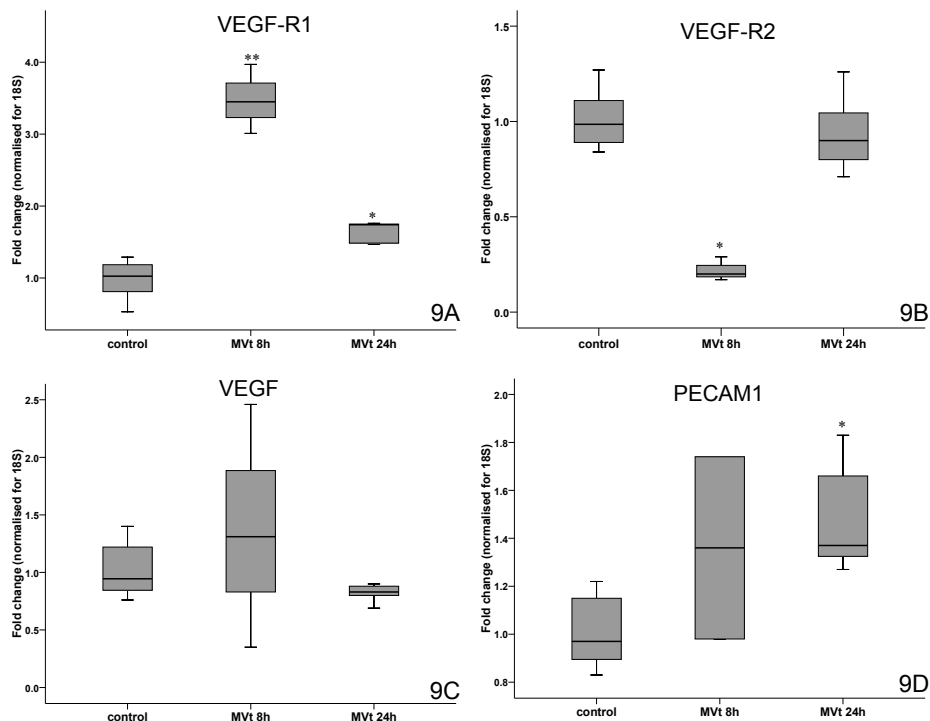


Figure 9. Effect of 8 and 24 hours of moderate V_T ventilation with room air and low V_T on the expression of matrix genes controlling vascularisation in lungs of 7-day old rats. Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles. **A)** * $p < 0.05$ vs. NV; ** $p < 0.05$ vs. NV, 24hours; **B)** * $p < 0.05$ vs. NV, 24hours; **D)** * $p < 0.05$ vs. NV.

the expression of VEGF-R1 *versus* non-ventilated animals (Fig. 9A). Again, this alteration was not as explicit as after 8 h MV_T -ventilation (Fig. 9A). The expression of VEGF and VEGF-R2 was not altered (Figs 9B, C). The expression of PECAM was significantly increased after 24 h MV_T -ventilation (Fig. 9D).

Vessel count: In animal models of mild BPD, the arrest in alveolar development is accompanied by a paucity of pulmonary capillaries. Small vessels are defined by vessels 20–65 μm in diameter. Ventilation with different tidal volumes for 8 h did not affect the vessel density (Table 3A). However, after 24 h MV_T ventilation of 7-day old rats a significant decrease in number of small vessels was observed *versus* non-ventilated 8-day old rats. The number of small vessels was not different from the number of small vessels in non-ventilated 7-day old rats (Table 3B).

TABLE 3A. Effect of low (LV_T), moderate (MV_T) and high (HV_T) ventilation for 8 hours on the vessel density in lungs of 7-day old rats.

Vessel density	Tidal Volume			
	NV (n=8)	LV_T 8h (n=4)	MV_T 8h(n=4)	HV_T 8h (n=4)
Vessels < 20 μ m	23.7 \pm 2.6	26.5 \pm 3.5	22.7 \pm 4.7	21.8 \pm 2.9
Vessels 20-60 μ m	25.0 \pm 7.9	28.5 \pm 2.6	21.0 \pm 9.7	22.8 \pm 4.6
Vessels > 60 μ m	48.7 \pm 7.0	55.0 \pm 6.1	43.7 \pm 8.8	44.6 \pm 5.1

Vessel density per 30 images (X40 high power fields). Values represent mean \pm SD.

TABLE 3B. Effect of prolonged (24h) ventilation on the vessel density in lungs of 7-day old rats.

Vessel density	Tidal Volume		
	NV day 7(n=4)	MV_T 24h (n=4)	NV day 8 (n=4)
Vessels < 20 μ m	29.5 \pm 3.1	39.3 \pm 3.8* ^s	23.7 \pm 2.6
Vessels 20-60 μ m	11.3 \pm 2.5 ^s	12.3 \pm 3.5 ^s	25.0 \pm 7.9
Vessels > 60 μ m	40.8 \pm 5.0	51.7 \pm 5.0*	48.7 \pm 7.0

Vessel density per 30 images (X40 high power fields). Values represent mean \pm SD.

* $p < 0.05$ vs. control day 7, ^s $P < 0.05$ vs. control day 8.

DISCUSSION

Arrested lung growth is one of the hallmarks of BPD. In the present study, we focused on the effect of mechanical ventilation on pulmonary expression of genes that regulate formation of alveoli and blood vessels in lungs of newborn rats. Because in rats, lungs at birth have a saccular appearance and alveolarization occurs exclusively postnatal, this model is relevant to the premature born infants developing BPD, although we acknowledge the major differences between mechanically ventilated newborn rats and premature born infants (18). Herein, we demonstrated an alteration in expression of molecules involved in alveolarization and angiogenesis in relation to tidal volume and duration of ventilation.

Mechanical ventilation and expression of genes implicated in alveolarization

Formation of new alveoli during the first phase of alveolarization ((postnatal days 4-21 in rat), starts with the development of new septa from immature preexisting septa. Elastogenesis is an essential step to this process of alveolar septation (24). Elastin is synthesized as tropoelastin. Both fibulin5 and lysyl oxidase play a key role during the assembly of tropoelastin into elastin fibers. Fibulin5 and elastin null mice exhibit abnormal alveolar development (24, 25). Lungs of LOX null mice display impaired development of distal and proximal airways with dilatation of the alveolar walls, sacs and primitive alveolar structures (25). Mechanical forces have been shown to induce

mRNA expression of tropoelastin *in vitro* (26) and *in vivo* (27). It has been suggested that ventilation with LV_T is less injurious. We previously reported decreased alveolar development in newborn rats after 12 and 24 h of mechanical ventilation, but not after 8 h of mechanical ventilation (15, 18). In accordance with these data we found in the present study that ventilation with LV_T and MV_T for 8 h did hardly influence the expression of tropoelastin, lysyl oxidase and fibulin5. Injurious ventilation (HV_T) increased the gene expression of all three elastogenic genes, in line with previous reports showing up-regulation of these genes during lung injury repair (28-30). A recent study has also reported increased gene expression of tropoelastin and lysyl oxidase, but not fibulin5 after 8 h ventilation of 2-4 days old mice (31). However, since tidal volume settings were not reported it is difficult to compare those data to ours. Next we studied the effect of eight hours LV_T ventilation combined with a systemic inflammation and/or oxygen and a prolonged period of MV_T ventilation for 12 and 24 h. LV_T ventilation (8 h) with 50% oxygen and MV_T ventilation for (12 h) caused a significant increase in fibulin5 mRNA, while LV_T ventilation (8 h) with room air or oxygen after pre-exposure to LPS and MV_T ventilation for 24 h reduced the mRNA levels of fibulin5. MV_T ventilation for 12 hours increased mRNA expression of tropoelastin and lysyl oxidase. After 24 hours of MV_T ventilation the increase of expression of these genes was less explicit. In newborn mice ventilated for 24 h with 40% oxygen, only the elastin assembly proteins fibulin5 and emilin-1 were decreased and the protein content of tropoelastin in the lung was increased (31). Our data demonstrate that inflammation accelerates the ventilation-induced effect on fibulin5 gene expression. Short-term ventilation after pre-exposure to LPS had the same effect on fibulin5 gene expression of as long-term ventilation with room air without pre-exposure to LPS. The influence of inflammation on fibulin5 expression seems to have more impact than 50% oxygen. The up-regulation of fibulin5 after LV_T ventilation (8 h) with oxygen is completely abolished after pre-exposure to LPS. For normal elastic fiber formation molecular interactions of tropoelastin and several extracellular matrix proteins, including fibulin5 and lysyl oxidase, are essential. Lysyl oxidase plays a critical role in the formation and repair of the extracellular matrix (ECM) by oxidizing lysine residues in elastin and collagen. As a result, covalent crosslinkages which stabilize these fibrous proteins are formed (32). Fibulin5 is an ECM protein that can bind integrins. Its role seems to be anchorage of elastic fibres to cells, thereby stabilizing and organizing elastic fibres in the lung (33). It is reasonable to assume that normal elastic fiber formation requires a certain balance between both assembly molecules (lysyl oxidase and-fibulin5) and tropoelastin. During the first 12 h of MV_T ventilation we found increases in tropoelastin, lysyl oxidase, and fibulin5 gene expression. Interestingly, while expression of both tropoelastin and lysyl oxidase were still increased after 24 h of MV_T expression of fibulin5 was decreased when compared to nonventilated controls. These data suggests that fibulin5 is prob-

ably one of the most important elastin assembly proteins, responsible for dysregulated elastin synthesis due to mechanical ventilation during the first 24 h.

ECM glycoprotein TN-C plays a morphoregulatory role during development and tissue remodeling. Fetal lungs of TN-C deficient mice have enlarged airspaces (34). In agreement with a previous study using newborn mice (35) we observed a significant down-regulation of TN-C expression after ventilation with low to moderate tidal volumes, with or without oxygen and/or pre-exposition to LPS. Both prolonged (24 h) MV_T ventilation with room air and LV_T -ventilation (8h) with oxygen after pre-exposure to LPS had the most negative impact on TN-C expression. Several findings support the idea that pulmonary TN-C is a mechanosensitive gene. Cyclic mechanical stretch (elongation 10% for 48 h) of human lung fibroblasts reduced TN-C expression and decreased differentiation into ECM producing myofibroblasts (36). In a murine model of pulmonary hypoplasia with co-existent diaphragmatic hernia TN-C expression is down-regulated (37). In contrast, cyclic mechanical strain of human airway smooth muscle cells (17–18.5% elongation for 1-5 days) has been shown to increase TN-C expression (38). Furthermore, TN-C is highly expressed in autopsied babies with BPD (39). Recent analysis of gene expression in repairing lungs of naphthalene-exposed mice showed a transient increase of TN-C expression in the lung whereas abortive repair in a transgenic model allowing ablation of all reparative cells resulted in a progressive accumulation of TN-C (49). Thus, the increase in TN-C expression during HV_T ventilation fits with a lung injury repair response. In contrast, the down-regulation of TN-C after 8-24 h of mechanical ventilation with low to moderate tidal volumes may negatively influence alveolar development, although we did not find significant differences at the TN-C protein level.

Matrix metalloproteinases (MMPs) represent a family of zinc-dependent proteases that degrade the ECM. These proteases are secreted by endothelial cells, alveolar cells, macrophages, fibroblasts, and other connective tissue cells (40). They have been shown to play a role in airway remodeling (41). By breaking down ECM proteins they facilitate cell migration, which is essential for normal wound repair (42). Since mechanical ventilation triggers the expression of acute phase cytokines (15) it is not surprising that MMPs are reportedly elevated in BPD (19-22). We did find an increase in *Mmp9* message after 12 h of mechanical ventilation, however, the exact ECM targets are yet unknown.

Mechanical ventilation and expression of genes implicated in angiogenesis

Ample evidence suggests that abnormal vascularization may contribute to disrupted alveolar development. Especially reduced signaling through angiogenic VEGF and PDGF pathways appear to affect alveologenesis. Blocking PDGF receptor signaling in neonatal rats causes abnormal alveolar development (43). Expression of VEGF and

its receptors are decreased in premature lambs, baboons and human infants, who developed BPD after a prolonged period of mechanical ventilation with oxygen (9, 44, 45). In the present study, non-injurious (LV_T and MV_T) ventilation with room air had no effect on VEGF expression. Even prolonged ventilation (24 h) and ventilation (8 h) with oxygen and pre-exposure to LPS did not change the expression of VEGF. We only observed a significant decrease of VEGF-R2 expression after MV_T ventilation. Although VEGF-R1 expression was up-regulated with LV_T and MV_T ventilation for 8 h, it is believed that VEGF-R2 mediates the major growth and permeability actions of VEGF. Thus, LV_T ventilation appears to have less influence on lung vascular development in comparison with MV_T ventilation. Our finding of reduced PDGF-B expression after MV_T and HV_T ventilation, but not LV_T ventilation, supports this conclusion. PDGF-B/PDGF-R β signaling has been shown to promote proliferation of vascular smooth muscle cells and pericytes during their recruitment to new vessels (46). Only after 24 h of MV_T ventilation we observed a decrease in small vessel density compared to non-ventilated controls. Proper PDGF-A/PDGF-R α signaling is crucial for alveolar septation (47). In contrast with a previous study using newborn mice (35) prolonged ventilation, independent of V_T , did not reduce the expression of PDGF-A in neonatal rats. Interestingly, independent of tidal volume, PDGF-R α expression was increased by ventilation. An increase in PDGF-R α has been shown to be up-regulated prior to the development of fibroproliferative lung lesions (48), while a decrease in PDGF-R α expressing lung fibroblasts coincided with alveolar thinning (49).

In conclusion, MV_T ventilation alters the expression of genes involved in alveolarization and angiogenesis in newborn rats. Our data suggest an imbalance between the expression of fibulin5 and tropoelastin as the first trigger for dysregulated elastin formation during the first 24 h of mechanical ventilation. This ventilatory negative effect on Fibulin5 and tropoelastin expression is accelerated by a systemic inflammation. We also show that altered PDGF and VEGF receptor expression after MV_T -ventilation affects pulmonary vessel formation. LV_T ventilation was protective, suggesting that the negative effect of ventilation on angiogenesis is tidal volume dependent. We are the first to demonstrate these alterations in gene expression in relation to tidal volume and duration of ventilation. This may be important for future therapeutic strategies to prevent BPD.

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Chapter 8

General discussion



INTRODUCTION

Bronchopulmonary dysplasia (BPD) is a chronic lung disease, one of the most important sequelae of oxygen and ventilation-mediated injuries following a premature birth. The pulmonary impairment begins *in utero*, progresses postnatally and continues into later childhood and adulthood. Treatment with antenatal corticosteroids, introduction of protective ventilation strategies, surfactant replacement therapy, progress in nutritional support and better nursing care has improved survival of premature born infants with Respiratory Distress Syndrome, but the incidence of BPD has hardly changed. BPD is characterized by an arrest in alveolar development: alveoli are fewer and larger, and the vasculature is dysmorphic. BPD remains a major complication in premature infants, who are mechanically ventilated with oxygen; most likely due to the ever-changing limit of viability resulting in higher survival rates of infants born at gestational age less than 26 weeks. Both mechanical ventilation and oxygen interfere with alveolar and vascular development. The main goal of this thesis was to identify molecular pathways that are activated by mechanical ventilation and interfere with normal lung development. To investigate these underlying pathogenic pathways we used a newborn rat model.

LIMITATIONS OF THE NEWBORN RAT MODEL

Morphologic and quantitative studies of the rat lung have greatly contributed to the understanding of the formation of alveoli. However, major differences exist between neonatal rats and premature infants. First, neonatal rats that receive mechanical ventilation are at term and consequently have a more developed surfactant system. With the use of stable isotopes it has been shown that the *de novo* synthesis and turnover rates of surfactant in preterm infants with respiratory distress syndrome are very low (1). Secondly, the anti-oxidant system in neonatal rats is more mature. Human studies have demonstrated that preterm infants produce high amounts of reactive oxygen species (ROS), while lacking appropriate amounts of antioxidant enzymes, including superoxide dismutase and glutathione peroxidase (2). The antioxidant system is upregulated during the last 15% of gestation (3). A major advantage is that at birth rat lungs mimic the saccular lung of very premature patients and alveolar formation occurs exclusively postnatally within a very short period of about 2 weeks (4). There are other animal models to study alveolarization, but some are very expensive (premature baboons and sheep) while others are technically a challenge to ventilate (newborn mice). Hence, despite its shortcomings, the newborn rat is an appropriate model to study ventilatory-induced pathways leading to arrested alveolarization. This is relevant to premature born infants at risk to develop BPD.

EFFECT OF DIFFERENT TIDAL VOLUMES AND DURATION OF MECHANICAL VENTILATION ON INFLAMMATORY RESPONSE

In **chapter 1** the different mechanisms of ventilator-induced lung injury (VILI) are described. High peak inspiratory pressures and repetitive end-expiratory collapse are considered to be important determinants of VILI. That's why high frequency oscillatory ventilation (HFOV) has been introduced in the neonatal care. HFOV delivers small tidal volumes at rapid rates. Multiple studies in animals have suggested that this mode of ventilation is less injurious compared to conventional ventilation. HFOV improved early lung function and decreased inflammation (5). However, clinical trials comparing HFOV with conventional ventilation showed only a very small reduction in BPD with HFOV and no longterm benefits (6, 7). In baboons with BPD impaired alveolarization and capillary development occurred in spite of appropriate oxygenation (FiO_2 : 0.2-0.5) and use of ventilator strategies to prevent volutrauma (8). Thus even minute stretching of a developing lung is enough to cause injury. In **chapter 3** we describe that both low V_T and moderate V_T ventilation with room air for 8 h induced an inflammatory response. The expression of IL-6 and CXCL-2 were increased. The expression of these genes was further increased following low V_T ventilation with 50% oxygen. IL-6 and CXCL-2 activate and attract leukocytes that are implicated in the pathogenesis of BPD (9, 10). In **chapter 7** we describe the effect of different tidal volumes on the expression of genes involved in alveolarization. We found an almost similar change in expression of these genes after 8 h of low or moderate V_T ventilation. Expression of tropoelastin and lysyl oxidase was increased while that of tenascin-C was decreased compared to nonventilated controls. Lysyl oxidase and tropoelastin message levels peaked at 12 hours of ventilation and remained elevated at 24 hours. In contrast, fibulin5 expression was increased after 12 hours of ventilation but decreased compared to nonventilated controls at the time (24 hours) we observed a disturbed elastin deposition. Short-term moderate V_T ventilation after pre-exposure to LPS had the same negative effect on fibulin5 expression as long-term ventilation with room air without pre-exposure to LPS. Although our prolonged (24 hrs) ventilation used a moderate V_T , it is reasonable to assume that the changes in gene expression after prolonged ventilation with low V_T will be the same as those seen with moderate V_T considering the changes in gene expression after 8 h. Uncoupled elastin synthesis and assembly could explain decreased septation (alveolarization), as seen in BPD lungs (11).

These data may explain why randomised controlled trials did not show beneficial effect of protective ventilation (HFOV) in preventing BPD. However, we have to consider the following issues: (a) the limitations of the model as described above; (b) during the period with low and moderate tidal volume ventilation it was not possible to optimize the lung volume ventilation strategy to recruit alveoli, eliminate atelectasis or prevent

overdistension because of lack of continuous measurement of O_2 saturation or PaO_2 ; ventilation strategies which have demonstrated to reduce VILI (12); (c) animals were not allowed to recover after the ventilation period.

It still has to be elucidated if the disturbed elastogenesis is a direct result of ventilation-triggered cell stretch or a secondary effect of increased expression of pro-inflammatory cytokines and chemokines. A systemic inflammation in fetal sheep increased the concentration of IL-6 in bronchoalveolar lavage, together with disturbed deposition of elastin in the lungs (13). This finding and our data suggest that the IL-6 pathway may be relevant in disturbed elastin deposition.

MECHANICAL VENTILATION INDUCES APOPTOSIS AND CELL CYCLE ARREST

Normal lung development is a highly orchestrated process of growth, differentiation and programmed cell death (14, 15). During normal lung development three peaks of apoptosis, preceded by peaks of cell proliferation are described (**chapter 1**). *In vitro* studies using isolated lung cells have demonstrated that mechanical overstretch inhibits proliferation and induces apoptosis (15, 16). Only stretch-induced apoptosis, but not stretch-induced cell cycle arrest, has been demonstrated *in vivo* (11). Such interference in cell proliferation and programmed cell death may affect alveolar formation in the developing lung. Consequently it is reasonable to hypothesize that dysfunctional apoptotic and cell proliferation mechanisms contribute to the pathophysiology of BPD. Our studies provide evidence for this hypothesis. After prolonged (24 hours) mechanical ventilation we found increased apoptosis and inhibited proliferation of lung cells together with an arrest in alveolar development.

Different pathways regulate both apoptosis and proliferation of cells. For a better understanding of the pathogenesis of BPD, it is essential to know which signaling pathway are involved. In **chapter 5** we show that prolonged mechanical ventilation for 24 h increased the number of TUNEL and cleaved caspase-3 positive lung cells via activation of the FasL/Fas (extrinsic apoptotic) pathway. This was confirmed by stretching of primary isolated rat fetal lung epithelial cells. Activation of the extrinsic apoptotic pathway was demonstrated by increased activity of caspase-8, while caspase-9 activity (intrinsic apoptotic pathway) was not increased. Also the expression of FasL mRNA and protein was increased. Inhibition by universal inhibitors of caspases abrogated stretch-induced caspase-3 activation and a neutralizing anti-rat FasL monoclonal antibody attenuated the stretch-induced levels of cleaved caspases-3 and -8. It is plausible that the extrinsic apoptotic pathway via the FasL/Fas pathway is directly activated by stretch. Tracheal occlusion of fetal rabbits resulted in an increase of FasL in alveolar type II cells, together with increased apoptosis of these cells (17). Other triggers for activation of the

extrinsic apoptotic pathway by mechanical ventilation are pro-inflammatory cytokines/chemokines. Again IL-6 seems to play an important part. IL-6 has been shown to induce apoptosis in some models, but it also exerts anti-apoptotic effect in others (18, 19). IL-6 is linked with the pathogenesis of emphysema. In IL-6 deficient mice treated with pancreatic elastase, hyperoxia and cigarette smoke, the increase of apoptotic cells was suppressed (18, 20, 21). Unfortunately, the FasL/Fas pathway was not studied. Although IL-6's involvement in ventilation/stretch-induced apoptosis remains to be determined, our finding of increased expression of IL-6 after 8 h of mechanical ventilation (**chapter 3**) is compatible with such a role. However, besides IL-6, mechanical ventilation upregulates other pro-inflammatory mediators (IL-1 β , IL-8 and TGF- β), that exhibit pro-apoptotic properties.

In **chapter 6** the effect of prolonged mechanical ventilation on lung cell proliferation is elucidated. The response of pulmonary epithelial cells to mechanical stretch *in vitro* has been shown to vary considerably depending on the cell type, culture conditions, pattern, and duration of stretch exposure (22). Mechanical stretch simulating normal fetal breathing movements increased type II cell proliferation, indicating that mechanical strain is necessary for normal lung growth (23, 24). From *in vitro* studies it is known that mechanical stretch and oxygen can also inhibit lung cell proliferation (25, 26). We are the first to describe that ventilation for 24 h inhibits uptake of BrdU in lung cells and decreases expression of Cyclin D1 and E1 mRNA and protein. Before a cell can proliferate, it needs to pass through four non-overlapping phases (**chapter 1**). We give evidence that the ventilation-induced arrest in lung cell proliferation takes place in G₁ phase. In this phase the cell is preparing for DNA synthesis. Expression and nuclear localization of Cdk inhibitor proteins p27^{Kip1} and p57^{Kip2} was increased. This prevents binding of Cyclin D1 and E1 to their CDK partner, which is essential for transition from G₁ to S phase of the cell cycle. It is not known how mechanical strain influences this binding. We could not identify transforming growth factor- β (TGF- β) as a trigger in our study. We do know that TGF- β can prevent the decline of p27^{Kip1} mRNA expression. TGF β -treated cells have p27^{Kip1} mRNA levels equal to those of proliferating cells (27). In the bronchoalveolar lavage fluid of preterm infants increased levels of TGF- β have been found in those patients who developed BPD (28, 29). In contrast, high tidal volume ventilation for 3 hours did not increase TGF- β content in newborn rat lung (30) and stretching subcutaneous tissue did decrease TGF- β protein (31). Thus, it is plausible that the level of TGF- β expression is important for long-term outcome by determining the intensity of tissue remodeling and repair (32). We found an arrest in lung cell proliferation, with no change in TGF- β expression, suggesting that after 24 h of moderate tidal volume ventilation remodeling or repair of lung tissue has not yet started. Undoubtedly this thesis and future research will bring us a step closer to elucidate the pathogenesis of BPD.

FUTURE PERSPECTIVES

The general aim of this thesis was to identify molecular pathways that are activated by mechanical ventilation and interfere with normal lung development. We were able to unmask two pathways in ventilated newborn rats. First, mechanical ventilation induces apoptosis of lung cell via the FasL/Fas pathway. Secondly, mechanical ventilation induces an arrest in lung cell proliferation by increased expression and nuclear localization of Cdk inhibitors from the Kip family. Normal lung development is a highly orchestrated process of growth, differentiation and programmed cell death. The imbalance we found between lung cell proliferation and programmed cell death may be the key to the disturbed alveolar formation in the developing lung.

A logical next step will be to study if inhibition of the FasL/Fas pathway and targeting protein p27^{Kip1} will prevent or at least attenuate the arrest in alveolarization. The ultimate goal will be to develop treatment strategies to prevent this ventilator-induced injury for the premature born infants based on our findings. However, treatment strategies based on inhibiting apoptosis or stimulating proliferation do carry the potential harm of inhibiting physiological apoptosis and inducing/enhancing proliferation in other cells such as fibroblasts and neutrophils. This can be even more harmful for the developing lung. Hence, a better understanding of the role of apoptosis and proliferation in the pathogenesis of ventilator-induced alterations in lung development and the signaling cascades are necessary before therapeutic strategies can be developed for the treatment.

Another promising direction for research will be stem/progenitor cell therapy. Studies in the adult lung suggest that bone marrow-derived stem cells (BMCs) can prevent adult lung injury. *In vitro* BMSCs prevent alveolar growth arrest in a BPD model. They adopt immunophenotypic and ultrastructural characteristics of alveolar type II cells (33). Also an increased number of circulating endothelial progenitor cells (EPCs) in patients with acute lung injury is correlated with improved survival (34). Given the fact that disrupted alveolarization is associated with a dysmorphic microvasculature and the importance of lung vasculature for repair after lung injury, EPCs may have therapeutic potential. Umbilical cord blood represents an important source for stem cells. The establishment of a biobank in which umbilical cord and cord blood can be collected will be a first step in a better understanding of stem cell biology and its therapeutical potential. At the same time trachea aspirates can be collected to find early biomarkers and potential mediators of ventilation-induced lung injury in preterm infants.

Besides basic research, clinical studies are needed. Good follow-up studies of infants with BPD are lacking. Adverse health consequences of BPD include long-term respiratory morbidity such as asthma and emphysema, pulmonary hypertension, undernutrition, growth failure, cognitive impairment, cerebral palsy and neurodevelopmental

deficits. Until now there is no specific follow-up program and no guidelines for therapy. This requires a multidisciplinary approach. Finally, to reduce the adverse effects associated with intubation and mechanical ventilation, non-invasive ventilation looks promising. There is evidence that non-invasive ventilation improves gas exchange and reduces extubation failure after mechanical ventilation in preterm born infants, but multicenter clinical trials are needed to demonstrate if this mode of respiratory support will decrease the incidence of BPD.

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Chapter 9

Summary



In **Chapter 1** – the general introduction – the current knowledge regarding BPD related to lung development and lung injury is discussed. The critical concept underlying the pathogenesis of BPD is that the lung is structurally immature in preterm born infants. Any antenatal or postnatal event that will take place during this period can adversely alter the developmental program of the immature lung. The pathological findings seen in BPD is the result of a complex interplay between perinatal insults (infection, chorioamnionitis), impairments in the premature lung (surfactant deficiency, less developed antioxidant system) and damage resulting from lifesaving therapies, such as mechanical ventilation and supplemental oxygen administration. Together they culminate in a chronic inflammation in the developing lung that impairs alveolarization and vascularization. Both processes are essential for normal lung development and without doubt play a role in the pathogenesis of BPD.

The aims of this thesis are described in **Chapter 2**.

In **Chapter 3** we assessed the effect of different tidal volumes (V_T), oxygen and endotoxin on cytokine/chemokine production in lungs of newborn rat that were ventilated for eight hours. We demonstrated that low-tidal volume ventilation resulted in a mild inflammatory response. Gene expression of pro-inflammatory cytokines/chemokines CXCL-2 and IL-6 was significantly increased. A further increase was observed with increasing V_T and ventilation with 50% oxygen, but not after pre-exposure to endotoxin. The increase in mRNA expression was accompanied by an increase of IL-1 β and/or IL-6 protein in the bronchoalveolar lavage fluid (BALF) after high- V_T ventilation and ventilation with oxygen and ventilation after exposure to endotoxin. Elevated concentrations of these cytokines/chemokines in amniotic fluid and BALF have been associated with BPD. The underlying mechanisms by which stretching of a developing lung may disturb lung development are described in the next chapters.

Chapter 4 reviews apoptotic cell death in lung development and BPD. Insight in lung development is crucial to study the pathogenesis of BPD. Throughout each stage of lung development apoptosis is important for remodelling of lung tissue. Apoptotic activity is genetically determined but can also be triggered by stretch/mechanical ventilation, hyperoxia and inflammation. Hence, it is not surprising that dysfunctional apoptotic mechanisms are believed to be important in the development of BPD. Recent studies provide strong evidence for Fas/FasL dependent epithelial apoptosis. Candidates for activation of apoptotic pathways by oxygen are: pro-apoptotic Bax protein, cell cycle regulator p53 and cytokine TGF- β . Neutrophils play a critical role in inflammation-induced apoptosis. They release pro-apoptotic mediators but also have a prolonged half-life due to diminished apoptosis with triggers an ongoing inflammatory response.

In **Chapter 5** we investigated the signalling mechanisms of ventilation (stretch)-induced apoptosis in newborn rat lung. Mechanical ventilation of newborn rats for 24 hours with moderate V_T induced apoptosis of alveolar type II cells. This coincided with activation of the Fas/FasL system. Involvement of the Fas/FasL system was confirmed by continuous cyclic stretching of fetal lung epithelial cells. Not only increased stretch expression of FasL by the cells, but neutralizing FasL antibodies abrogated this increase. Together with increased expression of FasL, we observed increased expression of cleaved caspase-3, -7 and -8, but not of caspase-9. These data indicate activation of the extrinsic death pathway via the FasL/Fas system by prolonged mechanical ventilation, resulting in apoptosis of alveolar type II cells.

Chapter 6 focuses on the effect of prolonged mechanical ventilation on lung cell proliferation. A decrease in the number of proliferating lung cells was observed in newborn rats with increase of duration of mechanical ventilation. After ventilating for 24 hours lung cell proliferation came to a halt in the G1 phase of the cell cycle. This was associated with an arrest in alveolar development as demonstrated by a decrease in secondary crests and larger alveoli. For transition of the cell cycle from G1 into S phase it is crucial that cyclin-dependent kinases (Cdk) 4/6 form a complex with cyclin D as well as Cdk2 with cyclin E. We found increased expression of Cdk inhibitor p27^{Kip1} and p57^{Kip2} what prevents binding of the Cdk to its Cyclin partner. These findings were confirmed *in vitro* by continuous cyclic stretching of fetal lung epithelial cells. All together, these experiments revealed a new mechanism by which mechanical ventilation inhibits cell proliferation.

The aim of the experiments described in **Chapter 7** was to investigate the influence of (prolonged) mechanical ventilation, inflammation and hyperoxia on the expression of genes regulating the formation of alveoli and blood vessels in lungs of newborn rats. The current view is that the development of the blood vessels in the lung passively follows that of the airways. However, increasing evidence suggests that lung angiogenesis actively promotes alveolar growth during development via the secretion of angiogenic growth factors. A better understanding of both developmental processes may have potential for the treatment of BPD and other lung diseases characterized by disrupted alveolarization. Our findings suggest an imbalance between the expression of Fibulin5 and tropoelastin due to mechanical ventilation resulting in a dysregulated elastin assembly. A systemic inflammation promoted this ventilation-induced imbalance in elastogenic gene expression. A disturbance in angiogenesis was initiated by altered PDGF and VEGF receptor gene expression. These changes in mRNA expression of angiogenic receptors was only observed after moderate tidal volume ventilation, not after ventilation with low tidal volumes.

Chapter 10

Appendices



NEDERLANDSE SAMENVATTING

In **hoofdstuk 1** – de algemene introductie - worden de huidige inzichten ten aanzien van BPD in relatie tot longontwikkeling en longschade bediscussieerd. De onderliggende gedachte over de ontstaanswijze van BPD is dat de long van te vroeg geboren kinderen structureel niet volgroeid is. Iedere gebeurtenis, welke voor of na de geboorte optreedt gedurende deze periode, kan het ontwikkelingsprogramma van de onvolgroeide long nadelig beïnvloeden. De pathologische bevindingen, welke gezien worden bij BPD, zijn het gevolg van een complex samenspel tussen trauma's voor de geboorte (infectie, chorioamnionitis), tekortkomingen van de onrijpheid van de long (onvoldoende surfactant, minder ontwikkeld anti-oxidant systeem) en beschadigingen ten gevolge van levensreddende behandelingen zoals beademing en extra zuurstof toediening. Gezamenlijk leidt dit tot een chronische ontsteking van de long, welke het ontstaan van longblaasjes en longvaten schaden. Beide processen zijn essentieel voor de normale ontwikkeling van de long en spelen zonder twijfel een rol in de ontstaanswijze van BPD.

De redenen waarom de studies werden uitgevoerd die beschreven zijn in dit proefschrift worden genoemd in **hoofdstuk 2**.

In **hoofdstuk 3** is het effect van verschillende teugvolumina (V_T), zuurstof en endotoxinen op de cytokine/chemokine productie bepaald in de long van pasgeboren ratten, die gedurende acht uur werden beademd. Het beademen met een klein teugvolume resulteerde in een geringe ontstekingsreactie. De gen expressies van pro-inflammatoire cytokines/chemokines CXCL-2 en IL-6 waren significant verhoogd. Een verdere toename werd vastgesteld met toename van het teugvolume en beademing met 50% zuurstof, maar niet na behandeling met endotoxine. De toename van mRNA expressie ging samen met een toegenomen hoeveelheid van het eiwit IL-1 β en/of IL-6 in de bronchoalveolaire lavage vloeistof (BALF) na het beademen met een groot teugvolume. Dit vond ook plaats bij het beademen met zuurstof of bij behandeling met endotoxine. Toegenomen concentraties van deze cytokines/chemokines (IL-1 β en/of IL-6) in amnion vloeistof en BALF zijn geassocieerd met BPD. De onderliggende mechanismen waardoor het oprekken van de zich ontwikkelende long kunnen leiden tot een verstoorde longontwikkeling, worden in de volgende hoofdstukken beschreven.

In **hoofdstuk 4** wordt een overzicht gegeven van de rol van apoptotische cel dood gedurende de normale longontwikkeling en de ontwikkeling van BPD. Kennis van de longontwikkeling is essentieel voor het bestuderen van het ontstaan van BPD. Gedurende ieder ontwikkelingsstadium van de long is apoptosis belangrijk. Apoptotische

activiteit is genetisch vastgelegd, maar kan ook gestimuleerd worden door rek/beademing, hyperoxie en ontsteking. Het is dus niet verrassend dat wordt verondersteld dat verstoorde apoptotische processen van belang zijn voor het ontstaan van BPD. Recente studies tonen overtuigend aan dat apoptose van de epitheliale cellen afhankelijk is van het Fas/FasL systeem. Kandidaten voor activatie van apoptose ten gevolge van zuurstof zijn: het pro-apoptotische eiwit Bax, cel cyclus regulator p53 en cytokine TGF- β . Witte bloedlichaampjes spelen een kritische rol in door ontsteking veroorzaakte apoptosis. Uit de witte bloedlichaampjes komen pro-apoptotische mediators vrij, maar ook hebben ze een verlengde levensduur ten gevolge van verminderde apoptosis. Dit geeft aanleiding tot een aanhoudende ontsteking.

In **hoofdstuk 5** hebben we mechanismen onderzocht hoe beademing kan leiden tot apoptose in de long van pasgeboren ratten. Beademing van pasgeboren ratten gedurende 24 uur met middelmatige teugvolumina veroorzaakte apoptosis van de alveolaire type II cellen. Dit ging gepaard met activatie van het Fas/FasL systeem. Betrokkenheid van dit systeem werd bevestigd door het continu cyclisch rekken van foetale epitheliale longcellen. Niet alleen versterkte het rekken de expressie van FasL door deze cellen, maar met het toedienen van neutraliserende FasL antilichamen was de apoptosis verminderd. Gelijktijdig met de toegenomen expressie van FasL, was ook de expressie van 'cleaved caspase-3, -7 en -8' toegenomen, maar niet die van caspase-9. Deze data tonen aan dat beademing de 'extrinsic death pathway' via het FasL/Fas systeem activeert met apoptose van de alveolaire type II cellen als gevolg.

Hoofdstuk 6 belicht het effect van langdurige beademing op het delen (proliferatie) van longcellen. Een afname in het aantal prolifererende longcellen van pasgeboren ratten werd waargenomen met toename van de duur van de beademing. Na een beademingsperiode van 24 uur stopte de proliferatie van de longcellen in de G1 fase van de celdeling. Dit ging samen met een remming van de ontwikkeling van de longblaasjes: het aantal 'secundaire crests' was afgenomen en de longblaasjes waren groter. Voor transitie van de cel cyclus van de G1 naar de S fase is het van belang dat cycline-afhankelijke kinases' (Cdk)4/6 een complex vormen met cycline D en Cdk2 met cycline E. Wij toonden een toegenomen expressie aan van de eiwitten p27^{Kip1} and p57^{Kip2}. Deze eiwitten voorkomen binding van Cdks met de bijbehorende cycline. Deze bevindingen werden bevestigd *in vitro* door het continu cyclisch rekken van foetale epitheliale longcellen. Door deze experimenten hebben we een nieuw mechanisme aangetoond hoe beademing de deling van longcellen remt.

Wat is de invloed van (langdurige) beademing, ontsteking en hyperoxie op de expressie van genen die de ontwikkeling van longblaasjes en bloedvaten in de longen

van pasgeboren ratten reguleren? Dit wordt beschreven in hoofdstuk 7. Het huidige inzicht is dat de ontwikkeling van de bloedvaten in de long de ontwikkeling van de longblaasjes passief volgt. Er is zijn echter toenemende aanwijzingen dat de bloedvat vorming in de long de groei van longblaasjes actief stimuleert. Een beter inzicht in beide ontwikkelingsprocessen kan mogelijk leiden tot de behandeling van BPD en andere longziekten die worden gekenmerkt door een gestoorde ontwikkeling van de longblaasjes. Onze bevindingen suggereren dat beademing de balans tussen de expressie van fibulin5 en tropoelastin verstoort met als gevolg een verstoorde samenstelling van de elastine vezels. Een systeem infectie stimuleert deze door de beademing veroorzaakte imbalance via genen die betrokken zijn bij de vorming van de elastine vezels. Een verstoring van bloedvat vorming werd veroorzaakt door een veranderde expressie van PDGF en VGF receptor genen. Deze veranderingen in mRNA expressie werd alleen waargenomen na het beademen met middelmatige teugvolumina, niet na beademen met kleine teugvolumina.

Tot slot wordt in de discussie (hoofdstuk 8) alle bevinding in perspectief geplaatst en worden aanbevelingen gedaan voor verder onderzoek.

CURRICULUM VITAE

André Kroon was born as 'Amsterdammer' on the 21st of December 1964. He went to secondary school at the S.G. Sint Nicolaas Lyceum in Amsterdam from 1977 to 1983. After graduating, he started his medical training at the Medical Faculty of The Vrije University of Amsterdam in 1984 and received his medical degree cum laude in 1990. He joined the military service and worked as first lieutenant physician at the ENT department of the Central Military Hospital in Utrecht from 1991 to 1992. There after a long career started as paediatric resident-not-in-training. First at the Sint Lucas Hospital in Amsterdam from 1992 to 1994, next at the Academic Medical Centre in Amsterdam from 1994 to 1996 and finally in the Erasmus MC-Sophia in 1996, where he became pediatric resident-in-training in 1997 (Prof. Dr. H.J. Neijens). The non-academic part of the training was followed in the Zuiderziekenhuis in Rotterdam (dr. R. Sukhai). In 2001 he started his fellowship Neonatology in the Erasmus MC-Sophia under supervision of dr. J.N. van den Anker. He finished it in 2003 under supervision of Prof. dr. L.J.I. Zimmerman, who made him enthusiastic for lung research. Since then he is staffmember of the Department of Neonatology, Erasmus MC-Sophia. In 2005 he started his PhD thesis, when he went for the first time to Toronto. He started a research fellowship in the laboratory of the Department of Lung Biology at the Hospital for Sick Children under guidance of Prof. dr. M. Post. In 2009 he went for another year as research fellow to Toronto.

André became 'Rotterdammer' in 2011. He lives happily together with George Damhuis.

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Kroon AA, Post M. Apoptotic Cell Death in Bronchopulmonary Dysplasia. *Current Pediatric Reviews*. 2011 Nov; 7(4):285-292.

PhD PORTFOLIO

Summary of PhD training and teaching

Name PhD student: A.A. Kroon PhD period: 2005-2011
 Erasmus MC Department: Neonatology Promotors: Prof. Dr. J.B. van Goudoever
 Research School: Erasmus MC Prof. Dr. M. Post

1. PhD training

	Year	Workload (hours)
General courses		
- Biological, Chemical and Radiation Safety Seminars Research Integrity	2005/2009	48
- Lab Animal Services General Orientation	2005	4
- Lab Animal Services Handling	2005	4
- Lab Animal Services Gas Anesthesia	2005	4
- Lab Animal Rodent Survival Surgery	2005	4
- Introduction to Data-analysis	2007	35
Seminars and workshops		
- Research meetings 'Moeder en Kind' Center	2006-2011	20
- Improving Mechanical Ventilation in Neonates, Gross Doelln	2008	16
- Research meetings 'Lung Biology Research, Physiology and Experimental Medicine Program'	2005/2009	150
Presentations		
- Prolonged mechanical ventilation alters gene expression in newborn rats (SPR, Washington)	2006	30
- Influence of mechanical stretch on lung development (Research in progress meeting, Toronto)	2006 2006	30 30
- Breathless in Toronto (Grand Round, Erasmus MC-Sophia, Rotterdam)	2007	30
- Schade ten tijde van de longontwikkeling (Onderzoeksdag, Erasmus MC-Sophia, Rotterdam)	2008	60
- Oxygen and Inflammation alter alveolarisation in newborn rats ventilated with low tidal volumes (SPR, Honolulu)	2009	60
- Ins and Outs of Bloodgas analysis (Boerhaave cursus, Leiden)	2009	60
- Oxygen and Inflammation alter alveolarisation in newborn rats ventilated with low tidal volumes (ESPR, Hamburg)	2009	60
- Prolonged ventilation causes p27 ^{KIP1} induced cell cycle arrest in newborn rat lung (ESPR, Copenhagen)	2010	30
(Inter)national conferences		
- Hot Topics in Neonatology, Denver	2007	28
- Nederlandse Nascholing Neonatologie, Tegelen	2006/2010	56
- SPR, Denver	2011	28
2. Teaching		
- Introduction training internship pediatrics, Rotterdam	2006-2011	150
- NICU nurses education, Rotterdam	2006-2011	50
- Medical training pediatric residents, Rotterdam	2006-2011	50

LIST OF ABBREVIATIONS

BALF	bronchoalveolar lavage fluid
BPD	bronchopulmonary dysplasia
BrdU	5-bromo-2-desoxyuridine
CDK	cyclin dependent kinase
ECM	extra cellular matrix
Egr	early growth response
FBM	fetal breathing movements
Fib5	fibulin5
FLF	fetal lung fibroblast
FLDE	fetal lung epithelial cel
GA	gestational age
HFOV	high frequency oscillatory ventilation
HV _T	high tidal volume
IL	interleukin
LOX	lysyl oxidase
LPS	lipopolysaccharide
LV _T	low tidal volume
MPO	myeloperoxidase
MV	mechanical ventilation
MV _T	moderate tidal volume
NV	non-ventilated
PDGF	platelet-derived growth factor
PECAM	platelet endothelial cell adhesion molecules
PEEP	positive end expiratory pressure
RDS	respiratory distress syndrome
ROS	reactive oxygen species
TN-C	tenascin-C
TNF	tumor necrosis factor
TO	tracheal occlusion
VEGF	vascular endothelial growth factor
VILI	ventilator-induced lung injury
V _T	tidal volume