Oxidative stress in experimental bronchopulmonary dysplasia

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Chapter

1

Introduction

INTRODUCTION

Bronchopulmonary dysplasia (BPD) continues to be a significant cause of mortality and morbidity in the neonatal intensive care unit (NICU) and affects an estimated 500 very preterm infants (often with gestational ages <28 weeks and birth weights <1,000 g) in the Netherlands each year. BPD is a chronic lung disease typical for preterm infants born when lung development is far from complete. Soon after birth these infants develop respiratory problems (respiratory distress syndrome, RDS). They may need surfactant replacement to open up their alveoli and assisted ventilation with extra oxygen to guarantee optimal gas exchange during lung development. These acute respiratory problems affect both alveolarization and vascular development of the immature lung and ultimately lead to chronic lung disease, i.e. BPD. Until recently, preterm infants with BPD were weaned from the ventilator using glucocorticoids, such as dexamethasone. Glucocorticoids accelerate lung development, but their main drawback in pediatric pulmonology is their negative effect on alveolarization by inhibiting secondary septation, ultimately leading to a permanent simplification of the airspaces with enlarged saccular-like alveoli. This results in a permanent reduction of the gas-exchange surface area and lung function. In addition, the realization that early postnatal administration of glucocorticoids may affect neurodevelopment, has led to the advice by the American Academy of Pediatrics not to use postnatal glucocorticoids any longer. This advice has created a therapeutic vacuum in the treatment of preterm infants with BPD in the NICU. This thesis investigates the pathophysiology of hyperoxia-induced neonatal lung injury in the rat as a model for very premature infants suffering from RDS with a high risk of developing BPD and explores the therapeutic potential of the methylxanthine pentoxifylline and inhaled nitric oxide in the treatment and/or prevention of BPD.

PERINATAL LUNG DEVELOPMENT

For a better understanding of the pathogenesis of BPD knowledge of normal lung development and surfactant metabolism and function is necessary.

Organogenesis

Organogenesis of the lung can be divided into 5 distinct phases: embryonic, pseudoglandular, acinar or canalicular, saccular, and alveolar (reviewed in 15, 24). During the *embryonic phase* (26 to 52 days of gestation) an endodermal outgrowth derived from the primitive foregut divides and branches dichotomously to form the early tracheobronchial tree. In the *pseudoglandular phase* (52 days to 16 weeks of gestation) the primitive airway epithelium starts to differentiate and neuroendocrine, ciliated, and goblet cells appear, whereas cartilage and smooth muscle cells emerge from the mesenchyme. The airway branching pattern is completed in the *canalicular or acinar phase* (16 to 24-26 weeks of gestation) and the prospective gas-exchange region starts to develop: respiratory bronchioli emerge, interstitial tissue decreases, vascularization of peripheral mesenchyme increases, and distal cuboidal epithelium differentiates into alveolar type I and II cells signaling the start of surfactant production. Development of the distal pulmonary circulation by vasculogenesis with capillaries is present at 20 weeks. The *saccular phase* (24-26 to 36 weeks of gestation) is characterized by maturation of the surfactant system, growth of the pulmonary parenchyma and thinning of the connective tissue or interstitium. The capillary network comes in close contact with the developing airway epithelium in the primitive alveoli, enabling gas-exchange between blood and the environment in case of a premature birth from 24 weeks of gestation onward. The *alveolar phase* extends from 36 weeks of gestation to at least 18 postnatal months in which true alveoli, with increased acinar complexity and increased gas-exchange surface area, are formed. Alveoli are formed due to secondary septation from the present airspaces of smooth-walled transitory ducts and saccules with primitive thick septa. In addition, microvascular maturation takes place with fusion of the double capillary layer into a single medial layer facing both alveolar lumens of the septum. Thus, the process of alveolarization and terminal microvascular development begins in the late fetal period and proceeds after birth in humans.

Pulmonary surfactant

Surfactant synthesis (reviewed in 42, 100) starts in alveolar type II cells at 24-28 weeks of gestation. After secretion into the alveoli, surfactant lowers surface tension in the alveoli and distal bronchioli which promotes

lung expansion during inspiration and prevents alveolar collapse at expiration, both mandatory for gas-exchange and oxygenation. Surfactant deficiency in immature lungs triggers a cascade of alveolar instability and collapse, capillary leakage, and hyaline membrane formation leading to decreased gas-exchange, atelectasis, increase of the functional right-to-left shunt, pulmonary hypertension, respiratory acidosis and pulmonary edema with further inactivation of surfactant by plasma contents. Besides surface tension reduction, surfactant also plays a critical role in innate host defense and inflammation in the lung.

Pulmonary surfactant consists of about 90% lipids and 10% proteins and its composition is similar across species including man and rodents. Phospholipids make up 80-90% of the surfactant lipids, of which 70-80% accounts for phosphatidylcholine (PC). PC is the most important component to lower surface tension. Approximately 50 to 60% of PC is disaturated and largely consists of dipalmitoyl phosphatidylcholine (DPPC). Of the surfactant proteins two are large and hydrophilic (surfactant protein A [SP-A] and D [SP-D]), the other two are small and hydrophobic (SP-B and SP-C).

The functions of the surfactant proteins are summarized in Table 1. SP-A is the most abundant surfactant protein in the alveoli, and constitutes approximately 50% of all pulmonary surfactant proteins. Human SP-A, encoded by two distinct genes (SFTPA-1 and -2) and located on chromosome 10 (43), is a 26-36-kDA (monomer) hydrophilic collagen/lectin hybrid. The collagen-like domain interacts with phospholipids and the C-terminal domain resembles lectins, which play a role in host defense mechanisms. SP-A is expressed and synthesized by alveolar type II cells and to a lesser extent by non-ciliated Clara cells. SP-A has multiple functions, but knock-out studies have shown that SP-A is not pivotal for lung function, but critical for the recognition, binding, opsonization, and killing of various bacterial, viral, and fungal pathogens in the lung (36, 51). SP-D is a 42-kDA collagenous glycoprotein that is encoded by a single human SFTPD gene on chromosome 10. Like SP-A, SP-D is predominately synthesized by alveolar type II cells and non-ciliated Clara cells, but unlike SP-A, SP-D is not associated with surfactant lipids. SP-D deficient mice are susceptible to pulmonary infection, indicating the important role of SP-D in innate host defense (59, 60).

The small hydrophobic surfactant proteins, SP-B and SP-C, play a pivotal enhancing role in the adsorption of phospholipids at the air-liquid interface in the alveoli that is critical for maintaining the stability and morphological integrity of the alveolus. Together they account for only 1-2% of the surfactant weight. SP-B and SP-C both require specialized intracellular processing events to produce their mature forms. SP-B is a polypeptide with a molecular mass of 8.7 kDa, which is expressed in alveolar type II cells and Clara cells. Human SP-B is encoded by a single gene on chromosome 2 (SFTPB). SP-B is the only surfactant protein essential for life (69). Mutations in the SP-B gene result in lethal SP-B deficiency. SP-B is a member of the saposin-like family of peptides and is always associated with surfactant phospholipids. SP-B influences the processing and secretion of SP-C, because SP-B deficient mice also lack mature SP-C.

Surfactant deficiency is a common problem in very preterm infants and typically presents as RDS with nonspecific tachypnea, expiratory grunting, nasal flaring, cyanosis, and substernal and intercostal retractions. The previously used term hyaline membrane disease (HMD) is synonymous with RDS, and refers to the pathologic finding of membranes that stain like hyaline cartilage. These hyaline membranes consist of necrotic alveolar cells, plasma transudate, aspirated squamae, and fibrin, and line terminal bronchioles and alveolar ducts. Surfactant replacement therapy was introduced in the early 1990s and has greatly improved perinatal lung function and reduced mortality and morbidity of very preterm infants.

Pulmonary surfactant proteins		
SP-A	Hydrophilic	• Formation of tubular myelin • Regulation of phosholipid insertion into the surface film • Modulation and secretion and uptake of surfactant lipids by type II cells • Protection of surfactant against inactivation by plasma proteins • Protection against oxidative damage • Role in innate lung defense o Activation of alveolar macrophages o Binding and clearance of bacteria and viruses o Binding to LPS
SP-B	Hydrophobic	• Promotion of phospholipid insertion into the surface film • Formation of tubular myelin • Protection of surfactant against inactivation by plasma proteins
SP-C	Hydrophobic	• Promotion of phospholipid insertion into the surface film • Protection of surfactant against inactivation by plasma proteins
SP-D	Hydrophilic	• Role in innate lung defense o Activation of alveolar macrophages o Agglutination of bacteria o Protection against viruses o Binding to pneumocystis carinii

Table 1. Functions of the four surfactant proteins (Adapted and modified from reference 100).

BRONCHOPULMONARY DYSPLASIA

Clinical presentation

'Classic' BPD was first reported by Northway and colleagues in 1967 as a severe lung disease resulting from mechanical ventilation and oxygen exposure in preterm infants with RDS or HMD (70). BPD was defined as the presence of persistent respiratory symptoms and need for supplemental oxygen and an abnormal chest radiograph at 28 days after birth (70). Chest radiographs demonstrated a pattern of heterogeneous aeration of severe hyperexpansion with a combination of cystic emphysema, volume loss and fibrosis, the so called 'bubbly lungs' (85). Pathologic findings included necrotizing bronchiolitis, vascular smooth muscle hypertrophy and pulmonary hypertension, inflammation, pulmonary edema, and alveolar changes of overinflation and atelectasis with pulmonary fibrosis. Long-term follow-up studies showed that many BPD infants have recurrent (especially respiratory syncytial virus [RSV]) respiratory infections during infancy and reactive airways disease, upper airway obstruction, pulmonary hypertension and exercise intolerance in childhood and adolescence (57).

Due to pharmacological, nutritional and technical advances the survival of younger and smaller preterm infants with BPD has changed the clinical picture and definition of BPD over the past 40 years. The last decade the overall incidence of BPD is still the same, but the clinical course and pathology of BPD developing in infants after premature birth has changed after the introduction of surfactant therapy (16, 39, 40, 76). Of the original series published in 1967, surviving infants with BPD were born at 34-weeks gestation, weighed 2,200 g and the mortality was 67% (70). Today, 75% of the affected newborns weigh less than 1000 g at birth (39, 76). The risk of BPD rises with decreasing birthweight, with an incidence reported as high as 85% in neonates between 500-699 g versus 5% in neonates with birthweights over 1500 g (39, 76). Chronic oxygen dependency may even develop in premature newborns without severe RDS (39). Survival rates have dramatically increased from less than 10% to presently over 50% in extremely preterm infants of 24-26 weeks' gestation (39). Also the histology features of classic progressive fibroproliferation are now generally less striking. In 1999 the term 'New' BPD was introduced by Jobe (40). 'New' BPD is a milder chronic disease in very preterm infants treated with less or no ventilatory support and lower inspired oxygen concentrations during the first postnatal days than in the days of 'classic' BPD. Their lung disease is more uniform and lung injury is milder with less inflammation and fibrosis, but histological studies show severely disturbed alveolar and vascular growth. This new BPD is fundamentally based on an inhibition of acinar and vascular growth during a vulnerable stage of lung development, whereas classic BPD was attributed primarily to the combination of oxygen injury and mechanical ventilation in prematurity. The differences in histologic findings of 'classic' BPD and 'new' BPD are shown in Table 2 (Adapted and modified from references 21 and 46). The chest radiographs of infants with BPD now

often appear hazy or dense and progress to a relatively uniform pattern of fine coarse reticular interstitial opacities with more uniform and smaller cystic lucencies (1). As a result of the shift in clinical and radiographic pulmonary changes new diagnostic criteria of BPD were developed based on time of clinical assessment and clinical severity (Table 3) (39).

Classic BPD	New BPD
Alternating atelectasis and hyperinflation	Less regional heterogeneity of lung disease
Severe airway epithelial lesions (e.g. hyperplasia, squamous metaplasia)	Rare airway epithelial lesions
Marked airway smooth muscle hyperplasia	Mild airway smooth muscle thickening
Extensive, diffuse fibroproliferation	Rare fibroproliferative changes
Hypertensive remodelling of pulmonary arteries, including endothelial edema, medial thickening, elastin deposition in normally non-muscularized pulmonary arterioles, and consequent right ventricular hypertrophy	Fewer arteries but 'dysmorphic'; Less severe arterial/arteriolar vascular lesions
Decreased alveolarisation and surface area	Fewer, larger and simplified alveoli (alveolar hypoplasia, decreased acinar complexity)

Table 2. Differences in pathological features of 'classic' and 'new' BPD (Adapted and modified from references 21 and 46).

Gestational age	< 32 weeks	> 32 weeks
Time point of Assessment	36 weeks post-menstrual age or discharge*	>28 days but <56 days postnatal age or discharge*
Treatment with oxygen	>21% for at least 28 days	>21% for at least 28 days
Bronchopulmonary Dysplasia		
Mild	Breathing room air at 36 weeks post-menstrual age, or discharge*	Breathing room air by 56 days postnatal age, or discharge*
Moderate	Need for <30% O_2 at 36 weeks post-menstrual age, or discharge*	Need for <30% O_2 at 56 days postnatal age, or discharge*
Severe	Need for >30% O_2, with or without positive pressure ventilation or continuous positive pressure at 36 weeks post-menstrual age, or discharge*	Need for >30% O_2 with or without positive pressure ventilation or continuous positive pressure at 56 days postnatal age, or discharge*

Table 3. NIH diagnostic criteria for bronchopulmonary dysplasia. [*Whichever comes first] (Adapted from reference 39).

Experimental Bronchopulmonary dysplasia

Animal models are critical to further unravel the pathophysiology of BPD and to test potential treatment options for BPD. Hyperoxia exposure of premature baboons (20, 22), neonatal mice (13, 96), and rats (17, 33, 75) induces a progressive chronic lung disease (experimental BPD), that closely resembles BPD in premature newborns. Nowadays in the postsurfactant era, premature infants at greatest risk for BPD are born at 24-28 weeks gestation during the late canalicular or saccular stage of lung development. The unique advantage of a rodent animal model is that lung injury is also induced during the saccular stage of lung development. In 2001 the National Institue of Child Health and Human Development (NICHD), the National Hearth, Lung and Blood Institute (NHLBI) and the Office of Rare Diseases (ORD) BPD workshop proposed to develop experimental BPD animal models for better characterization of the pathophysiology and new treatment strategies for BPD (39).

Pathophysiology: general

The three key factors in the pathogenesis of BPD are lung immaturity, lung injury and inadequate repair. BPD represents the response of the lung to injury during the critical period of lung growth at the end of the canalicular and beginning of the saccular stage in which airspace septation and the vascular system are developing. After premature birth, multiple stimuli including oxidative stress, barotrauma, surfactant deficiency, inflammation, alveolar fibrin deposition, vascular maldevelopment, fluid management, patent ductus arteriosus (PDA), nutrition, and genetic background (39, 40), act at the susceptible lung in a critical stage of development. Mechanical injury and oxygen toxicity were the two main factors invoked in classic BPD, whereas new or current BPD is associated with immaturity, perinatal infection and inflammation, PDA and disrupted alveolar and capillary development. The (im)balance between initiating factors and host characteristics probably determines whether BPD will occur in a preterm infant.

Supplemental oxygen given to preterm infants with respiratory failure challenges them to oxidative stress. Oxygen treatment induces the production of cytotoxic reactive oxygen species (ROS), that regulate signal transduction pathways and transcription factors, and aggravate inflammation. Preterm infants are highly susceptible to oxidative stress, because the antioxidant defense system is underdeveloped. Higher oxygen levels are related to worsening of BPD in humans. Resuscitation of depressed infants with 21% or 100% oxygen results in a decreased defense of the anti-oxidant glutathione and increased mortality after brief exposure to 100% oxygen (77, 93).

Mechanical ventilation leads to barotrauma and volutrauma in the preterm lung. Barotrauma and volutrauma overstretch or overdistend the underdeveloped lung parenchyma and lead to tissue disrupture that induces

a reparative mechanism including cellular influx, inflammation, elastosis, and distorted acinar and vascular growth (87). Excessive and disordered elastin is a key feature in the fibroproliferative changes in the histology of BPD. Increased elastin has been found in infants who died of BPD (87). Mechanical ventilation in newborn mice with room air results in expression of elastin-related genes. Moreover, mechanical ventilation with 40% oxygen not only leads to an upregulation of genes related to elastin, but also to reductions in lung abundance of proteins that affect the formation of alveoli and lung capillaries (12). Pulmonary complications from the treatment of RDS with ventilatory and oxygen support include pulmonary air leaks (pulmonary interstitial emphysema, pneumo-mediastinum, and pneumothorax), pulmonary edema, and concurrent pulmonary infection. Pulmonary complications due to mechanical ventilation and oxygen supplementation will lead to prolonged ventilatory and oxygen support, further increasing the risk of developing BPD. Chronic inflammation and edema as a complication of barotrauma also suppresses surfactant function. As surfactant reduces surface tension and minimizes alveolar collapse, surfactant deficiency or inactivation requires even more aggressive ventilation leading to more lung tissue damage.

Lung inflammation is defined by an increased amount of inflammatory cells in the airspaces and lung tissue producing pro-inflammatory mediators. The inflammatory response can be triggered by infectious factors and a number of non-infectious factors, including oxygen, free radicals, positive-pressure ventilation, ventilation with an excessive tidal volume and increased pulmonary blood flow caused by a PDA. Both intra-uterine (chorioamnionitis or antenatal infection) and extra-uterine inflammation (nosocomial infection) contribute to the development and severity of BPD. Chorioamnionitis, a common clinical problem associated with preterm delivery, or antenatal exposure to pro-inflammatory cytokines may prime the fetal developing lung for minimal postnatal injury, resulting in abnormal alveolarization and pulmonary vascular development (83).

An arrest in both the formation of the alveolar and vascular system of the lung is the key characteric of BPD. The abnormal growth of the developing pulmonary microcirculation results in elevation of the pulmonary artery pressure. In normal lung morphogenesis development of the distal epithelial and capillary networks are very closely related. Inhibition of vascular development in fetal mouse lung explants resulted in abrogation of epithelial branching morphogenesis (92). Treatment with three different anti-angiogenic agents, including fumagillin and thalidomide, attenuated both vascular growth and alveolarization in the lungs of newborn rats (38). These data suggest a close interaction between epithelial and endothelial cells in the developing lung. Lung epithelium probably stimulates capillary morphogenesis through elaboration of angiogenic factors, and the vascular system likely has a regulatory role on the epithelium. A major factor in lung angiogenesis is vascular endothelial growth factor (VEGF), and its two receptors Flk-1 (also known as VEGF recepter-2) and Flt-1 (also known as VEGF receptor-1). VEGF is produced by both alveolar type 2 and bronchiolar cells, and stimulates vascularization at the leading edge of branching

airways. Lung VEGF expression is reduced in hyperoxia induced BPD in animal models (62, 63). Recombinant human VEGF treatment improves alveolarization and vascular growth in hyperoxia-exposed newborn rats (55). Transgenic mice overexpressing IL-13 stimulate pulmonary VEGF expression and improved survival in hyperoxia (25). In conclusion, vascular maldevelopment results in pulmonary hypertension and contributes to the development of BPD. Primary injury to either the airspace or lung circulation may have profound secondary effects on the other.

Pulmonary edema is an important factor in preterm infants with BPD. Interstitial and intra-alveolar protein rich pulmonary edema is the result of increased permeability of the alveolar-capillary membrane in the preterm lung. Pulmonary edema is mainly due to immaturity and probably aggravated by barotrauma (41), activated plasma proteins, including fibrin, and inflammatory cells (14). The plasma proteins in intra-alveolar edema contribute to the formation of hyaline membranes. As mentioned previously, pulmonary edema inactivates surfactant. Pulmonary edema may also be the result of a PDA or excessive fluid administration, which both negatively influence pulmonary function, and are associated with BPD (72). PDA induces systemic-to-pulmonary shunting, thereby increasing the pulmonary circulation leading to pulmonary edema and endothelial injury (7). Increased inflammatory parameters have been measured in preterm infants with a PDA (30). The incidence of BPD decreased after the implementation of indomethacin therapy for ductal closure (19).

Inadequate nutrition and certain genetic factors also predispose to development of BPD. Adequate nutrition is essential for normal lung development and repair. Nutritional deficits in animal models lead to impaired alveolarization and thickened septa for age (64). Variants of polymorphisms for surfactant proteins are related to BPD development (31).

Pathophysiology: inflammation and coagulation

The inflammatory response and an imbalance of the coagulation and fibrinolytic cascades, leading to pulmonary fibrin deposition, play pivotal roles in the pathophysiology of BPD. The contribution of inflammation and coagulation seems to be of crucial importance in the arrest in alveolarization and vascular development. Inflammation and coagulation are closely related processes, but the exact mechanism of their relationship in BPD is not clear.

Activation of the inflammatory response has been detected in both preterm infants and animal models with (experimental) BPD. Persistence of neutrophils in bronchoalveolar lavage fluid (BALF) correlated with the development of BPD (3, 71). Increased levels of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β and mediators reflecting neutrophil recruitment and activation, including soluble intercellular adhesion molecule (s-ICAM), chemokines IL-8 and MCP-1, and neutrophil elastase have been observed in tracheal aspirates of infants developing BPD (52-54). Moreover, in premature baboons (22), mice (96) and rats (10) with experimental BPD pro-inflammatory cytokines and inflammatory cells are elevated in BALF or

lung tissue. Antichemokine treatment with anti-CINC-1 (4, 27), anti-MCP-1 (94) and anti-MIP-2 (27) attenuates neutrophil and/or alveolar macrophage accumulation in BALF and preserves alveolar development of hyperoxia-exposed newborn rats. These studies indicate the impact of activation of the inflammatory response on alveolar enlargement, one of the key findings of BPD.

Pro-inflammatory cytokines create a procoagulant and antifibrinolytic state that may lead to fibrin deposition in the airspaces and microvasculature of the lungs. IL-6 is an important intermediate factor in coagulation activation in endotoxemia (91) and anti-IL-6 infusion in low grade endotoxemia in chimpanzees results in attenuation of coagulation. The procoagulant state caused by pro-inflammatory cytokines is frequently accompanied by inhibition of the fibrinolytic system or natural anticoagulants. Fibrinolysis decreases after TNF-α infusion (89, 90). During inflammation the fibrinolytic system is stimulated by increasing levels of plasminogen activators, which are released from the endothelium. TNF-α and IL-1 are able to reduce the levels of tissue-type plasminogen activators. Moreover, both pro-inflammatory cytokines have an additional antifibrinolytic effect by increasing PAI-1 release (11, 78). The natural anticoagulant APC system is impaired by the pro-inflammatory cytokines TNF-α and IL-1β through downregulation of the key mediator thrombomodulin on the endothelial cell surface (67, 68).

The inflammatory response can activate the coagulation cascade, and, in turn, coagulation may influence inflammation (Figure 1). Tissue Factor (TF), a transmembrane bound protein, is the physiologic initiator of the extrinsic coagulation pathway with fibrin deposition as ultimate result. Fibrin is degraded into fibrin degradation products after the conversion of plasminogen into plasmin by plasminogen activators, i.e. tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA) bound to its receptor uPAR. The generation of plasmin is inhibited by plasminogen activator inhibitors (PAI-1,-2 and -3), of which PAI-1 is believed to be the most powerful regulator. Fibrinolysis is also regulated by two other antifibrinolytic factors, α2-antiplasmin and thrombin-activatable fibrinolysis inhibitor (TAFI). α2-antiplasmin inactivates free plasmin not bound to the fibrin network, whereas TAFI inhibits the binding of plasmin on the fibrin network by removing the carboxyterminal lysine and arginine residues.

Excess procoagulant and decreased fibrinolytic activity in the lung lead to fibrin deposition in alveoli, interstitium and capillaries (8, 34, 35). In PAI-1 deficient mice exposed to hyperoxia fibrin deposition is decreased, leading to a less severe phenotype and increased survival (8). In baboons with sepsis-induced acute lung injury TF blockage reduces intra-alveolar fibrin deposition and early collagen formation (98). Fibrin is not only the result of coagulation activation initiated by TF, but also has profibrotic (34, 35) and pro-inflammatory properties via activation of NF-κB and AP-1 (82). In addition, fibrin deposition can hamper gas-exchange by inactivating lung surfactant (80) and thus favoring alveolar collapse. Therefore, intra-alveolar fibrin deposition may function as a key marker for the severity of BPD with respect to coagulation activation and impaired fibrinolysis.

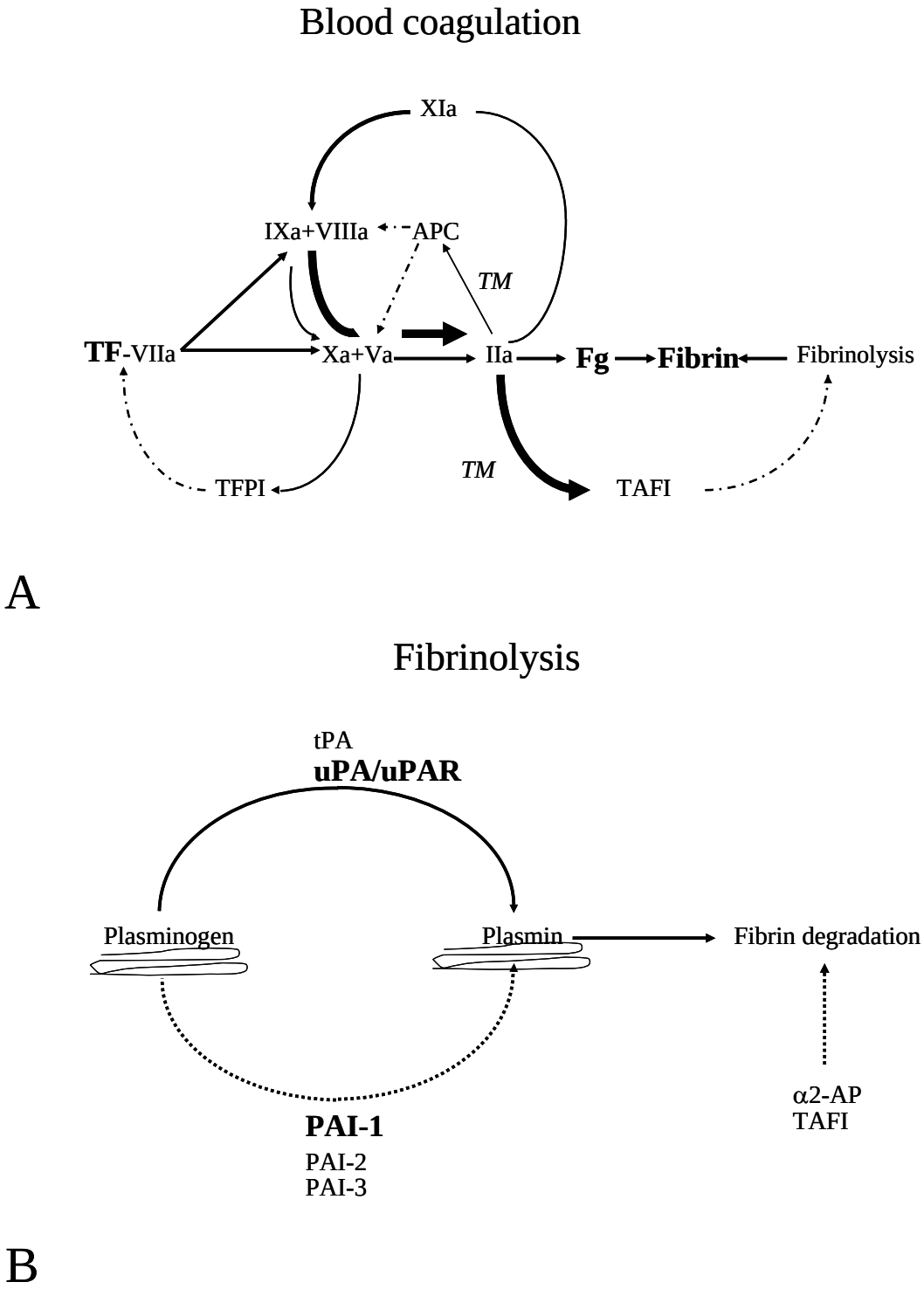

Figure 1. Schematic representation of the coagulation and fibrinolytic cascades. (*A*) Taken from Wagenaar et al., Free Radic Biol Med; 2004. Tissue damage results in the local expression of the physiological activator of the coagulation cascade tissue factor (TF). TF binds to factor VII/VIIa. (activated factor VII). This complex activates factors IX and X. Factor Xa activates prothrombin (factor II), resulting in thrombin (factor IIa) generation. Also, generation of factor Xa results in an inhibition of the extrinsic pathway by tissue factor pathway inhibitor (TFPI). At low concentration thrombin acts as an anticoagulant. After binding to its cofactor thrombomodulin (TM), thrombin activates protein C. Activated protein C (APC) inhibits the coagulation cascade by inactivation of factors VIIIa and Va, which act as cofactors of factors IXa and Xa, respectively. High concentrations of thrombin are

procoagulant. It results in even higher thrombin concentrations via the factor XIa feedback loop. Proteolytic cleavage of fibrinogen (Fg) results in fibrin formation. Hyperoxia results in a local upregulation of TF and fibrinogen expression and a downregulation of TM expression, resulting in a procoagulant environment. High concentrations of thrombin are antifibrinolytic via the activation of thrombin-activatable fibrinolysis inhibitor (TAFI), bound to its cofactor TM. (*B*) Fibrinolysis is the process by which fibrin degradation takes place. Fibrin is degraded by plasmin after proteolytic cleavage of plasminogen by plasminogen activators, i.e., tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA) bound to its receptor uPAR. Plasmin formation is regulated by plasminogen activator inhibitors (PAI-1, -2, and –3) of which PAI-1 is believed to be the most important PAI in fibrinolysis. Plasmin bound to the fibrin network is protected from inactivation by α2-antiplasmin. Binding of plasmin to the fibrin network is prevented by TAFI, which removes the carboxyterminal lysines from the fibrin network that serve as binding sites for plasmin. Hyperoxia results in a moderate upregulation of the profibrinolytic factor uPA and its receptor uPAR, but not of tPA expression, and a tremendous upregulation of the inhibitor of fibrinolysis PAI-1. This will probably result in an antifibrinolytic environment. Hyperoxia results in a local procoagulant and antifibrinolytic environment, ultimately resulting in fibrin deposition in the developing neonatal lung. Solid lines indicate activation and dotted lines indicate inhibition. Factors in bold are upregulated and factors in italic are downregulated in our experiments.

INTERVENTION

The importance of extravascular fibrin deposition through an imbalance in the interrelated processes of activation of the inflammatory response and coagulation and/or fibrinolytic cascades in BPD suggests a potential role for compounds with both anti-inflammatory and anticoagulant properties.

Pentoxifylline

The methylxantine derivative pentoxifylline (PTX) is a nonspecific phosphodiesterase inhibitor and acts as an immunomodulation agent. PTX has been used clinically in the treatment of peripheral arterial disease because it enhances the deformability of red blood cells and thereby improves the microcirculatory blood flow (73, 95). PTX increases intracellular cAMP levels and decreases the TNF-α production with beneficial effects on the inflammatory response. PTX attenuates neutrophil sequestration, prevents pulmonary vascular permeability to protein to the alveolar lumen (97), and inhibits the production of free oxygen radicals (88). The anti-inflammatory and anticoagulant properties of PTX have been shown in baboons suffering from lipopolysaccharide(LPS)-induced endotoxemia (58). In severe acute alcoholic hepatitis PTX improves the short-term survival (2). Positive effects of PTX have also already been reported in septic preterm infants, in whom administration of PTX reduces the treatment requirements after the first month of life (56). In a recent LPS-induced endotoxemia rat model, PTX administration resulted in a significant decreased production of IL-8, MMP-2 and MMP-9 in combination with a reduction of NF-κB and ICAM-1 activity in lung tissue leading to less severe lung injury (23).

Inhaled nitric oxide

A novel therapeutic strategy for infants with respiratory failure is administration of inhaled nitric oxide (iNO). NO is an important mediator of biological processes in the pulmonary epithelium, such as neurotransmission, pulmonary vasodilatation, smooth muscle contraction, inflammatory mechanisms, ciliary motility, mucin secretion and plasma exudation (9, 29, 37, 74). It exerts its biological effects via the activation of guanylate cyclase resulting in the production of cyclic 5'-guanose monophosphate (cGMP) (66). Endogenous NO is synthesized from arginine and oxygen by three NO synthases (neuronal [nNOS], inducible [iNOS] and endothelial [eNOS]), that have been identified in the human and animal lung (29, 49, 81). NO is able to attenuate the procoagulant activity induced by acute lung inflammation in rats (44) and plays an important role in the regulation of the pulmonary vascular tone and lung liquid production (26). Moreover, NO seems to promote the formation of alveoli and branching morphogenesis in the developing lung (5, 61, 99). In animals with chronic lung disease, NO also reduces abnormal elastin deposition (65, 87), decreases lung neutrophil accumulation (47), and has a positive effect on early pulmonary function (47, 65). In the NICU iNO is used as a vasodilatator to alleviate persistent pulmonary hypertension of the newborn, a complication in full-term infants with respiratory failure. These data indicate that iNO treatment of preterm infants with respiratory distress syndrome may be beneficial to improve oxygenation and reduce the survival rate and/or development of BPD.

Several clinical trials do support this hypothesis. Unfortunately, the results are controversial. After treatment with early low-dose iNO many clinical studies reported an improvement in oxygenation (32, 48, 50, 84), and a reduction of the need for extracorporeal membrane oxygenation (18) and a shorter stay at the NICU (86). The study of Schreiber et al. is the only clinical trial showing both a decrease in the incidence of neonatal chronic lung disease and death (79). Therefore, Field advised in 2005 that near-term and term infants with respiratory failure should enter a trial with iNO, but that preterm infants should be treated conventionally (28). Recently, two large multicenter, randomized and placebo-controlled iNO trials have been conducted in preterm infants (6, 45). In the study of Kinsella et al. preterm infants were treated with 5 particles per minute (ppm) iNO after birth for 21 days. The incidence of BPD or death was not reduced, but iNO did diminish the risk of brain injury (45). Ballard et al. treated preterm infants between day 7 and 21 of age with decreasing iNO concentrations beginning at 20 ppm, resulting in a better pulmonary outcome (6).

AIMS AND OUTLINE OF THIS THESIS

Due to a lack of patient materials and ethical reasons animal models of BPD are critical for characterization the pathophysiology of BPD and testing of potential treatment options. In chapter 2 of this thesis we characterize a rat model for experimental BPD, induced in neonatal pups by prolonged exposure to hyperoxia, by investigating histopathology and differential gene expression profiles in the lung and demonstrate its significance for studying BPD in premature infants. In chapter 3 we describe the spatial and temporal expression of surfactant proteins in this experimental BPD model.

Since inflammation and unbalanced coagulation and fibrinolysis, leading to extravascular fibrin deposition in the lung, are two interrelated processes that play a pivotal role in the pathophysiology of inflammatory lung disease, we investigated whether the pathophysiology of experimental BPD could be improved by interrupting the vicious cycle of inflammation and coagulation. Fibrin deposition can be prevented directly via inhibition of the coagulation cascade and/or stimulation of the fibrinolytic cascade or indirectly via inhibition of the inflammatory response, thereby preventing activated leucocytes to perform their procoagulant and antifibrinolytic activity. In chapters 4 and 5 intervention studies in experimental BPD are described which study the potential therapeutic effect of agents with anti-inflammatory and/or anticoagulant activity for premature infants who are at risk of developing BPD. The role of pentoxifylline, a methylxantine derivative and weak non-selective phosphodiesterase inhibitor with anti-inflammatory and anticoagulant properties, and with positive effects on capillary blood flow in experimental BPD is presented in chapter 4. The role of nitric oxide, a gas that is involved in multiple (patho)physiological processes in the injured lung, including pulmonary vasodilatation, inflammation and plasma exudation, is presented in chapter 5. In chapter 6 the presented studies of chapters 2-5 and the future perspectives are discussed. In chapter 7 a summary is given of this thesis.

REFERENCES

1. **Agrons GA, Courtney SE, Stocker JT and Markowitz RI.** From the archives of the AFIP: Lung disease in premature neonates: radiologic-pathologic correlation. *Radiographics* 25: 1047-1073, 2005.
2. **Akriviadis E, Botla R, Briggs W, Han S, Reynolds T and Shakil O.** Pentoxifylline improves short-term survival in severe acute alcoholic hepatitis: A double-blind, placebo-controlled trial. *Gastroenterology* 119: 1639-1648, 2000.
3. **Arnon S, Grigg J and Silverman M.** Pulmonary inflammatory cells in ventilated preterm infants: effect of surfactant treatment. *Arch Dis Child* 69: 44-48, 1993.
4. **Auten RL Jr, Mason SN, Tanaka DT, Welty-Wolf K and Whorton MH.** Anti-neutrophil chemokine preserves alveolar development in hyperoxia-exposed newborn rats. *Am J Physiol Lung Cell Mol Physiol* 281: L336-L344, 2001.
5. **Balasubramaniam V, Tang JR, Maxey AM, Plopper CG and Abman SH.** Mild hypoxia impairs alveolarization in the endothelial nitric oxide synthase-deficient mouse. *Am J Physiol Lung Cell Mol Physiol* 284: L964-L971, 2003.
6. **Ballard RA, Truog WE, Cnaan A, Martin RJ, Ballard PL, Merrill JD, Walsh MC, Durand DJ, Mayock DE, Eichenwald EC, Null DR, Hudak ML, Puri AR, Golombek SG, Courtney SE, Stewart DL, Welty SE, Phibbs RH, Hibbs AM, Luan X, Wadlinger SR, Asselin JM and Coburn CE.** Inhaled nitric oxide in preterm infants undergoing mechanical ventilation. *N Engl J Med* 355: 354-364, 2006.
7. **Bancalari E, Claure N and Gonzalez A.** Patent ductus arteriosus and respiratory outcome in premature infants. *Biol Neonate* 88: 192-201, 2005.
8. **Barazzone C, Belin D, Piguet PF, Vassalli JD and Sappino AP.** Plasminogen activator inhibitor-1 in acute hyperoxic mouse lung injury. *J Clin Invest* 98: 2666-2673, 1996.
9. **Barnes PJ.** Nitric oxide and airway disease. *Ann Med* 27: 389-393, 1995.
10. **Ben Ari J, Makhoul IR, Dorio RJ, Buckley S, Warburton D and Walker SM.** Cytokine response during hyperoxia: sequential production of pulmonary tumor necrosis factor and interleukin-6 in neonatal rats. *Isr Med Assoc J* 2: 365-369, 2000.
11. **Biemond BJ, Levi M, ten Cate H, van der Poll T, Buller HR, Hack CE and ten Cate JW.** Plasminogen activator and plasminogen activator inhibitor I release during experimental endotoxaemia in chimpanzees: effect of interventions in the cytokine and coagulation cascades. *Clin Sci (Lond)* 88: 587-594, 1995.
12. **Bland RD.** Neonatal chronic lung disease in the post-surfactant era. *Biol Neonate* 88: 181-191, 2005.
13. **Bonikos DS, Bensch KG, Ludwin SK and Northway WH Jr.** Oxygen toxicity in the newborn. The effect of prolonged 100 per cent O2 exposure on the lungs of newborn mice. *Lab Invest* 32: 619-635, 1975.
14. **Brus F, van Oeveren W, Heikamp A, Okken A and Oetomo SB.** Leakage of protein into lungs of preterm ventilated rabbits is correlated with activation of clotting, complement, and polymorphonuclear leucocytes in plasma. *Pediatr Res* 39: 959-965, 1996.
15. **Burri PH.** Structural aspects of prenatal and postnatal development and growth of the lung. *New York: Marcel Dekker* 1-35, 1997.
16. **Charafeddine L, D'Angio CT and Phelps DL.** Atypical chronic lung disease patterns in neonates. *Pediatrics* 103: 759-765, 1999.
17. **Chen Y, Martinez MA and Frank L.** Prenatal dexamethasone administration to premature rats exposed to prolonged hyperoxia: a new rat model of pulmonary fibrosis (bronchopulmonary dysplasia). *J Pediatr* 130: 409-416, 1997.
18. **Clark RH, Kueser TJ, Walker MW, Southgate MW, Huckaby JL, Perez JA, Roy BJ, Keszler M and Kinsella JP.** Low-dose nitric oxide therapy for persistent pulmonary hypertension of the newborn. Clinical Inhaled Nitric Oxide Research Group. *N Engl J Med* 342: 469-474, 2000.
19. **Clyman RI.** Recommendations for the postnatal use of indomethacin: an analysis of four separate treatment strategies. *J Pediatr* 128: 601-607, 1996.

20. **Coalson JJ.** Experimental models of bronchopulmonary dysplasia. *Biol Neonate* 71 Suppl 1: 35-38, 1997.
21. **Coalson JJ.** Pathology of new bronchopulmonary dysplasia. *Semin Neonatol* 8: 73-81, 2003.
22. **Coalson JJ, Winter VT, Siler-Khodr T and Yoder BA.** Neonatal chronic lung disease in extremely immature baboons. *Am J Respir Crit Care Med* 160: 1333-1346, 1999.
23. **Coimbra R, Melbostad H, Loomis W, Porcides RD, Wolf P, Tobar M and Hoyt DB.** LPS-induced acute lung injury is attenuated by phosphodiesterase inhibition: effects on proinflammatory mediators, metalloproteinases, NF-kappaB, and ICAM-1 expression. *J Trauma* 60: 115-125, 2006.
24. **Copland I and Post M.** Lung development and fetal lung growth. *Paediatr Respir Rev* 5 Suppl A: S259-S264, 2004.
25. **Corne J, Chupp G, Lee CG, Homer RJ, Zhu Z, Chen Q, Ma B, Du Y, Roux F, McArdle J, Waxman AB and Elias JA.** IL-13 stimulates vascular endothelial cell growth factor and protects against hyperoxic acute lung injury. *J Clin Invest* 106: 783-791, 2000.
26. **Cummings JJ.** Nitric oxide decreases lung liquid production in fetal lambs. *J Appl Physiol* 83: 1538-1544, 1997.
27. **Deng H, Mason SN and Auten RL Jr.** Lung inflammation in hyperoxia can be prevented by antichemokine treatment in newborn rats. *Am J Respir Crit Care Med* 162: 2316-2323, 2000.
28. **Field DJ** Nitric oxide-still no consensus. *Early Hum Dev* 81: 1-4, 2005.
29. **Gaston B, Drazen JM, Loscalzo J and Stamler JS.** The biology of nitrogen oxides in the airways. *Am J Respir Crit Care Med* 149: 538-551, 1994.
30. **Gonzalez A, Sosenko IR, Chandar J, Hummler H, Claure N and Bancalari E.** Influence of infection on patent ductus arteriosus and chronic lung disease in premature infants weighing 1000 grams or less. *J Pediatr* 128: 470-478, 1996.
31. **Hallman M and Haataja R.** Genetic influences and neonatal lung disease. *Semin Neonatol* 8: 19-27, 2003.
32. **Hamon I, Fresson J, Nicolas MB, Buchweiller MC, Franck P and Hascoet JM.** Early inhaled nitric oxide improves oxidative balance in very preterm infants. *Pediatr Res* 57: 637-643, 2005.
33. **Han RN, Buch S, Tseu I, Young J, Christie NA, Frndova H, Lye SJ, Post M and Tanswell AK.** Changes in structure, mechanics, and insulin-like growth factor-related gene expression in the lungs of newborn rats exposed to air or 60% oxygen. *Pediatr Res* 39: 921-929, 1996.
34. **Idell S, James KK, Levin EG, Schwartz BS, Manchanda N, Maunder RJ, Martin TR, McLarty J and Fair DS.** Local abnormalities in coagulation and fibrinolytic pathways predispose to alveolar fibrin deposition in the adult respiratory distress syndrome. *J Clin Invest* 84: 695-705, 1989.
35. **Idell S, Koenig KB, Fair DS, Martin TR, McLarty J and Maunder RJ.** Serial abnormalities of fibrin turnover in evolving adult respiratory distress syndrome. *Am J Physiol* 261: L240-L248, 1991.
36. **Ikegami M, Korfhagen TR, Whitsett JA, Bruno MD, Wert SE, Wada K and Jobe AH.** Characteristics of surfactant from SP-A-deficient mice. *Am J Physiol* 275: L247-L254, 1998.
37. **Jain B, Rubinstein I, Robbins RA, Leise KL and Sisson JH.** Modulation of airway epithelial cell ciliary beat frequency by nitric oxide. *Biochem Biophys Res Commun* 191: 83-88, 1993.
38. **Jakkula M, Le Cras TD, Gebb S, Hirth KP, Tuder RM, Voelkel NF and Abman SH.** Inhibition of angiogenesis decreases alveolarization in the developing rat lung. *Am J Physiol Lung Cell Mol Physiol* 279: L600-L607, 2000.
39. **Jobe AH and Bancalari E.** Bronchopulmonary dysplasia. *Am J Respir Crit Care Med* 163: 1723-1729, 2001.
40. **Jobe AJ.** The new BPD: an arrest of lung development. *Pediatr Res* 46: 641-643, 1999.
41. **Jobe A, Ikegami M, Jacobs H, Jones S and Conaway D.** Permeability of premature

lamb lungs to protein and the effect of surfactant on that permeability. *J Appl Physiol* 55: 169-176, 1983.
42. **Johansson J, Curstedt T and Robertson B.** The proteins of the surfactant system. *Eur Respir J* 7: 372-391, 1994.
43. **Katyal SL, Singh G and Locker J.** Characterization of a second human pulmonary surfactant-associated protein SP-A gene. *Am J Respir Cell Mol Biol* 6: 446-452, 1992.
44. **Kermarrec N, Zunic P, Beloucif S, Benessiano J, Drouet L and Payen D.** Impact of inhaled nitric oxide on platelet aggregation and fibrinolysis in rats with endotoxic lung injury. Role of cyclic guanosine 5'-monophosphate. *Am J Respir Crit Care Med* 158: 833-839, 1998.
45. **Kinsella JP, Cutter GR, Walsh WF, Gerstmann DR, Bose CL, Hart C, Sekar KC, Auten RL, Bhutani VK, Gerdes JS, George TN, Southgate WM, Carriedo H, Couser RJ, Mammel MC, Hall DC, Pappagallo M, Sardesai S, Strain JD, Baier M, and Abman SH.** Early inhaled nitric oxide therapy in premature newborns with respiratory failure. *N Engl J Med* 355: 354-364, 2006.
46. **Kinsella JP, Greenough A and Abman SH.** Bronchopulmonary dysplasia. *Lancet* 367: 1421-1431, 2006.
47. **Kinsella JP, Parker TA, Galan H, Sheridan BC, Halbower AC and Abman SH.** Effects of inhaled nitric oxide on pulmonary edema and lung neutrophil accumulation in severe experimental hyaline membrane disease. *Pediatr Res* 41: 457-463, 1997.
48. **Kinsella JP, Walsh WF, Bose CL, Gerstmann DR, Labella JJ, Sardesai S, Walsh-Sukys MC, McCaffrey MJ, Cornfield DN, Bhutani VK, Cutter GR, Baier M and Abman SH.** Inhaled nitric oxide in premature neonates with severe hypoxaemic respiratory failure: a randomised controlled trial. *Lancet* 354: 1061-1065, 1999.
49. **Kobzik L, Bredt DS, Lowenstein CJ, Drazen J, Gaston B, Sugarbaker D and Stamler JS.** Nitric oxide synthase in human and rat lung: immunocytochemical and histochemical localization. *Am J Respir Cell Mol Biol* 9: 371-377, 1993.
50. **Konduri GG, Solimano A, Sokol GM, Singer J, Ehrenkranz RA, Singhal N, Wright LL, van Meurs K, Stork E, Kirpalani H and Peliowski A.** A randomized trial of early versus standard inhaled nitric oxide therapy in term and near-term newborn infants with hypoxic respiratory failure. *Pediatrics* 113: 559-564, 2004.
51. **Korfhagen TR, Bruno MD, Ross GF, Huelsman KM, Ikegami M, Jobe AH, Wert SE, Stripp BR, Morris RE, Glasser SW, Bachurski CJ, Iwamoto HS and Whitsett JA.** Altered surfactant function and structure in SP-A gene targeted mice. *Proc Natl Acad Sci USA* 93: 9594-9599, 1996.
52. **Kotecha S.** Cytokines in chronic lung disease of prematurity. *Eur J Pediatr* 155 Suppl 2: S14-S17, 1996.
53. **Kotecha S, Chan B, Azam N, Silverman M and Shaw RJ.** Increase in interleukin-8 and soluble intercellular adhesion molecule-1 in bronchoalveolar lavage fluid from premature infants who develop chronic lung disease. *Arch Dis Child Fetal Neonatal Ed* 72: F90-F96, 1995.
54. **Kotecha S, Wilson L, Wangoo A, Silverman M and Shaw RJ.** Increase in interleukin (IL)-1 beta and IL-6 in bronchoalveolar lavage fluid obtained from infants with chronic lung disease of prematurity. *Pediatr Res* 40: 250-256, 1996.
55. **Kunig AM, Balasubramaniam V, Markam NE, Morgan D, Montgomery G, Grover TR and Abham SH.** Recombinant human VEGF treatment enhances alveolarization after hyperoxic lung injury in neonatal rats. *Am J Physiol Lung Cell Physiol* 289: L529-L535, 2005.
56. **Lauterbach R, Pawlik D, Kowalczyk D, Ksycinski W, Helwich E and Zembala M.** Effect of the immunomodulating agent, pentoxifylline, in the treatment of sepsis in prematurely delivered infants: a placebo-controlled, double-blind trial. *Crit Care Med* 27: 807-814, 1999.
57. **Lemons JA, Bauer CR, Oh W, Korones SB, Papile LA, Stoll BJ, Verter J, Temprosa M, Wright LL, Ehrenkranz RA, Fanaroff AA, Stark A, Carlo W, Tyson JE, Donovan EF, Shankaran S and Stevenson DK.** Very low birth weight outcomes of the National Institute of Child health and human development neonatal research network, January 1995 through December 1996. NICHD Neonatal Research Network.

Pediatrics 107: E1, 2001.
58. **Levi M, ten Cate H, Bauer KA, van der Poll T, Edgington TS, Buller HR, van Deventer SJ, Hack CE, ten Cate JW and Rosenberg RD.** Inhibition of endotoxin-induced activation of coagulation and fibrinolysis by pentoxifylline or by a monoclonal anti-tissue factor antibody in chimpanzees. *J Clin Invest* 93: 114-120, 1994.
59. **LeVine AM and Whitsett JA.** Pulmonary collectins and innate host defense of the lung. *Microbes Infect* 3: 161-166, 2001.
60. **LeVine AM, Whitsett JA, Gwozdz JA, Richardson TR, Fisher JH, Burhans MS and Korfhagen TR.** Distinct effects of surfactant protein A or D deficiency during bacterial infection on the lung. *J Immunol* 165: 3934-3940, 2000.
61. **Lin YJ, Markham NE, Balasubramaniam V, Tang JR, Maxey A, Kinsella JP and Abman SH.** Inhaled Nitric Oxide Enhances Distal Lung Growth after Exposure to Hyperoxia in Neonatal Rats. *Pediatr Res* 58: 22-29, 2005.
62. **Maniscalco WM, Watkins RH, D'Angio CT and Ryan RM.** Hyperoxic injury decreases alveolar epithelial cell expression of vascular endothelial growth factor (VEGF) in neonatal rabbit lung. *Am J Respir Cell Mol Biol* 16: 557-567, 1997.
63. **Maniscalco WM, Watkins RH, Pryhuber GS, Bhatt A, Shea C and Huyck H.** Angiogenic factors and alveolar vasculature: development and alterations by injury in very premature baboons. *Am J Physiol Lung Cell Mol Physiol* 282: L811-L823, 2002.
64. **Maritz GS, Cock ML, Louey S, Joyce BJ, Albuquerque CA and Harding R.** Effects of fetal growth restriction on lung development before and after birth: a morphometric analysis. *Pediatr Pulmonol* 32: 201-210, 2001.
65. **McCurnin DC, Pierce RA, Chang LY, Gibson LL, Osborne-Lawrence S, Yoder BA, Kerecman JD, Albertine KH, Winter VT, Coalson JJ, Crapo JD, Grubb PH and Shaul PW.** Inhaled NO improves early pulmonary function and modifies lung growth and elastin deposition in a baboon model of neonatal chronic lung disease. *Am J Physiol Lung Cell Mol Physiol* 288: L450-L459, 2005.
66. **Moncada S and Higgs A.** The L-arginine-nitric oxide pathway. *N Engl J Med* 329: 2002-2012, 1993.
67. **Nawroth PP, Handley DA, Esmon CT and Stern DM.** Interleukin 1 induces endothelial cell procoagulant while suppressing cell-surface anticoagulant activity. *Proc Natl Acad Sci USA* 83: 3460-3464, 1986.
68. **Nawroth PP and Stern DM.** Modulation of endothelial cell hemostatic properties by tumor necrosis factor. *J Exp Med* 163: 740-745, 1986.
69. **Nogee LM, Garnier G, Dietz HC, Singer L, Murphy AM, deMello DE and Colten HR.** A mutation in the surfactant protein B gene responsible for fatal neonatal respiratory disease in multiple kindreds. *J Clin Invest* 93: 1860-1863, 1994.
70. **Northway WH Jr., Rosan RC and Porter DY.** Pulmonary disease following respirator therapy of hyaline-membrane disease. Bronchopulmonary dysplasia. *N Engl J Med* 276: 357-368, 1967.
71. **Ogden BE, Murphy SA, Saunders GC, Pathak D and Johnson JD.** Neonatal lung neutrophils and elastase/proteinase inhibitor imbalance. *Am Rev Respir Dis* 130: 817-821, 1984.
72. **Oh W, Poindexter BB, Perritt R, Lemons JA, Bauer CR, Ehrenkranz RA, Stoll BJ, Poole K, Wright LL: Neonatal Research Network.** Association between fluid intake and weight loss during the first ten days of life and risk of bronchopulmonary dysplasia in extremely low birth weight infants. *J Pediatr* 147: 786-790, 2005.
73. **Porter JM, Cutler BS, Lee BY, Reich T, Reichle FA, Scogin JT and Strandness DE.** Pentoxifylline efficacy in the treatment of intermittent claudication: multicenter controlled double-blind trial with objective assessment of chronic occlusive arterial disease patients. *Am Heart J* 104: 66-72, 1982.
74. **Robbins RA, Hamel FG, Floreani AA, Gossman GL, Nelson KJ, Belenky S and Rubinstein I.** Bovine bronchial epithelial cells metabolize L-arginine to L-citrulline: possible role of nitric oxide synthase. *Life Sci* 52: 709-716, 1993.
75. **Roberts RJ, Weesner KM and Bucher JR.** Oxygen-induced alterations in lung vascular development in the newborn rat. *Pediatr Res* 17: 368-375, 1983.
76. **Rojas MA, Gonzalez A, Bancalari E, Claure N, Poole C and Silva-Neto G.**

Changing trends in the epidemiology and pathogenesis of neonatal chronic lung disease. *J Pediatr* 126: 605-610, 1995.
77. **Saugstad OD, Ramji S en Vento M.** Resuscitation of depressed newborn infants with ambient air or pure oxygen: a meta-analysis. *Biol Neonate* 87: 27-34, 2005.
78. **Sawdey MS and Loskutoff DJ.** Regulation of murine type 1 plasminogen activator inhibitor gene expression in vivo. Tissue specificity and induction by lipopolysaccharide, tumor necrosis factor-alpha, and transforming growth factor-beta. *J Clin Invest* 88: 1346-1353, 1991.
79. **Schreiber MD, Gin-Mestan K, Marks JD, Huo D, Lee G and Srisuparp P.** Inhaled nitric oxide in premature infants with the respiratory distress syndrome. *N Engl J Med* 349: 2099-2107, 2003.
80. **Seeger W, Grube C and Gunther A.** Proteolytic cleavage of fibrinogen: amplification of its surfactant inhibitory capacity. *Am J Respir Cell Mol Biol* 9: 239-247, 1993.
81. **Shaul PW, Afshar S, Gibson LL, Sherman TS, Kerecman JD, Grubb PH, Yoder BA and McCurnin DC.** Developmental changes in nitric oxide synthase isoform expression and nitric oxide production in fetal baboon lung. *Am J Physiol Lung Cell Mol Physiol* 283: L1192-L1199, 2002.
82. **Sitrin RG, Pan PM, Srikanth S and Todd RF 3rd.** Fibrinogen activates NF-kappa B transcription factors in mononuclear phagocytes. *J Immunol* 161: 1462-1470, 1998.
83. **Speer CP.** Inflammation and bronchopulmonary dysplasia: A continuing story. *Semin Fetal Neonatal Med* 11: 354-362, 2006.
84. **Subhedar NV and Shaw NJ.** Changes in oxygenation and pulmonary haemodynamics in preterm infants treated with inhaled nitric oxide. *Arch Dis Child Fetal Neonatal Ed* 77: F191-F197, 1997.
85. **Swischuk LE, Shetty BP and John SD.** The lungs in immature infants: how important is surfactant therapy in preventing chronic lung problems? *Pediatr Radiol* 26: 508-511, 1996.
86. **The Franco-Belgium Collaborative NO Trial Group.** Early compared with delayed inhaled nitric oxide in moderately hypoxaemic neonates with respiratory failure: a randomised controlled trial. *Lancet* 354: 1066-1071, 1999.
87. **Thibeault DW, Mabry SM, Ekekezie II and Truog WE.** Lung elastic tissue maturation and perturbations during the evolution of chronic lung disease. *Pediatrics* 106: 1452-1459, 2000.
88. **Thiel M, Bardenheuer H, Poch G, Madel C and Peter K.** Pentoxifylline does not act via adenosine receptors in the inhibition of the superoxide anion production of human polymorphonuclear leucocytes. *Biochem Biophys Res commun* 180: 53-58, 1991.
89. **van der Poll T, Buller HR, ten Cate H, Wortel CH, Bauer KA, van Deventer SJ, Hack CE, Sauerwein HP, Rosenberg RD and ten Cate JW.** Activation of coagulation after administration of tumor necrosis factor to normal subjects. *N Engl J Med* 322: 1622-1627, 1990.
90. **van der Poll T, Levi M, Buller HR, van Deventer SJ, de Boer JP, Hack CE and ten Cate JW.** Fibrinolytic response to tumor necrosis factor in healthy subjects. *J Exp Med* 174: 729-732, 1991.
91. **van der Poll T, Levi M, Hack CE, ten Cate H, van Deventer SJ, Eerenberg AJ, de Groot ER, Jansen J, Gallati H and Buller HR.** Elimination of interleukin 6 attenuates coagulation activation in experimental endotoxemia in chimpanzees. *J Exp Med* 179: 1253-1259, 1994.
92. **van Tuyl M, Liu J, Wang J, Kuliszewski M, Tibboel D and Post M.** Role of oxygen and vascular development in epithelial branching morphogenesis of the developing mouse lung. *Am J Physiol Lung Cell Mol Physiol* 1288: L167-L178, 2005.
93. **Vento M, Asensi M, Sastre J, García-Sala F, Pallardó FV and Viña J.** Resuscitation with room air instead of 100% oxygen prevents oxidative stress in moderately asphyxiated term neonates. *Pediatrics* 107: 642-647, 2001.
94. **Vozzelli MA, Mason SN, Whorton MH and Auten RL Jr.** Antimacrophage chemokine treatment prevents neutrophil and macrophage influx in hyperoxia-exposed newborn rat lung. *Am J Physiol Lung Cell Mol Physiol* 286: L488-L493, 2004.
95. **Ward A and Clissold SP.** Pentoxifylline. A review of its pharmacodynamic and

pharmacokinetic properties, and its therapeutic efficacy. *Drugs* 34: 50-97, 1987.
96. **Warner BB, Stuart LA, Papes RA and Wispe JR.** Functional and pathological effects of prolonged hyperoxia in neonatal mice. *Am J Physiol* 275: L110-L117, 1998.
97. **Welsh CH, Lia MH, Chen SJ and Yen MH.** Pentoxifylline decreases endotoxin-induced pulmonary neutrophil sequestration and extravascular protein accumulation in the dog. *Am Rev Respir Dis* 138: 1106-1114, 1988.
98. **Welty-Wolf KE, Carraway MS, Miller DL, Ortel TL, Ezban M, Ghio AJ, Idell S and Piantadosi CA.** Coagulation blockade prevents sepsis-induced respiratory and renal failure in baboons. *Am J Respir Crit Care Med* 164: 1988-1996, 2001.
99. **Young SL, Evans K and Eu JP.** Nitric oxide modulates branching morphogenesis in fetal rat lung explants. *Am J Physiol Lung Cell Mol Physiol* 282: L379-L385, 2002.
100. **Zimmermann LJI and van Golde LMG.** Lung metabolism in the fetus/neonate. *In Cowett RW, ed. Principles of perinatal-neonatal metabolsim. New-York, Springer-Verslag Inc.* 567-600, 1998.