Novel treatment options for bronchopulmonary dysplasia

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Chapter 7

General discussion

Premature birth interrupts development of the immature lung. The most common complication after very preterm birth is neonatal chronic lung disease (CLD) or bronchopulmonary dysplasia (BPD). Lung immaturity and treatment for respiratory distress syndrome (RDS) with mechanical ventilation and supplemental oxygen may result in lung injury and aberrant alveolar development that ultimately lead to BPD. BPD is characterized by simplified and enlarged alveoli caused by arrested alveolar and vascular development [1, 2], which persistently reduces the surface area of the respiratory membrane. In addition, severe BPD is complicated by oxidative stress-induced tissue damage, inflammation, alveolar and vascular remodeling, fibrosis, coagulation, pulmonary arterial hypertension (PAH) and right ventricular hypertrophy (RVH) [3]. Treatment options for BPD are restricted to alleviation of symptoms of respiratory distress and include surfactant therapy, neurological stimulation of the central respiratory center, fluid restriction and diuretics to reduce edema, and mechanical ventilation with supplemental oxygen [4]. BPD pathology is not only limited to the early neonatal period, because survivors of BPD are also affected later in life and have an increased risk for developing asthma, COPD at relatively young ages, recurrent viral respiratory infections and brain damage caused by hypoxia and cerebral and cerebellar bleedings [5]. Therefore, research is needed to develop novel treatment options to stimulate arrested angiogenesis-driven alveolar development and repair of damaged lung tissue, shorten the prolonged injurious period of mechanical ventilation with supplemental oxygen in the perinatal period and prevent morbidity in childhood and adulthood in survivors of BPD.

Advantages and limitations of animal models in BPD research.

Because the availability of patient materials of very preterm infants with BPD is limited and ethical regulations prevent intervention studies for testing of drugs in children, alternative treatment options for BPD are needed. The lung is a very complex multicellular organ and the convoluted BPD pathology in the developing lung (and heart) cannot be mimicked by cell cultures. Therefore, animal models are needed that mimic BPD, including rodents (mice, rats and rabbits), sheep and primates. Sheep and primates have mainly been used in mechanical ventilation studies [6]. Although BPD pathology in premature primates is almost identical to human BPD, making them the preferred animal model to study BPD *in vivo*, the use of large animal models, including baboons and sheep, in research is limited due to technical, ethical, and financial issues [6]. Mice are preferred to investigate the role of gene deficiency in BPD pathology, and mice and rats are frequently used to investigate the therapeutic potential of novel compounds in BPD pathology. Although lung anatomy and pathology in developing rats and mice is less complex than in humans, alveolar and vascular development and BPD pathology are similar in mammals [7, 8]. The many advantages of rats in neonatal lung research include their small size, large litter size, short cycle and duration of pregnancy, accuracy of timed-pregnancies (in contrast to mice), a developmental stage of the lungs at birth resembling that of very premature infants at risk of developing BPD, and relatively low costs compared to larger animal models [7]. In most rodents, experimental BPD is induced by exposing neonatal pups to hyperoxia (60-100% oxygen) for a period of 9 days to 1 month. In the experiments described in this thesis,

experimental BPD was induced in Wistar rats by exposing neonatal pups to 100% oxygen for 9-10 days [6]. The advantages of this severe hyperoxia-induced BPD rat model are: (a) similar to preterm infants with severe BPD, alveolarization in newborn rat pups occurs after birth, (b) the short time period needed to study the effects of an intervention in severe experimental BPD induced with 100% oxygen, (c) severe BPD pathology in newborn infants and hyperoxia-induced BPD in rats is similar, and (d) reproducibility.

Survivors of BPD are not only affected in the early neonatal period, but also later in life. Although neonatal hyperoxia has no impact on adult allergic asthma [9], an aggravated response to other stimuli after neonatal hyperoxia was demonstrated for bleomycin-induced fibrosis, and virus- and cigarette smoke-induced lung injury in survivors of experimental BPD in mice [10-12]. Therefore, we translated a well-established second-hit strategy in mice to rats by studying the effect of BPD on adult lung disease by exposing newborn rat pups to 90% oxygen for 9 days and challenging them after 6 weeks of recovery in room air with a single intraperitoneal injection of LPS. We observed an aggravated response to LPS in adult survivors of experimental BPD. In addition, LPAR1-deficient rat pups were not only protected against hyperoxia-induced BPD in the neonatal period, but also against the aggravated inflammatory response induced by LPS in adulthood [13].

Although the rat model mimics the important clinical pathological features of severe BPD, there are still limitations that hamper a swift translation of experimental findings to the clinic. In the clinical setting, surfactant treatment, mechanical ventilation- and oxidative stress-induced lung injury after intubation contributes to BPD pathogenesis [14]. Unfortunately, lung injury by prolonged mechanical ventilation cannot be studied in neonatal rats. In the rat, severe BPD was induced by a single stimulus of 100% oxygen, which may reduce the complexity of experimental BPD, but increase BPD severity, because the concentration of oxygen used in the experimental setting is much higher with a shorter period of exposure than in the clinic in the absence of surfactant. The severe experimental BPD model resembles “old” BPD, described by Northway in 1967, in which BPD developed in relatively old premature infants that were treated with hyperoxia for a prolonged period before the introduction of clinical surfactant treatment. To better mimic the clinical situation, we reduced the severity of the BPD model by lowering the oxygen concentration to 90% for 15 days in Chapter 6. Due to technical limitations, experimental compounds were administered by subcutaneous injection. This is different from treatment strategies in the clinic where oral, inhalation, intratracheal, intravenous and intramuscular administration are used.

Intervention studies in BPD research.

Intervention studies in animal models of BPD focus on improving: (a) one or multiple factors contributing to BPD pathology, such as aberrant alveolar and vascular development, inflammation, coagulation, fibrosis, vascular remodeling-induced PAH and RVH, and (b) ventilation strategies to reduce ventilator-induced lung injury. In this thesis, we investigated the therapeutic potential of metformin, BMP9, and inhibition of LPA-LPAR1- and intervening in AT2-dependent signaling in a rat model of hyperoxia-induced BPD in newborn rat pups.

The crucial role of inflammation in BPD and its potential interaction with other contributors to BPD pathology has been demonstrated *in vivo* and in newborns infants through the beneficial effects of anti-inflammatory treatment with neutralizing antibodies against pro-inflammatory cytokines and anti-inflammatory compounds on BPD pathology [15-19]. Furthermore, the beneficial effects of steroids and mesenchymal stem cells in BPD are, at least in part, due to reduced inflammation in BPD [20, 21]. Metformin, a registered drug that is widely used in the clinic for the treatment of type II diabetes [22], exerts its anti-inflammatory effects via the adenosine monophosphate (AMP)-activated protein kinase (AMPK), which exerts an anti-inflammatory role in multiple cell types under different experimental conditions. Inhibition of AMPK aggravated LPS-induced vascular leakage and lung injury in mice [23], and stimulation of AMPK with metformin, alleviates asthma, ventilator- and LPS-induced pulmonary injury, fibrosis and pulmonary hypertension [24-28]. AMPK decreases leukocyte infiltration by reducing the production of chemokine and adhesion molecules [28]. A potent anti-inflammatory effect of AMPK activation by metformin was also confirmed in this thesis, but the detailed mechanisms underlying the protective role of AMPK activation are still under investigation. Several pathways are reported to be involved in the protective effect of AMPK activation, including inhibition of pro-inflammatory NF-κB, JAK-STAT, MAP Kinases (p38/JNK/ERK pathway) and ROS generation, enhancement of protective cytokine IL-10 production, influence on polarization of macrophages and activation of eNOS [29-32]. In addition, we observed a potent anti-inflammatory and anti-fibrotic effect in all the interventions described in this thesis (Figure 1) by targeting the AT2 pathway, the LPA-LPAR1 pathway and the BMP/TGFβ pathway, demonstrating again the important role of inflammation in BPD pathology. Fibrin is the end-product of coagulation. Extravascular fibrin deposition is a complex process in BPD, caused by oxidative stress- and inflammation-driven endothelial injury, vascular leakage of fibrinogen into the alveolar lumen, and conversion of soluble fibrinogen into insoluble fibrin by thrombin on the epithelial layer of the alveolar membrane. Fibrin deposition on the luminal side of the respiratory membrane severely reduces the gas-exchange capacity of the lung and contributes to life-threatening respiratory distress in BPD patients. Experimental evidence has proven that expression of genes involved in coagulation and fibrinolysis, like plasminogen activator inhibitor 1(PAI-1), tissue factor (TF) and urokinase-type plasminogen activator receptor 1 (uPAR-1), is modulated in BPD [33]. Inhibitors of AMPK increase pulmonary endothelial permeability and edema, which can be reversed by metformin [26]. In addition, metformin also reduces coagulation and improves fibrinolysis [34], probably via inflammation-related mechanisms [35]. Furthermore, LPA has been suggested to induce platelet aggregation and expression of the initiator of the coagulation cascade tissue factor [36, 37]. In this thesis, metformin and Ki16425 showed moderate prevention against fibrin deposition.

In fibrosis, an important pathological feature of severe BPD in which inflammation plays an important role, many signaling pathways are involved, including RAS, endothelin-1 signaling and LPA-LPAR1 signaling [38-40]. The role of LPA-LPAR1 signaling in pulmonary fibrosis has been studied extensively [41-44]. LPA is a small glycerophospholipid that exerts multiple biological effects after binding to its receptors, among which LPAR1 or endothelial differentiation gene (EDG) family member 2 (EDG2) is the most important one. Experimental

and clinical evidence strongly suggests that LPA-LPAR1 signaling is involved in lung pathology and disease, including fibrosis, inflammation, airway repair and remodeling [42, 43, 45-47], via several downstream cascades: Rho-ROCK pathway, phospholipase C pathway, Ras- MAPK pathway, Akt pathway, and adenylyl cyclase inhibition [48]. The pro-fibrotic role of LPA-LPAR1 signaling involves attraction of fibroblasts to the lung and promotion of collagen deposition [41, 43, 44]. However, the role of LPA-LPAR1 signaling in pulmonary inflammation is still controversial with both stimulation [49-51] and inhibition [52] of LPA signaling resulting in an anti-inflammatory effect. In Chapter 3, we demonstrate significant anti-inflammatory and anti-fibrotic effects in the absence of LPA-LPAR1 signaling in experimental BPD. Furthermore, a protective role of LPAR1 deficiency against an acute LPS challenge was demonstrated in adult survivors of BPD in Chapter 5, demonstrating the anti-inflammatory role of LPA-LPAR1 blocking in neonatal and adult lung disease. Because pulmonary fibrosis is also a common feature of interstitial lung disease in adults, it will be worthwhile to study the role of LPAR1 deficiency in bleomycin-induced lung fibrosis in adult survivors of BPD.

The hallmark of BPD is aberrant alveolar and vascular development of the immature lung [1]. *In vivo* studies have identified multiple mediators of alveolarization in BPD, involved in multiple regulatory pathways, such as the NO pathway, the TGF-β/BMP signaling pathway, by stimulating multiple downstream targets, including NF-κB, and receptors for glycation end products (RAGE) [53, 54]. TGF-β- and BMP-dependent signaling are crucial in modulating many processes, including differentiation, proliferation, migration and host defense [55-57], that are important for normal lung development and cardiopulmonary disease, including BPD by improving aberrant vascular and alveolar development, PAH and RVH [54, 55, 58-61]. Although many experimental studies demonstrate that TGF-β is an important mediator in lung development and that inhibition of TGF-β-dependent signaling protects against BPD, experimental evidence suggests that direct intervention in the BMP arm of the pathway may be beneficial for BPD as well [62]. In this thesis, we investigated the role of the TGF-β/BMP signaling pathway by stimulating BMP9-ALK1-BMPR2-dependent signaling and found that BMP9 protects against impaired alveolarization and angiogenesis, inflammation and fibrosis in experimental BPD, thereby demonstrating that restoring the balance by both lowering TGF-β- and stimulating BMP9-dependent signaling has therapeutic potential for BPD by affecting multiple contributing factors. However, the mechanism by which BMP9 improves BPD is still unknown. We speculate that improved angiogenesis-driven alveolar development is caused by a direct stimulation of angiogenesis by BMP9 or indirectly by attenuating lung injury via a reduction of oxidative stress-induced and inflammation-driven fibrosis. We conclude that BMP9 protects against aberrant alveolarization, angiogenesis and inflammation. This makes intervention in the TGF-β/BMP signaling pathway a valuable toolbox for discovering compounds with strong therapeutic potential for BPD.

Pulmonary hypertension is a late complication of BPD, caused by vascular remodeling, resulting in PAH and RVH, a reduction in the pulmonary vascular bed and increased vascular tone [63], which is still considered to be an important pathological feature of very premature birth and “new” BPD in the post-surfactant era [64]. Experimental *in vivo* studies demonstrated that interventions in the prostacyclin, nitric oxide and endothelin pathways

with vasoactive compounds, including PDE-4- and -5- inhibitors, NO, and sGC activators, have strong therapeutic potential [65-67]. However, the most promising interventions with inhaled NO and the PDE-5 inhibitor sildenafil failed in clinical trials [68, 69]. The renin angiotensin system (RAS) is another important pathway in the regulation of vascular tone. The most important effector molecule in RAS is Ang II. Binding of Ang II to the angiotensin type 1 receptor (AT1) results in many adverse effects, including inflammation, fibrosis and heart disease [70-72], while binding to its type 2 receptor (AT2) leads to beneficial effects, such as vasodilation, reducing inflammation and fibrosis, reducing vascular remodeling and a cardio-protective effect. Our research focuses on the role of activation of receptors that can dimerize with AT1, including the apelin receptor APJ, the MAS oncogene receptor (MAS), and the angiotensin type 2 receptor (AT2), to reduce the adverse effects of AT1 stimulation [73-79]. Although we demonstrated the therapeutic potential of stimulation of APJ, MAS and AT2, a major drawback of these interventions was the narrow therapeutic window of the ligands used. This is demonstrated by the compound PD12339, a well-known inhibitor of AT2 that acts as an antagonist at a high dose (0.5 and 2 mg/kg/day), and as an agonist at low dose (0.1 mg/kg/day). Furthermore, LPA and BMP/TGF-β signaling are also reported to be involved in pulmonary hypertension, vascular injury response and vascular smooth muscle cell phenotyping [80-84], but this could not be confirmed in this thesis.

Crosstalk between signaling pathways.

The premature lung is a complex, growing, developing and functional multicellular organ. During growth, development, and pathogenesis cell-cell and ligand-receptor interaction play an important role. Ligand-stimulated receptors trigger biological processes via multiple pathways in a cell that may interfere with each other. For instance, BMP9-ALK1-BMPRII, LPA-LPAR1-dependent signaling and metformin stimulate MAPKs in various cells [85-90], while solid evidence also shows that AMPK activation inhibits the MAPK pathway [29]. These conflicting findings may be due to the experimental setup, making the mechanism studies extremely cell type- and context-specific. Furthermore, AT2-, AMPK- and BMP-signaling are reported to modulate nitric oxide (NO) synthesis [91-93], an important regulator in vascular tone, alveolarization and vascularization [94, 95], all of which are important contributing factors to BPD pathology. Besides, direct evidence shows that the TGF-β-activated kinase 1 (TAK1) can also be activated by AMPK in endothelial cells [96]. Crosstalk between RAS and TGF–β pathways has been suggested in many studies [97-99] and endothelin 1 (ET-1) was reported to interact with TGF-β signaling in fibrosis and vascular remodeling, both *in vivo* and *in vitro* [100-102]. These interactions between different pathways make it difficult to extrapolate *in vitro* and *in vivo* data, obtained from different cell types and experimental diseases, to experimental BPD.

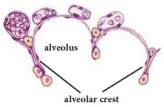
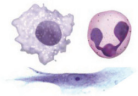
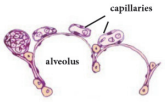
	PD123319	Metformin	LPAR1 deficiency	Ki16425	BMP9
 Alveolarization	–	–	–	–	+++
 Inflammation and fibrosis	+++	++	+++	++	+++
 Vascular remodeling	++	–	–	+	–
 Coagulation	–	++	–	+	–
 Alveolarization	–	++	–	–	++

Figure 1. General overview of treatment options in experimental BPD. +: stimulatory effect; -: no effect.

Future perspective for treatment of BPD.

Lung immaturity due to premature birth is an important contributing factor to BPD. If premature birth cannot be avoided, prenatal steroids, surfactant replacement and gentle ventilation management are promising strategies to prevent or alleviate BPD and are now widely used in neonatal intensive care centers. However, the clinical use of prenatal steroids before preterm birth is still under dispute. Although prenatal steroids accelerate lung development effectively by reducing thickness of the respiratory membrane, they also inhibit alveolar development by reducing secondary septation, thereby aggravating alveolar enlargement, and they are neurotoxic. However, effective treatment for BPD is still lacking. In this thesis, we have tested different compounds targeting multiple important signaling pathways involved in BPD pathology using a neonatal rat pup model. After effective translation to the clinic, these promising findings may lead to new treatment options for BPD.

Although beneficial effects in LPAR1 mutant rats and after metformin or Ki16425 treatment were observed in rat pups with BPD, these interventions do not improve arrested alveolar and vascular development, which is considered the hallmark of “new” BPD. BMP9 improves experimental BPD effectively by reducing inflammation and fibrosis and stimulating aberrant alveolar and vascular development, demonstrating its excellent therapeutic potential for BPD. However, additional research is needed to exclude potential adverse effects of BMP9 in humans that may hamper a swift translation of BMP9 to the clinic. Besides, the mechanisms of anti-inflammation, anti-fibrosis and stimulation of alveolarization and vascularization properties of BMP9 in this thesis are preliminary. Investigation on the detailed mechanism might be difficult to accomplish but is still necessary.

Based on the various effects on different pathological aspects of BPD (Figure 1 in discussion), a single remedy might not be sufficient for BPD. Combination therapy could lead to a better outcome for BPD patients. However, caution should be exercised on the crosstalk between different signaling pathways when combination therapy is used. We have confirmed the susceptibility of the lung to endotoxin in adult survivors of BPD and validated the beneficial effect of long term interfering with LPA-LPAR1 signaling pathway. This gives pulmonologists some hints about the dosing period of certain interventions.

Overall, the intervention studies in this thesis are still preliminary and there is still a long way before they go to clinical application, or even be rejected, like most of their pioneers, due to invalidation of the efficacy or severe side effects, such as inhaled nitric oxide and sildenafil. However, animal experiments contribute to a swift translation of promising findings obtained by valuable basic research to the clinic. Efforts in basic research are still necessary, since BPD patients are still suffering and causing an extremely heavy burden to their families and society.

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