Structural and functional changes of the pulmonary vasculature after hypoxia exposure in the neonatal period: a new swine model of pulmonary vascular disease

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ABSTRACT

Pulmonary vascular disease (PVD) represents an underestimated and increasing clinical burden not only in the neonatal period but also later in life, when exercise-tolerance is decreased. Animal models performing long-term follow-up after a perinatal insult are lacking. This study aimed to develop and characterize a neonatal swine model with hypoxia-induced PVD during long-term follow-up after reexposure to normoxia and to investigate the exercise response in this model. Piglets were exposed to a normoxic (N=10) or hypoxic environment (N=9) for 4 weeks. Neonatal hypoxia exposure resulted in pulmonary hypertension. Mean pulmonary artery pressure was elevated 1 day after reexposure to normoxia (30.2 ± 3.3 mmHg vs. 14.3 ± 0.9 mmHg), and remained significantly higher in the second week (32.8 ± 3.8 mmHg vs. 21.4 ± 1.2 mmHg), accompanied by decreased exercise tolerance. Exercise resulted in a trend toward an exaggerated increase of pulmonary artery pressure in hypoxia-exposed animals (week 6, P=0.086). Although pulmonary hypertension was transient, thickening of pulmonary arterioles was found at the end of follow-up. Furthermore, right ventricular dilation, and lower right ventricular fractional area change (RVFAC week 8, 40.0 ± 2.7% vs. 29.5 ± 4.7%) and tricuspid annular plane systolic excursion (TAPSE week 8, 27.0 ± 2.5 mm vs. 22.9 ± 2.1 mm) persisted during follow-up. Male animals showed more severe PVD than female animals. In conclusion, we developed a neonatal swine model that allows examination of the long-term sequelae of damage to the developing neonatal lung, the course of the disease and the effect of therapy on long-term outcome.

New and noteworthy

The swine model of neonatal pulmonary vascular disease developed in the present study is the first that allows exercise testing and examination of long-term sequelae of a perinatal hypoxic insult, the course of the disease, and the effect of therapy on long-term outcome.

INTRODUCTION

The improvement of neonatal care, including antenatal corticosteroid administration, improved ventilation strategies and surfactant therapy, has dramatically increased the survival of premature infants.[1-3] However, preterm birth is associated with a variety of short– and long-term health problems, including pulmonary vascular disease (PVD). In children, PVD is strongly associated with a number of complicated childhood diseases, such as bronchopulmonary dysplasia (BPD), and respiratory problems in later life.[4]

Premature infants are born in a critical stage of lung development (saccular or alveolar stage). After birth, their immature lungs are exposed to several injurious stimuli, including hypoxia and/or hyperoxia, ventilator-induced lung injury, infection, inflammation, and oxidative stress. In addition, antenatal risk factors, including maternal hypertension and smoking, are known to be injurious to the developing lung.[5] This leads to disruption of normal lung development, both in terms of the impaired alveolarization and dysmorphic vascular growth.[6] The impairment in pulmonary vascular development, resulting in significant PVD and pulmonary hypertension (PH), is often underestimated or neglected but contributes significantly to the morbidity and mortality of patients with BPD, with up to 50% mortality within 2 years of diagnosis.[7-9]

PVD and PH represent an increasing clinical burden not only in the neonatal period but also later in life. Despite decades of research, the mechanisms underlying PVD as well as to what extent PVD contributes to a decreased lung function, decreased exercise tolerance, and cardiovascular mortality later in life are currently unknown.[10,11] To understand the mechanisms and to develop new therapies, several animal models for neonatal PVD and PH, including a neonatal swine model of hypoxia-induced PH, have already been established.[12-19] Yet (large) animal models of neonatal PVD performing long-term follow-up are lacking. To fulfill the need to examine long-term sequelae of damage to the developing neonatal lung, we developed a swine model for neonatal PVD with long-term follow-up. This model allows 3-wk follow-up after reexposure to normoxia with hemodynamic measurement at rest and during exercise in awake piglets. By placing the cardiopulmonary system under stress with exercise testing, subtle dynamic abnormalities that are not apparent on conventional static tests may be revealed. Additionally, exercise testing will help to assess the severity of the disease. The aim of the present study is to characterize this swine model in terms of growth and systemic and pulmonary hemodynamics at rest and during exercise, as well as in terms of right ventricular (RV) function and structure, using echocardiography and histology.

METHODS

In vivo animal experiments

Studies were performed in accordance with the "Guiding Principles for the Care and Use of Vertebrate Animals in Research and Training" as approved by the American Physiological Society and with approval of the Animal Care Committee of the Erasmus MC, University Medical Center Rotterdam. Thirty-four crossbred Landrace x Yorkshire piglets, 48 h of age (18 male and 16 female piglets), entered the study. Shortly after arrival, all piglets received a single dose of artificial colostrum (Colo-active Plus, Schippers, Bladel, The Netherlands) and were placed in an incubator, in which the fraction of inspired oxygen (FiO_2) could be regulated, for 4 wk. Piglets assigned to the control group were exposed to a normobaric normoxic environment (FiO_2: 21%; male piglets: n=8 and female piglets: n=8), whereas piglets assigned to the intervention group were exposed to a normobaric hypoxic environment (FiO_2: 10-12%, male piglets: n=10 and female piglets: n=8). Piglets in the 10-12% FiO_2 group were exposed to 10% FiO_2 for at least 1 wk, and FiO_2 was adjust to higher levels (max. FiO_2: 12%) on the basis of clinical signs of severe PH (severe dyspnea, growth retardation, septal shift on echocardiography). Piglets were fed age-appropriately (Lactowean Extra, Babywean, or Topwean, Denkavit, Voorthuizen, The Netherlands) and received a supplementary feed for piglets, based on egg yolk (MS Pig Pusher Oral, Schippers, Bladel, The Netherlands) from days 1 to 3 to support the immune system and increase the vitality of the newborn piglets, especially in case of insufficient colostrum. Animals were weighed daily.

After 4 wk in the incubator, piglets were chronically instrumented as described below and subsequently placed in a normoxic environment. A schematic representation of the methods is presented in Figure 1.

Surgical procedures

Piglets were sedated with tiletamine-zolazepam (3 mg/kg ic), xylazine, (1.75 mg/kg iv), and atropine (0.5 mg), intubated, and ventilated with a mixture of O_2 and N_2 (1:2) to which 2.0% (vol/vol) isoflurane was added for adequate anesthesia. The depth of anesthesia was checked regularly using a pain stimulus (toe pinch). Piglets were instrumented under sterile conditions as previously described.[20] Briefly, the chest was opened via the fourth left intercostal space and fluid-filled polyvinylchloride catheters were inserted into the aortic arch, pulmonary artery, left atrium, and right ventricle (RV) for pressure measurements, blood sampling, and infusion of drugs. In a subset of animals (n=13), a flow probe (16 mm, Transonic Systems, Ithaca, NY) was positioned around the pulmonary artery for measurement of cardiac output (CO). Catheters and electrical wires were tunneled subcutaneously to the back, and the chest was closed in layers. All animals were subsequently placed in a normoxic environment and allowed to recover while receiving analgesia (buprenorphine 0.015 mg/kg im and fentanyl slow-release patch 6 μg/hr) and antibiotic prophylaxis (Augmentin 25/5

Figure 1. Schematic representation of the experimental design of the study. AOP, aortic pressure; CO, cardiac output; LAP, left atrial pressure; PAP, pulmonary arterial pressure; RVP, right ventricular pressure.

mg/kg iv) for 7 days. The catheters were flushed daily with heparinized saline (1000-5000 IU/ml).

Experimental protocols

Studies were performed 1-3 wk after surgery with piglets exercising on a motor-driven treadmill. Fluid-filled pressure transducers were positioned on the backs of the animals and calibrated at mid-chest level. With piglets (male piglets: normoxia n=4 and hypoxia n=4; female piglets: normoxia n=6 and hypoxia n=5) standing on the treadmill, resting hemodynamic measurements, consisting of heart rate, (CO), aortic pressure, pulmonary artery pressure, left atrial pressure, and RV pressure (RVP), were obtained. Rectal temperature was measured, and arterial and mixed venous blood samples were collected. Subsequently, a four-stage treadmill exercise protocol was started (1-4 km/h); each exercise stage lasted 3 min. Hemodynamic parameters were continuously recorded, and blood samples were collected during the last 45 s of each exercise stage, at a time when hemodynamics had reached a steady state. After completing the exercise protocol, animals were allowed to rest on the treadmill. Excellent reproducibility of consecutive exercise trials has been previously reported.[21]

Digital recording and offline analysis of hemodynamics have been described previously.[22] Pulmonary vascular conductance (PVC) was defined as CO divided by mean pulmonary artery pressure (PAP) minus mean left atrial pressure (LAP).[23] Systemic vascular conductance (SVC) was calculated as CO divided by mean aortic pressure (MAP). To accommodate for

the varying weights between animals and groups, CO, PVC, and SVC were indexed to body weight.

Blood gas measurements

Blood samples were kept on iced syringes until the conclusion of each exercise trial. Measurements of arterial pO_2 (in mmHg), arterial pCO_2 (in mmHg), pH, O_2 saturation (in %), and hemoglobin concentration (in g/100ml) were then immediately performed with a blood gas analyzer (ABL 820, Radiometer) and corrected for body temperature.

Echocardiography

Weekly, echocardiography (Aloka ProSound SSD-4000, Tokyo, Japan) was performed in conscious piglets. Two-dimensional echocardiographic recordings of the RV long-axis four-chamber view were obtained and stored for offline analysis.[24] RV end-diastolic cross-sectional area (EDA) and end-systolic cross-sectional area (ESA) were determined, and RV fractional area change (RVFAC) was calculated as follows: RVFAC (in %) = (RV EDA – RV ESA)/RV EDA x 100%. Tricuspid annular plane systolic excursion (TAPSE) was obtained using an M-mode cursor passed through the tricuspid lateral annulus in a four-chamber view, by measuring the amount of longitudinal displacement of the annulus at peak-systole. TAPSE has been shown to correlate well with isotopically derived RV ejection fraction.[25,26]

Histology

After completing all experimental protocols, animals were reanesthetized and ventilated, and the thorax was opened. The right lung of all animals was removed and weighed. Physiologic saline (0.9% NaCl) was first infused through a main bronchus of the middle or accessory lobe to flush the airways from blood, sputum and surfactant. The lobe was then fixed by tracheal installation of 3.5-4% buffered formaldehyde at constant physiological pressure (25 cm H_2O). After fixation, airway inflation pressure was maintained for at least 24 hours by tying off the bronchus without leaks, and the lobe was submerged in fixative.[27]

Transverse sections were obtained from the base, middle, and tip of the formaldehyde-fixed right middle or accessory lobe for morphometric analysis. Sections from each animal were processed and embedded in paraffin wax. Paraffin sections (4.5 μm) were cut from each block and stained with resorcin-fuchsin - van Gieson stain. Sections were evaluated by light microscopy using the Hamamatsu NanoZommer Digital Patholy (NDP) slide scanner (Hamamatsu Nanozoomer 2.0HT, Hamamatsu Photonics) for evidence of lung injury caused by chronic exposure to hypoxia.

To determine alveolar simplification, alveolar structure was analyzed by a custom-made routine using Clemex Vision PE version 7.0 image analysis software measuring alveolar area, septal area, and septal length. Septal thickness was calculated as septal area divided by septal

length. Quantitation of histological findings was performed by evaluation of the alveolar structure at six different locations per lung section per animal.

Morphometric measurements of pulmonary arteries were performed using NDP viewer (Hamamatsu). Panoramic screening of whole tissue sections was performed, and lumen area and area enclosed by the external elastic lamina of the pulmonary arteries were assessed by planimetry. To ensure that pulmonary veins were excluded for analysis, only transversely cut vessels close to bronchi of different diameters (<100, 100-200, 200-400 μm) were analyzed. Assuming circularity of the vessels, inner and outer radii were calculated as radius = $\sqrt{}$ (area/π). The wall-to-lumen ratio was calculated as (outer radius – inner radius)/inner radius.

Data analysis and statistical analysis

Statistical analysis was performed using SPSS version 21.0 (IBM, Armonk, NY). Data comparing survival in the different study groups were analyzed using Kaplan-Meier analysis. Data comparing resting hemodynamic measurements in the different study groups were analyzed using one-way (hypoxic) or two-way (hypoxic and sex) ANOVA with Bonferroni's multiple-comparison post hoc test.

Nonlinear growth curve analysis was used to compare growth between animals exposed to normoxia and hypoxia. To test for the effect of hypoxia (vs. normoxia) on exercise response, regression analysis was performed with FiO_2, treadmill speed and sex, as well as their interaction(s), as independent variables and animal number as case label. Echocardiographic data were compared using general linear model (GLM) – repeated-measures analysis. Histological data comparing alveolar structure were also analyzed using GLM-repeated-measures analysis. To test for the effect of hypoxia (vs. normoxia) on wall-to-lumen ratio, regression analysis was performed with FiO_2 and inner radius, as well as their interaction, as independent variables and animal number as case label. To test for the effect of perinatal transient PH on the response of systolic PAP to high-altitude exposure in male and female subjects, GLM-repeated-measures analysis was used. Statistical significance was accepted at $P \leq 0.05$. Grouped data are presented as means ± SE.

RESULTS

Survival

A total of 34 newborn piglets (2 days old) entered the study and were exposed to normoxia (FiO_2: 21%, n=16) or hypoxia (FiO_2: 10-12%, n=18) for 4 wk. During the study period, one animal that was raised in normoxia died of cardiac tamponade (overall survival in this group: 92.9%; Figure 2). Seven animals in the hypoxia group died of PH-related disease during the study period, either in the hypoxia period or shortly after surgery (overall survival in this group: 51.5%; Figure 2). In addition, surgical complications (bleeding or ventilation

Figure 2. Kaplan Meier survival curve showing the risk of death caused by hypoxia-induced pulmonary hypertension-related disease. Causes of death, other than hypoxia-induced pulmonary hypertension-related disease, are marked as "lost to follow-up" (black ticks without decrease in survival).

problems, normoxia: n=2 and hypoxia: n=2; Figure 2, marked as "lost to follow-up") and infection of the catheters (normoxia: n=3 and hypoxia: n=0; Figure 2, marked as "lost to follow-up") resulted in a final inclusion of 19 animals (male piglets: normoxia n=4 and hypoxia n=4; female piglets: normoxia n=6 and hypoxia n=5) for analysis of pulmonary hemodynamics.

Resting PAP

A significantly elevated PAP was found in animals raised in hypoxia as compared with control in the first 2 wk after surgery (30.2 ± 3.3 vs. 14.3 ± 0.9 mmHg and 32.8 ± 3.8 vs. 21.4 ± 1.2 mmHg, respectively, $P < 0.05$; Figure 3). Also, in the last 2 wk of follow-up, PAP was above 25 mmHg (26.2 ± 3.4 and 28.1 ± 6.1 mmHg; Figure 3), suggestive for PH, although there was no significant difference compared with animals raised under normoxic conditions.

Growth

The weight gain of animals raised in normoxia was slightly lower than of farm-raised piglets[28] and comparable to early weaned piglets.[29] Although the weight of the animals raised in hypoxia tended to be slightly lower after the first 4 wk (5.9 ± 0.3 vs. 6.8 ± 0.4kg, P=0.16), the relative growth rate (k) was similar in both groups (normoxia: k=0.326 ± 0.012 and hypoxia: k=0.324 ± 0.010, P=0.89; Figure 4). After surgery, and thus after reexposure to normoxia, the k value of animals in the hypoxia group was similar to that of animals in the normoxia group (normoxia: k=0.215 ± 0.037 and hypoxia: k=0.214 ± 0.032; P=0.99; Figure 4).

Hemodynamics during incremental exercise

In animals raised in normoxia, exercise up to 4 km/h produced a significant increase in cardiac index (Figure 5, C, F, and I). Because of the small vasodilator capacity of the lung

Figure 3. Mean pulmonary artery pressure (PAP) at rest in the different study groups over time. A: week 5 (postoperative day 1). B: week 6. C: week 7. D: week 8. Numbers per study group are presented in the boxes in the bars. Values are mean ± SE. * P ≤ 0.05 vs normoxia.

Figure 4. Growth curves of animals raised in normoxia and hypoxia. A: growth curves of the entire study period (weeks 0 – 8). B: growth curves of the period in the incubator (weeks 0 – 4). There were no significant differences in growth between the study groups.

vasculature, the pulmonary vascular conductance index (PVCi) increased only slightly during exercise (week 6: 24 ± 9%, week 7: 26 ± 14%, week 8: 17 ± 17%; Figure 5, B, E, and H). As a consequence of the marked increase in cardiac index, which caused an increase in the

pressure drop across the pulmonary vasculature, in combination with the minimal change in PVCi, PAP increased significantly during incremental exercise (Figure 5, A, D, and G).

Under resting conditions, PAP was higher in hypoxic compared with normoxic animals, as mentioned above, reaching significance in weeks 6 and 7 (Figure 5, A, D, and G), whereas LAP tended to be lower in week 8 (Table 1) and cardiac index was unchanged in both groups during the entire follow-up period (Figure 5, C, F, and I). This resulted in a significant reduction in PVCi in animals raised in hypoxia, indicative for pulmonary vasoconstriction and/or vascular remodeling (Figure 5, B, E, and H). Despite these changes in pulmonary hemodynamics, no alterations in blood oxygenation were found (Table 1), indicating that diffusion capacity was not compromised.

Figure 5. Changes in pulmonary hemodynamics during graded treadmill exercise in normoxic and hypoxic piglets. Relation between treadmill speed and mean pulmonary arterial pressure (PAP; A, D, and G), pulmonary vascular conductance index (PVCi; B, E and H), and cardiac index (CI; C, F, and I). Week 6: n=8 normoxia-exposed and 9 hypoxia-exposed piglets; week 7: n=10 normoxia-exposed and 9 hypoxia-exposed piglets; week 8: n=8 normoxia-exposed and 6 hypoxia-exposed piglets. Values are means ± SE. * $P \leq 0.05$ vs. normoxia; ** $P \leq 0.1$ vs. normoxia; † $P \leq 0.05$ effect of exercise; ‡‡ $P \leq 0.1$ vs. response in normoxia.

Table 1. Blood gas and hemodynamic measurements in normoxia- and hypoxia-exposed piglets.

		Normoxia		Hypoxia	
		Rest	Maximum Exercise	Rest	Maximum exercise
Heart Rate, beats/min	Week 5	171 ± 6		202 ± 21	
	Week 6	190 ± 8	320 ± 18	201 ± 13	313 ± 13
	Week 7	188 ± 7	296 ± 14	181 ± 26	287 ± 16
	Week 8	166 ± 6	287 ± 14	172 ± 14	266 ± 14
Left atrial pressure, mmHg	Week 5	2.2 ± 1.4		5.3 ± 1.1	
	Week 6	2.8 ± 1.5	6.3 ± 1.8	4.6 ± 1.2	9.4 ± 1.1
	Week 7	3.9 ± 2.4	5.7 ± 2.1	3.1 ± 1.3	7.0 ± 1.7
	Week 8	6.6 ± 1.3	8.7 ± 2.3	4.2 ± 2.3	7.9 ± 1.9
Systemic vascular conductance index, ml/min/kg/mmHg	Week 5	3.8 ± 1.1		5.5 ± 1.3	
	Week 6	3.7 ± 0.7	6.0 ± 0.7	3.4 ± 0.5	6.0 ± 0.3
	Week 7	3.6 ± 0.4	6.1 ± 0.5	4.4 ± 1.0	6.2 ± 1.5
	Week 8	3.4 ± 0.2	5.3 ± 0.2	3.2 ± 0.5	4.7 ± 0.3
Arterial pO_2, mmHg	Week 5	92.0 ± 4.9		93.3 ± 6.4	
	Week 6	110.0 ± 10.0	79.1 ± 4.8	98.3 ± 5.3	80.1 ± 3.3
	Week 7	100.0 ± 4.5	86.6 ± 7.3	95.7 ± 4.0	88.5 ± 3.6
	Week 8	99.8 ± 3.5	89.0 ± 5.9	110.8 ± 3.9	93.5 ± 5.5
O_2 saturation, %	Week 5	96.8 ± 0.6		95.6 ± 1.1	
	Week 6	98.5 ± 0.7	91.3 ± 2.3	96.9 ± 1.1	93.0 ± 3.2
	Week 7	96.5 ± 0.8	93.2 ± 2.0	97.7 ± 0.6	96.5 ± 0.7
	Week 8	96.8 ± 0.8	93.6 ± 1.7	98.4 ± 0.5	96.5 ± 1.6
Arterial pCO_2, mmHg	Week 5	32.7 ± 1.4		31.2 ± 1.4	
	Week 6	38.2 ± 2.1	34.7 ± 1.9	37.6 ± 1.4	37.2 ± 0.6
	Week 7	38.8 ± 1.1	36.8 ± 1.4	38.7 ± 1.5	33.8 ± 1.0
	Week 8	40.6 ± 1.5	36.4 ± 1.4	36.8 ± 2.8	33.5 ± 1.3
Hb, g/dl	Week 5	8.9 ± 0.5		7.7 ± 0.7	
	Week 6	7.5 ± 0.4	8.2 ± 0.3	6.6 ± 0.5	6.8 ± 0.4 *
	Week 7	8.1 ± 0.4	8.5 ± 0.4	7.4 ± 0.4	7.2 ± 0.4 *
	Week 8	8.3 ± 0.3	9.0 ± 0.4	8.4 ± 0.4	8.8 ± 0.4

Values are means ± SE; n=9 normoxia-exposed and n=8 hypoxia-exposed piglets at week 5, n=8 normoxia-exposed and n=8 hypoxia-exposed piglets at week 6, n=10 normoxia-exposed and n=8 hypoxia-exposed piglets at week 7, n=9 normoxia-exposed and n=5 hypoxia-exposed piglets at week 8. * $P \leq 0.05$ vs the normoxia-exposed animals.

The cardiac index increased to a similar extent in hypoxic and normoxic animals during incremental exercise up to 4 km/h (Figure 5, C, F, and I). In contrast to the normoxic group, the pulmonary vasculature lost its vasodilator capacity completely, as shown by a stable or even slightly decreasing PVCi during exercise in the hypoxic group (week 6: 3 ± 15%, week

7: –6 ± 8%, and week 8: –21 ± 4%; Figure 5, B, E, and H). Similar to animals raised in normoxia, PAP increased with incremental exercise in animal raised in hypoxia (Figure 5, A, D, and G). The increase in PAP in response to exercise tended to be larger in hypoxic animals compared with normoxic animals at week 6 (P=0.086) and week 8 (P=0.06; Figure 5).

The exercise-induced increase in cardiac index (Figure 5, C, F, and I) minimally affected MAP in both the normoxic and hypoxic group, becaue of a significant increase in systemic vascular conductance index (Figure 6). Although MAP was slightly, but significantly, lower in hypoxia-raised piglets compared with normoxia-raised piglets, the exercise-induced systemic vasodilation was comparable between the two groups.

Figure 6. Changes in systemic hemodynamics during progressive levels of exercise in normoxic and hypoxic piglets. A, C, and E; relation between treadmill speed and mean aortic pressure (MAP). B, D, and F: relation between tredsmill speed and systemic vascular conductance index (SVCi). Week 6: n=8 normoxia-exposed and 9 hypoxia-exposed piglets; week 7: n=10 normoxia-exposed and 9 hypoxia-exposed piglets; week 8: n=8 normoxia-exposed and 6 hypoxia-exposed piglets. Values are means ± SE. * P ≤ 0.05 vs. normoxia; ** P ≤ 0.1 vs. normoxia; † P ≤ 0.05 effect of exercise.

Echocardiography

Chronic exposure to hypoxia resulted in significant RV dilation (Figure 7, A and B). Although the size of the RV lumen area corrected for body weight decreased significantly over time in animals raised in normoxia and hypoxia, the change in size of the RV corrected for bodyweight compared with baseline was only minimal in hypoxic animals in the first 4 wk of the study (hypoxic period; Figure 7, C and D). Reexposure to normoxia resulted in a significant decrease in lumen of the RV corrected for body weight, but RVs of animals in the hypoxic group remained dilated (Figure 7). These results suggest that chronic exposure to hypoxia leads to RV dilation, which does not fully recover after reexposure to normoxia.

Not only the structure but also the function of the RV was altered after chronic exposure to hypoxia. In contrast to the normoxia-exposed piglets, RVFAC did not increase over time in hypoxia-exposed piglets (week 8: normoxia: 40.0 ± 2.7% and hypoxia: 29.5 ± 4.7%, time x FiO_2, P=0.02). TAPSE was significantly lower in animals exposed to hypoxia (week 8: 22.9 ± 2.1mm) compared with normoxia (week 8: 27.0 ± 2.5mm, P=0.05). Both parameters suggest an impaired RV function in animals exposed to chronic hypoxia.

Figure 7. Size (area) of the right ventricular lumen, obtained by echocardiography, corrected for bodyweight over time. A and B: end-diastolic area (A) and end-systolic area (B) of the right ventricular lumen. C and D: chance in right ventricular lumen area compared with baseline (week 1) at end-diastole and end-systole, respectively. n=8 normoxia-exposed and 9 hypoxia-exposed piglets; Values are means ± SE. * P ≤ 0.05 vs. normoxia.

Histology

In accordance with the normal diffusion capacity of the lung, there were no signs of alveolar simplification in piglets raised in hypoxia. Thus, no significant differences were found in alveolar area, septal length, or septal thickness between the study groups (Table 2).

Wall-to-lumen ratio was significantly higher in the distal arterioles (i.e., arterioles close to bronchi with diameters of <100 and 100-200 μm) of hypoxia-exposed piglets compared with normoxia-exposed piglets (Figure 8), particularly in the small arterioles (inner lumen radius <25 μm, P=0.004; inner lumen radius > 25 μm, P=0.69). In contrast, the wall-to-lumen ratio of the proximal arteries (i.e., vessels close to bronchi with a diameter of 200-400 μm) was similar between groups (P=0.66, data not shown).

Figure 8. Histological analysis of the lung vasculature. A and B: typical examples of pulmonary arterioles of normoxia-exposed and hypoxia-exposed animals (magnification: x20). C and D: there was a higher wall-to-lumen ratio in hypoxia-exposed animals (blue lines, n=8) compared with normoxia-exposed animals (red lines, n=5) in correlation to the radius of the inner lumen. E and F: shift toward thicker walled vessels (P < 0.05) in histograms of frequency distribution of the wall-to-lumen ratio of pulmonary arterioles.

Table 2. Histological findings of alveolar structure in normoxia-exposed and hypoxia-exposed piglets.

	Normoxia	Hypoxia	P-value
Alveolar area fraction, %	0.82 ± 0.02	0.79 ± 0.02	0.48
Septal length, nm/ μm^2	10.2 ± 0.8	9.7 ± 0.3	0.51
Septal thickness, µm	19.29 ± 2.40	22.34 ± 2.58	0.47

Values are means ± SE; n=5 normoxia-exposed and n=8 hypoxia-exposed piglets. No significant differences in alveolar structure were observed.

Sex differences

The prevalence of BPD has been previously reported to be higher in male than female premature infants[30], and being male is also associated with more severe disease and thus a higher risk for the development of PVD.[31] Therefore, we investigated sex differences in our neonatal piglet model of PVD.

In week 5, PAP at rest was significantly elevated both in male and female hypoxia-exposed animals (Figure 9A). At weeks 6 and 7, PAP had normalized in female hypoxia-exposed piglets. In contrast, in male hypoxia-exposed piglets, PAP remained elevated (Figure 9, B

Figure 9. Mean pulmonary artery pressure (PAP) at rest in male and female piglets raised in normoxia or hypoxia (fraction of inspired O_2 (FiO_2): 10-12%) over time. A: week 5 (postoperative day 1). B: week 6. C: week 7. D: week 8. Numbers per study group are presented in boxes in the bars. Values are means ± SE. * P ≤ 0.05 vs. normoxia; ** P ≤ 0.1 vs. normoxia; † P ≤ 0.05 vs. female piglets; †† P ≤ 0.1 vs. female piglets.

and C). Furthermore, PAP was significantly higher in male compared with female hypoxia-exposed animals in the last week of follow-up (Figure 9D).

The exercise-induced increase in PAP was larger in both hypoxia-exposed male and female piglets compared with normoxia-exposed piglets at week 6. Furthermore, PAP was significantly higher in hypoxia-exposed male piglets compared with hypoxia-exposed female

Figure 10. Changes in pulmonary and systemic hemodynamics during progressive levels of exercise in male and female piglets raised in normoxia or hypoxia. A, C, and E: relation between treadmill speed and mean aortic pressure (MAP). B, D, and F: relation between treadmill speed and mean pulmonary arterial pressure (PAP). Week 6: n=3 normoxia-exposed male piglets, 5 normoxia-exposed female piglets, 4 hypoxia-exposed male piglets and 5 hypoxia-exposed female piglets. Week 7: n=4 normoxia-exposed male piglets, 6 normoxia-exposed female piglets, 4 hypoxia-exposed male piglets and 5 hypoxia-exposed female piglets. Week 8: n=4 normoxia-exposed male piglets, 4 normoxia-exposed female piglets, 2 hypoxia-exposed male piglets and 4 hypoxia-exposed female piglets. Values are means ± SE. * $P \leq 0.05$ vs. normoxia; ** $P \leq 0.1$ vs. normoxia; † $P \leq 0.05$ effect of exercise. ‡ $P \leq 0.05$ vs. response in normoxia; ‡‡ $P \leq 0.1$ vs. response in normoxia. § $P \leq 0.05$ hypoxic male vs. hypoxic female piglets.

Figure 11. Size (area) of the right ventricular lumen in male and female piglets raised in normoxia or hypoxia (fraction of inspired O_2 (FiO_2): 10-12%), obtained by echocardiography, corrected for body-weight over time. A-H: end-diastolic size (A-D) and end-systolic size (E-H) of the right ventricular lumen. A, C, E, and G show data for male piglets; B, D, F, and H show data for female piglets. C and D as well as G and H show the difference in size of the right ventricular lumen compared with baseline (week 1) during end diastole and end systole respectively. n=3 normoxia-exposed male piglets, 5 normoxia-exposed female piglets, 4 hypoxia-exposed male piglets, and 5 hypoxia-exposed female piglets. Values are means ± SE. * $P \leq 0.05$ vs. corresponding sex in normoxia; ** $P \leq 0.1$ vs. corresponding sex in normoxia; † $P \leq 0.05$, male vs. female hypoxia-exposed piglets; †† $P \leq 0.1$, male vs. female hypoxia-exposed piglets.

Figure 12. Systolic pulmonary artery pressure (sPAP) in control subjects (6 men and 4 women) and subjects with transient perinatal pulmonary hypertension (PH; 7 men and 3 women). White bars represent sPAP (as measured with echocardiography) baseline values at sea levels; grey bars represent sPAP values at high altitude (hypoxia). Values are means ± SE. There were no significant differences in baseline sPAP values between the control and PH group and between male and female subjects. * $P \leq 0.05$ vs. male control subjects and vs. female PH subjects (only at high altitude).

piglets at all levels of exercise (Figure 10B), and male hypoxia-exposed swine were unable to complete the exercise protocol at 4 km/h. In the following weeks, PAP of female hypoxia-exposed animals normalized not only at rest but also during exercise (Figure 10, D and F). However, in male hypoxia-exposed animals, PAP levels remained elevated, indicative for chronic PH (Figure 10, D and F). Although MAP was significantly lower in hypoxic animals in week 6 and 7 (–12.8 ± 4.6 and –13.4 ± 5.6 mmHg, respectively), there were no sex differences in MAP during the follow-up period of the present study (Figure 10, A, C, and E).

The more severe disease in males, as described above, was confirmed by the echocardiography data. There was significant RV dilation in male, but not female, animals raised in hypoxia compared with normoxia (Figure 11 A, B, E, and F). The change in the size of the RV compared with baseline was significantly smaller in both male and female hypoxia-raised animals compared with control (Figure 11 C, D, G, and H). However, this change in RV size was even smaller in male compared with female hypoxia-raised piglets, reaching statistical significance at end systole (Figure 11 C, D, G, and H). RV function (RVFAC and TAPSE) did not differ between male and female animals (data not shown). Histological analysis did not show any significant differences between male and female piglets.

DISCUSSION

In the present study, a neonatal swine model for neonatal PVD was developed and characterized during long-term follow-up. The main findings of the present study a.re as follows. First, exposure to chronic hypoxia in early life leads to PH at rest and during exercise, even after reexposure to normoxia. Second, PH reversed ~2 wk after reexposure to normoxia.

Third, thickening of the distal pulmonary arterioles was found at the end of the follow-up period. Fourth, dilation of the RV persisted during follow-up. Finally, similar to clinical observations, male animals showed more severe and more persistent PH than female animals. The implications of the present findings are discussed below.

Methodological considerations

Animal model

To develop clinical therapies and interventions to improve human health, translational research and preclinical testing using animal models are necessary. Swine are widely used as a large animal model, both in cardiovascular research[32] and as a model for the developing human lung.[33-36] Swine lungs share many anatomical, histological, biochemical, and physiological features with human lungs[37], and the relevance of the developing pulmonary circulation of neonatal piglets to human infants was established already in the early 1980s.[38,39] Although alveolar multiplication occurs faster in piglets (2-4 wk compared with 3 yr in human infants), the morphological development of pulmonary architecture in swine is comparable to that in humans.[37] Therefore, in the present study, neonatal piglets were used to develop a model for neonatal PVD. In this large animal model, long-term follow-up is feasible by chronic instrumentation of the animals after 4 wk of exposure to hypoxia, thereby opening new opportunities to investigate the long-term consequences of injury to the developing lung and novel therapeutic strategies. The drawback of this model is that mortality rates are relatively high compared with those reported in studies with older (adolescent) swine. This higher mortality is associated with inherent problems due to premature weaning of the piglets (48 h after birth), leading to a decreased immunity and risk for infections both in the hypoxic period and, especially, after chronic instrumentation (infection of the catheters). In the present study, we experienced that obtaining piglets from a breeder with a high health status in combination with good hygienic conditions, age-appropriate diet, and supplementary feed to support the immune system did reduce the mortality rates. Furthermore, chronic instrumentation of such young piglets is challenging as the surgical area and anatomic structures are smaller, and it is more difficult to wean the piglets off the ventilator during recovery from surgery. Finally, in the present study, we chose not to acquire hemodynamic data during the neonatal period, as this has previously been studied by Camelo et al.[19] and would preclude prolonged follow-up because of the large weight gain of the piglets in the first weeks of life, which would result in catheter failure.

Flowprobe

To measure CO, a flow probe was placed around the pulmonary artery. Unfortunately, the flow probe caused local tissue fibrosis, resulting in narrowing of the pulmonary artery. Hemodynamic signs of pulmonary artery stenosis (more rounded flow profile) started 4 wk after chronic instrumentation. Indeed, at euthanasia 6 wk after chronic instrumentation,

pulmonary artery narrowing was found at the site of the flow probe, thereby increasing RV afterload and contributing to RV hypertrophy. Therefore, a subset of animals without a flow probe was included, and hemodynamic follow-up was limited to 4 wk after chronic instrumentation (total of 8 wk). Although technically challenging, placing the flow probe around the aorta in future studies will allow measurement of CO while circumventing this problem.

Characterization of the neonatal swine model for neonatal PVD

Several animal models of neonatal PVD and PH, including a neonatal swine model with hypoxia-induced PH, have been previously established.[12-19] In the present study, resting PAP decreased over time after reexposure to normoxia in hypoxia-exposed animals, and overt PH (defined as mean PAP ≥ 25 mmHg) was no longer consistently present 2 wk after reexposure to normoxia. Because resting PAP in the control normoxic group increased slightly over time (Figure 3), there was no significant difference in resting PAP between animals raised in normoxic and hypoxic conditions at the end of follow-up. However, although PH is an important diagnostic criterion for PVD, PVD also encompasses abnormalities in vascular growth, structure, tone, and reactivity without overt PH.[40,41] Morbidity and mortality associated with this condition strongly depend on the degree of adaptation of the RV to the high pulmonary load.[42] Consequently, RV function and hypertrophy as well as systolic PAP, measured with echocardiography, are presently the most important parameters in the diagnosis and follow-up of patients with PVD.[40,41] Consistent with these criteria, we observed echocardiographically determined dilation of the RV, which persisted during the entire follow-up. Furthermore, abnormalities in the pulmonary vascular structure, found in histological sections, also confirm the diagnosis of PVD.

Chronic exposure to a hypoxic environment is thought to be a key element in the development of neonatal PVD in this swine model. However, in the clinical setting, premature infants experience episodes of intermittent hypoxia and normoxia (relative hyperoxia). These hypoxic and relative hyperoxic episodes in the neonatal period result in the activation of various signal transduction pathways.[43] Hypoxia-inducible factors (HIFs) are transcription factors that play a critical role in regulating the responses to hypoxia. These transcription factors activate the transcription of sets of genes essential for cell survival and angiogenesis, including vascular endothelial growth factor (VEGF).[44,45] Under hypoxic conditions, both HIF-1α and HIF-2α accumulate instantaneously, whereas HIF-1α, but not HIF-2α, protein disappears when hypoxia is sustained (>12h).[46] Reoxygenation results in the rapid degradation of both HIF-1α and HIF-2α (51). In the present study, the incubator was opened once or twice a day for up to 1 h to provide appropriate animal care, resulting in daily reexposure to normoxia for a short period of time. Not only will this period of reoxygenation result in rapid degradation of HIF-2α, but also subsequent reexposure to hypoxia will provide a "new" acute decrease in pO_2, leading to a rapid increased in HIF-1α and HIF-2α. Both HIF

isomers have distinct roles in the pulmonary vascular response to hypoxia; HIF-1α promotes pulmonary vascular smooth muscle cell proliferation, whereas HIF-2α promotes pulmonary vascular endothelial cell proliferation.[47] Both HIF-1α and HIF-2α can therefore contribute to the pulmonary vascular remodeling and PH in chronic hypoxic conditions.[47-49]

The present study shows that exposure of neonatal piglets to a hypoxic environment (FiO_2: 10-12%) for 4 wk results in PH as evidenced by a significant increase in PAP and a decrease in pulmonary vascular conductance compared with age-matched piglets maintained under normoxic conditions. These findings are consistent with previous reports of other investigators demonstrating PAPs of ~25-30 mmHg after exposure to chronic hypoxia.[12-18] In contrast to the present study, in which hemodynamic measurements were obtained in conscious piglets, in those previous studies, hemodynamic measurements were all obtained under anesthesia. Only Camelo et al.[19] performed hemodynamic measurements in conscious piglets for up to 6 days of hypoxia (FiO_2: 12%), and they found that PAP levels were ~35 mmHg and 45 mmHg after 2 and 6 days of hypoxia, respectively. These levels of PAP during hypoxia are much higher than the PAP values found in the present study 1 wk after reexposure to normoxia fafter a 2-wk period of hypoxia. It is likely that the measurements by Camelo et al. after 6 days of hypoxia principally reflect hypoxic pulmonary vasoconstriction, while our measurements after reexposure to normoxia probably reflect pulmonary vascular remodeling.

An important new finding of the present study is that incremental exercise results in exacerbated elevations of PAP, which were due to a loss of pulmonary vasodilator capacity after chronic exposure to hypoxia. Particularly in the first week after surgery, exercise capacity was limited in male animals exposed to hypoxia. This observation corresponds well with clinical studies in long-term survivors of prematurity and/or neonatal PVD, who also demonstrated significantly impaired exercise performance.[50-54]

To our knowledge, the present study is the first to comprehensively investigate long-term consequences of injury to the developing lung. It confirms clinical observations that PH induced by chronic hypoxia is transient, with PAP normalizing within 2 wk after reexposure to normoxia. However, despite normalization of PAP, structural and functional changes in the RV and lung vasculature were still present, as evidenced by weekly echocardiography and lung histology. In accordance with these findings, Lewandowski et al.[55] showed with cardiovascular magnetic resonance imaging that preterm birth is associated with global myocardial structural and functional differences even in adult life, with potentially clinically significant impairments in RV systolic function. In contrast to our study, Lewandowski et al. found a smaller RV size, which may be explained by the longer follow-up period compared with the present study. Sartori et al.[56] also showed long-term effects of transient perinatal pulmonary hypertension on the pulmonary vasculature. These authors found an exaggerated altitude-induced increase in systolic PAP and hence exacerbated pulmonary vasoconstriction in young adults with perinatal transient PH compared with age-matched controls [56]. These

data are consistent with perinatal programming of the pulmonary vasculature and right ventricle in which a transient perinatal insult to the pulmonary circulation has persistent effects into adulthood. This possibly results in an increased risk for cardiovascular events later in life, such as RV failure, thereby contributing disproportionately to the burden of adult cardiovascular disease in the future.[57]

Future studies are necessary to determine whether these long-term pulmonary consequences of early exposure to hypoxia are also emulated in our swine model of perinatal PH.

Sex differences

The prevalence of BPD is higher in male compared with female premature infants[30], and being male is also associated with more severe disease and thus a higher risk for the development neonatal PVD.[31] Little is known about sex differences in the long-term outcome of neonatal PVD. As mentioned above, Sartori et al.[56] showed a greater altitude-induced increase in systolic PAP in young adults who had had transient PH. Reanalyses of these data showed that men with perinatal transient PH displayed significantly higher systolic PAP at high altitude than both male controls and women with perinatal transient PH, whereas baseline systolic PAP levels were not significantly different between all groups (Figure 12). These data suggest that a transient perinatal insult to the pulmonary circulation results in exaggerated pulmonary vasoreactivity in men but not in women. Although the numbers were small, significant sex differences were present in our study. Confirming the clinical data mentioned above, male hypoxia-exposed piglets demonstrated more severe disease than female hypoxia-exposed piglets, as evidenced by a limitation of their exercise capacity, particularly in week 6, that was accompanied by a higher PAP, which persisted for a longer period (chronic PH), and more pronounced RV dilation in male hypoxia-exposed piglets compared with female hypoxia-exposed piglets.

Conclusion and clinical implications

There is evidence that neonatal PVD may lead to an increased risk for cardiovascular events later in life, including RV failure, thereby contributing significantly to the future burden of adult cardiovascular disease.[41] Our neonatal swine model shows clinical features resembling those found in patients with neonatal PVD (including transient PH), in terms of pulmonary hemodynamics, abnormalities in the structure of the RV, and disruptions in normal lung development (vascular remodeling). Consistent with clinical practice, male swine develop more severe and more persistent PVD, have a limited exercise capacity, and exhibit more pronounced RV remodeling during follow-up in normoxia.

In the present study, only the cardiovascular responses to acute exercise were assessed. Future studies should also investigate the effect of exercise training on long-term outcome, as exercise training has been shown to be beneficial in adult patients with pulmonary arterial hypertension of any cause.[58,59] Training is associated with improved pulmonary perfusion[60],

blood gas exchange[61] and RV function[62-64]. Furthermore, exercise training has been shown to reduce smooth muscle cell proliferation[59]. Altogether, these beneficial effects improve exercise capacity and may reduce PAP, thereby improving quality of life.[58,59]

Several interventions in the neonatal period, including oral L-citrulline[12,14], thromboxane inhibition[15], angiotensin II type 1 receptor blockade[19] and endothelin-A receptor antagonists[16], have been shown to ameliorate PH and/or pulmonary vascular remodeling in a similar porcine model. However, the long-term outcome of these interventions remains to be established. Our model is an excellent model to examine long-term sequelae of damage to the developing lung and the effect of exercise training and/ or pharmacotherapy on long-term outcome as well as the molecular mechanisms underlying potential beneficial effects

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