Injury to the developing pulmonary vasculature
Short- and long-term effects

Daphne de Wijs-Meijler

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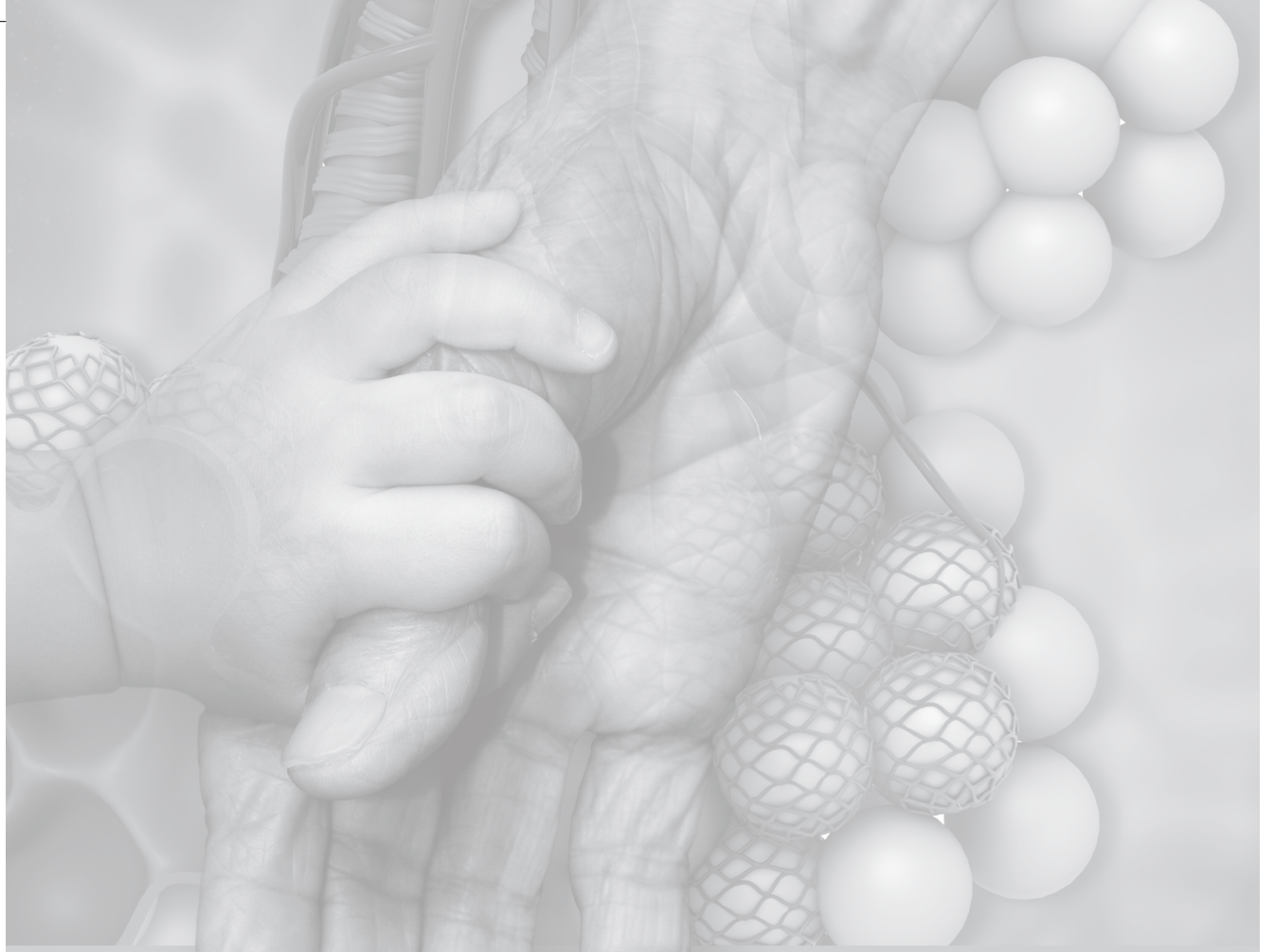

Chapter 1

General introduction and outline of the thesis

1. GENERAL INTRODUCTION

Prematurity is defined as childbirth that occurs before 37 completed weeks or 259 days of pregnancy. Worldwide, an estimated 15 million babies are born prematurely each year. That is more than 1 in 10 babies, affecting families all around the world.[1,2] In the Netherlands, about 7% of all live births were preterm (in 2015).[3]

Prematurity is the leading cause of newborn deaths (babies in the first 4 weeks of life) and death in children under the age of 5. Over 1 million children die each year due to complication of preterm birth.[2] However, improved neonatal care has dramatically increased the survival of premature babies. Furthermore, neonatal survival is extending to lower and lower extremes of gestational age. Survivors of prematurity, in turn, face specific health problems, many of which are unique to the preterm population. Because they are born before they are physically ready to face the world, these babies often require special care and are at risk for developing complications that result from anatomic and functional immaturity, like feeding difficulties, brain injury, severe infections and respiratory illnesses.[4,5]

The morbidity associated with preterm birth often extends beyond the neonatal period and throughout the life cycle. Premature babies are at greater risk for significant health problems later in life, such as neurodevelopmental disabilities and cognitive impairments, hypertension, metabolic syndrome and diabetes, respiratory abnormalities and exercise intolerance.[6]

Because they develop late in the embryo and are even far from mature at birth, the lungs appear to be most susceptible to damage in premature babies. Chronic respiratory morbidity is the most common serious adverse outcome affecting premature infants born prior to 32 weeks pregnancy, with up to 40% (23-73%) of preterm survivors having bronchopulmonary dysplasia (BPD). BPD, a severe chronic lung disease defined as supplemental oxygen requirement for at least 28 days,[7] is recognized a consequence of disrupted lung development, and is characterized by an arrest in vascular and alveolar growth,[7-9] as will be outlined in more detail in the next paragraphs.

1.1 Lung development

The lungs are the primary organs of the respiratory system. Already in the 4th week of gestation the development of the lungs starts with the appearance of a primitive lung bud from the ventral surface of the foregut. Lung development continues in five histological stages, namely embryonic (week 4-7), pseudoglandular (week 8-16), canalicular (week 17-25), saccular (week 26-38) and alveolar stages (week 38 – 3 year). During these stages, the respiratory tract develops by a branching process, which forms the bronchi, bronchioles, and ultimately the alveoli. The alveoli are thin walled small air sacs, located in the respiratory zone of the lungs and representing the smallest units in the respiratory tract, where air exchange occur. The number of alveoli in each lung increases from zero at 32 weeks' gestation to 50-150 million alveoli in term infants and 300-500 million in adults.[10-12]

At the same time and in the same spatial pattern, the pulmonary vasculature develops.[10,11,13,14] The target of bronchopulmonary development is the formation of an effective gas exchange organ where blood and air are in intimate contact of a large surface area. The major function of the lungs is to oxygenate blood and to clear carbon dioxide (CO_2) from the blood by gas exchange; the process of diffusion of oxygen from the inspired air into the blood and carbon dioxide out of the blood into the expired air. The blood gases in the pulmonary capillaries equilibrate with those in the alveolar air across the blood-air barrier, a very thin (≈ 2μm) diffusion membrane consisting of the walls of the alveoli and the endothelial cells of the pulmonary capillaries. Once oxygen is passively diffused to the blood, it binds to hemoglobin in red blood cells or dissolves in the plasma, is spread evenly throughout the body by the left ventricle, where it is used to sustain aerobic metabolism.

Premature infants are born in a critical stage of lung development (saccular or alveolar stage). At this stage, the immature lung has poorly developed airways with a much smaller surface area and relatively thick septa, insufficient for gas exchange. Furthermore, there is a surfactant deficiency, a decreased compliance, underdeveloped antioxidant mechanisms and inadequate fluid clearance. After birth, lungs of premature infants are exposed to several injurious stimuli, including hypoxia and/or hyperoxia, mechanical ventilation, infection and inflammation. Early injury to the developing lung can impair alveolarization, which result in simplification of the distal lung airspace and clinical manifestations of BPD.[14]

1.2 Pulmonary circulation

Pulmonary circulation refers to the movement of blood from the right ventricle, to the lungs via the pulmonary arteries and back to the left atrium via the pulmonary veins. Before birth, only 10% of the blood pumped out by the right ventricle enters the lungs, since the placenta, and not the lung, function as the organ of gas exchange.[15] This is due to a high pulmonary vascular resistance (PVR), driving the flow of blood away from the pulmonary circulation to the systemic and placental circulation, leading to a right-to-left shunt through the ductus arteriosus and foramen ovale. The high PVR in the fetus is maintained by compression of pulmonary vessels by the fluid-filled lungs, lack of rhythmic distention of the lungs (breathing) and hypoxic pulmonary vasoconstriction due to low alveolar oxygen tension. Humoral mediators such as endothelin-1 and lack of vasodilators such as nitric oxide (NO) also contribute to the high PVR.[16,17]

At birth, an impressive fall in PVR and an increase in systemic vascular resistance results in the transition from fetal to an adult circulation, including the closure of the ductus arteriosus and foramen ovale. Various mechanical factors and vasoactive agent signaling pathways contribute to this fall in PVR. Of these factors, pulmonary endothelial NO, acting via the cyclic guanosine monophosphate (cGMP) pathway, mediate pulmonary vasodilation and has a great importance in normal physiological pulmonary transition.[18,19]

As mentioned before, the development of the pulmonary vasculature is closely related to that of the airways. Vasculogenesis (de novo formation of blood vessels from angioblasts or endothelial progenitor cells) and angiogenesis (formation of blood vessels by direct extension of pre-existing vasculature) are the principal mechanisms governing the formation of the pulmonary vasculature.[20] Premature birth not only have deleterious impact on the development of the airways and alveoli, as mentioned before, but also cause early disruption of angiogenesis and vasculogenesis. Such disruption leads to a decreased vessel density, and thus to a reduction in the cross-sectional area of the pulmonary vasculature. Furthermore, hypoplasia of the pulmonary vasculature in combination with the underdeveloped airways results in hypoxic vasoconstriction. Chronic hypoxia in the lung tissues also alters vasoreactivity. Together, vascular simplification, hypoxic vasoconstriction and increased vasoreactivity lead to an increased PVR and causes structural remodeling with intimal hyperplasia and increased muscularization of small pulmonary arteries (pulmonary vascular disease; PVD).[21] Moreover, premature birth is associated with exposure to several injurious stimuli after birth. Intermittent hypoxia and hyperoxia, mechanical ventilation and infection/inflammation aggravate pulmonary vascular remodeling. If not prevented from progression, structural remodeling can result in pulmonary hypertension (mean pulmonary artery pressure ≥ 25mmHg), and ultimately right heart failure and death.[21,22]

The incompletely understood pathogenic cascade, as well as the absence of an effective treatment for neonatal pulmonary vascular disease (PVD) and PH renders neonatal PVD an urgent call for research. Additionally, with the increase in longevity of preterm infants with neonatal PVD and/or PH it is of critical significance to study long-term outcomes of this disease. Until now, most studies concerning long-term health outcomes have focused on respiratory outcomes. However, less is known about cardiovascular function in survivors of neonatal PVD.

1.3 Pulmonary vascular tone

Pulmonary hypertension, irrespective of the cause, is characterized by an increase in PVR. PVR is defined as mean pulmonary artery pressure minus mean pulmonary backpressure divided by cardiac output. The regulation of PVR occurs by changing the diameter of blood vessels, and the changes in vascular diameter are the sum of both passive (structural and mechanical) and active (smooth muscle tone) influences.

1.3.1 Passive influences

In the pulmonary circulation, there are two passive mechanisms at work being recruitment and distension of the small vessels.[23] Under normal conditions, when pulmonary artery pressure is low, perfusion pressures of pulmonary vessels vary between different lung segments. As pressure increases, vessels that were open but not conducting blood or were even closed are recruited simultaneously, thereby decreasing PVR. Moreover, the wall of the pulmonary

vessels is relatively thin, resulting in a large compliance that allows the pulmonary vessels to distend in response to increases in pulmonary pressure, leading to a further reduction of PVR. Although passive influences in the regulation of vascular diameter and resistance are important, this thesis focuses mainly on active regulation of pulmonary vascular tone.

1.3.2 Active regulation

Pulmonary vessels, like other blood vessels, have an inner lining of endothelial cells, which are surrounded by vascular smooth muscle cells (except the capillaries). Pulmonary vascular tone refers to the state of contraction of these vascular smooth muscle cells. It is the result of a complex interplay between a multitude of contracting (vasoconstrictor) and relaxing (vasodilator) factors that influence smooth muscle cell contraction or relaxation and thus the vascular diameter, thereby determining PVR. The factors that regulate pulmonary vascular tone can be divided in neurohumoral, mechanical, metabolic, endocrine, paracrine and endothelial influences. In addition, many other vasoactive factors have been shown to influence pulmonary vascular tone, including reactive oxygen species (ROS) and phosphodiesterases (PDE).[24,25]

In this thesis, we mainly focus on endothelial control of pulmonary vascular tone, and particularly the nitric oxide pathway (nitric oxide, PDE and ROS).

1.3.2.1 Nitric oxide

Nitric oxide was firstly described as an endothelial derived relaxing factor. It is synthetized in the endothelium from L-arginine by endothelial NO synthase (eNOS). eNOS is activated by mechanical forces (i.e. an increase in shear stress exerted by the blood flow on the endothelium) as well as by a host of chemical factors such as bradykinin, acetylcholine, substance P and noradrenaline acting on their respective receptors on the endothelium.[24] NO diffuses to the underlying smooth muscles, where it activates soluble gyanylyl cyclase (sGC), resulting in the production of cGMP. cGMP causes smooth muscle cell relaxation by activating protein kinase G (PKG), resulting in lowering intracellular Ca^{2+} and activation of myosin phosphatase, leading to a decrease in the sensitivity of the contractile apparatus to Ca^{2+}.[26]

NO has been implicated in normal pulmonary vascularization by stimulating endothelial proliferation through the VEGF-NO pathway. NO is an important downstream target for the proliferative effects of VEGF and for the differentiation of developing pulmonary artery endothelial cells. Furthermore, NO plays a critical role in the rapid fall in PVR during normal pulmonary perinatal transition. At birth, oxygenation and shear stress acutely increase NO production by increasing eNOS activity and by upregulating its expression. These mechanisms are likely to be involved in sustained reduction in PVR. Therefore, disruption of the NO pathway leads to impairment of pulmonary microvascular formation and has been implicated in the pathogenesis of (neonatal) PVD and PH.[10,21,27] However, the exact underlying pathophysiologic mechanisms remain incompletely understood.

Since there is increasing evidence that alterations in the NO-cGMP signaling pathway play an important role in the pathogenesis of neonatal PVD and PH, inhaled NO (iNO) was widely used in neonatal intensive care units as rescue therapy for preterm infants with respiratory disease undergoing ventilation. However, iNO treatment in premature infants (≤ 34 weeks) shows equivocal effects on pulmonary outcomes and survival and its use for preterm infants with respiratory failure is currently controversial.[28-30]

1.3.2.2 Phosphodiesterases

PDEs are enzymes responsible for the degradation of cyclic nucleotide second messengers cAMP and cGMP. Therefore, inhibition of PDEs in vascular smooth muscle has been recognized a powerful tool to reduce vascular tone by prolonging the half-life of cAMP and/or cGMP. To date, at least 11 different families of PDEs have been identified, all with different kinetic properties, localization and function.[31] The PDE isoform that are predominately present in vascular smooth muscle cells are PDE1, 3, 4, 5, 7 and 9.[32] Because the expression of PDE5 is 10 times more abundant in the pulmonary as compared to the systemic circulation, PDE5 inhibition preferentially dilates the pulmonary vasculature, with relatively little systemic vasodilation, and has been clinically validated as an effective treatment for PH.[33-35]

Oral sildenafil, a selective PDE5 inhibitor, was approved by the U.S. Food and Drug Administration (FDA) in 2005 for the treatment of PH in adults. Despite the safety and efficacy in pediatric patients had not been established, the drug has become a major component in the treatment of pediatric PH. However, there is a lack of licensing for its use in children below 1 year of age, meaning a significant number of patients are outside the approved remit including children with BPD-PH.[36] In 2013, the U.S. Food and Drug Administration (FDA) cautioned against the use of sildenafil in children with PH in light of the increases in mortality in children receiving high doses.[37] Despite such setbacks, sildenafil continues to be used off-license. Several recent studies, both in human and animals, are encouraging; sildenafil treatment in patient with BPD-PH was associated with improvement in clinical and hemodynamic parameters and a low mortality rate,[38,39] and sildenafil promoted adequate lung angiogenesis, decreased PVR, right ventricle hypertrophy and arterial medial wall thickness in newborn rats.[40]

1.3.2.3 Reactive oxygen species

Reactive oxygen species (ROS) are highly reactive oxygen-containing molecules with an unpaired electron.[41] Because of their highly reactive nature, ROS can react with various intracellular proteins and alter their structure and function. Small amounts of ROS are continuously produced in the human body, mainly during ATP production in the mitochondria, and have been shown to play a role in many signaling processes.[42-47] However, in order to prevent deleterious effects of ROS and to maintain proper cellular function, the amount of ROS needs to be carefully controlled. Under normal physiological conditions, most ROS

are scavenged by the anti-oxidant systems of the body (antioxidant vitamins and endogenous antioxidants such as superoxide dismutase, catalase and glutathione peroxidase).[42-48]

Because the pulmonary vasculature is by nature exposed to high levels of oxygen, production of ROS is likely to be more prominent in the pulmonary vasculature as compared to the systemic vasculature.[48] Modest variations in the balance between production and scavenging of ROS may contribute to regulation of normal function of the pulmonary vasculature (redox signaling).[49,50] The increase in ROS production results in pulmonary vasoconstriction and increase in PVR in vitro and in vivo.[51-54]

Neonates, and especially those who are born premature, are particularly vulnerable to oxygen toxicity, as their levels of antioxidant enzymes are inadequate and unable to protect the rapidly growing tissues, including the developing lung, from oxidative injury.[44,55-58] In the developing lung, oxidative stress lead to inactivation of surfactant, cellular dysfunction, and impaired cell survival, thereby playing a critical role in the pathogenesis and pathophysiology of neonatal PVD.[14,57-60]

1.4 Developmental Origins of Health and Disease

Nowadays, there is growing evidence that disruption of normal pulmonary vascular development in early life contributes to the development of PVD in adult life. In the late 1990s, it was already shown that a transient perinatal insult to the pulmonary circulation increases the risk of developing pulmonary hypertension.[61] It has also been shown that pulmonary artery pressure is elevated in offspring of mothers with pre-eclampsia, demonstrating that placental hypoxia causes pulmonary vascular dysfunction.[62] Underlying mechanisms of this so-called "fetal or perinatal programming" are currently unknown.

In view of the growing cohort of adult survivors of prematurity and/or neonatal PVD, more research into the long-term consequences of perinatal pulmonary vascular events is imperative. Little is known about the cardiovascular function in this is relatively new patient population. More research to the long-term cardiovascular outcomes is necessary in order to improve the health of prematurely born survivors of neonatal PVD and to reduce the burden of adult cardiopulmonary morbidity and mortality.

2. AIMS AND OUTLINE OF THE THESIS

The general aim of this thesis is 1) to study peri- and neonatal (mal)adaptation, and 2) to investigate endothelial function in the adolescent pulmonary vasculature, both in an intact animal model of swine as well as in isolated small pulmonary arteries.

2.1 Peri- and neonatal (mal)adaptation

The main focus of this section is the effect of injurious stimuli in the peri-and neonatal period to the pulmonary vasculature. Both premature birth—with incomplete vascular growth, immature vascular function, and decreased host defenses—as well as exposure to injurious stimuli after birth, contribute to an abnormal development of the lung circulation.

Reactive oxygen species play a key role in the pathogenesis of neonatal PVD and can be caused by hyperoxia, mechanical ventilation, hypoxia, and inflammation. **Chapter 2** gives an overview of short- and long-term consequences of oxidative injury to the perinatal lung for the human cardiovascular system.

Failure of normal lung development will lead to neonatal PVD due to an altered function of the pulmonary vessels (with an increased vasomotor tone), as well as an altered structure of the pulmonary vasculature, i.e. vascular remodeling (including smooth muscle cell proliferation). PVD represents an underestimated and increasing clinical burden in the neonatal period, but also later in life. Despite decades of research, the exact mechanisms underlying PVD as well as to what extend PVD contributes to long-term cardiovascular morbidity and mortality are currently unknown. Consequently, we developed a new swine model for neonatal PVD allowing follow-up. In **Chapter 3** we demonstrate the surgical placement of catheters for long-term cardiovascular follow-up at rest and during exercise testing. **Chapter 4** describes the development and characteristics of the swine model of neonatal PVD, which 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.

It is well known that neonatal PVD is associated with multiple disruptions in the NO-cGMP signaling pathway, such as a decreased eNOS activity and reduced vasodilator response to NO.[63-68] However, little is known about disruptions more downstream in this pathway, including sGC- and cGMP-dependent mechanisms. Therefore, we investigated in **Chapter 5** the functionality of different parts of the NO-cGMP signaling pathway in the long-term, in vivo (at rest and during incremental exercise) and in vitro.

2.2 Endothelial function in the adolescent pulmonary vasculature

Endothelial function is a key factor in vascular development as well as in maintenance of vascular structure and function throughout life. Besides endothelial dysfunction is a crucial factor in neonatal PVD, it plays a crucial role in the pathogenesis of adult PVD, includ-

ing PH.[69-71] While the endothelial function of the systemic and coronary circulation is extensively investigated, studies into the endothelial function of the pulmonary vasculature received less attention. Therefore, in the second part of this thesis we present the results of studies concerning pulmonary endothelial function and vascular control.

Although the incidence of PH is higher in females, the severity and prognosis of PVD have been shown to be worse in male patients.[72,73] Until now, studies concerning sex differences in PH have mainly focused on the role of sex hormones. As it is unknown whether intrinsic sex-related differences in the NO-cGMP signaling pathway contributes to these difference between males and females, we investigated pulmonary vascular function in male and female swine in vivo and in vitro (**Chapter 6**).

By mimicking some aspects of endothelial dysfunction using hemoglobin-based oxygen carrier (HBOC)-201, an important pathogenic factor in PVD can be studied. HBOC-201 administration resulted in pulmonary (and systemic) vasoconstriction and thus elevated blood pressures. In **Chapter 7**, we determined the potential roles of NO, ROS and endothelin (ET) in mediating the observed vasoconstriction in resting and exercising swine.

As described earlier, PDE5 inhibition with sildenafil has been used as a therapeutic tool in treating patients with PH. ET receptor blockade has also been shown to induce pulmonary vasodilation and is also clinically used in patients with PH. However, little is known about whether the combination if those two treatments may have additional therapeutic effects. Therefore, in **Chapter 8**, we studied the effects of combined treatment of PDE5 inhibition and ET receptor blockade in the pulmonary circulation, as well as the mechanisms of interaction between the PDE5 and ET systems.

In the summary and general discussion (**Chapter 9**) the overall findings of this thesis, general considerations, recommendations and future perspectives will be addressed. Finally, a Dutch summary is provided in **Chapter 10**.

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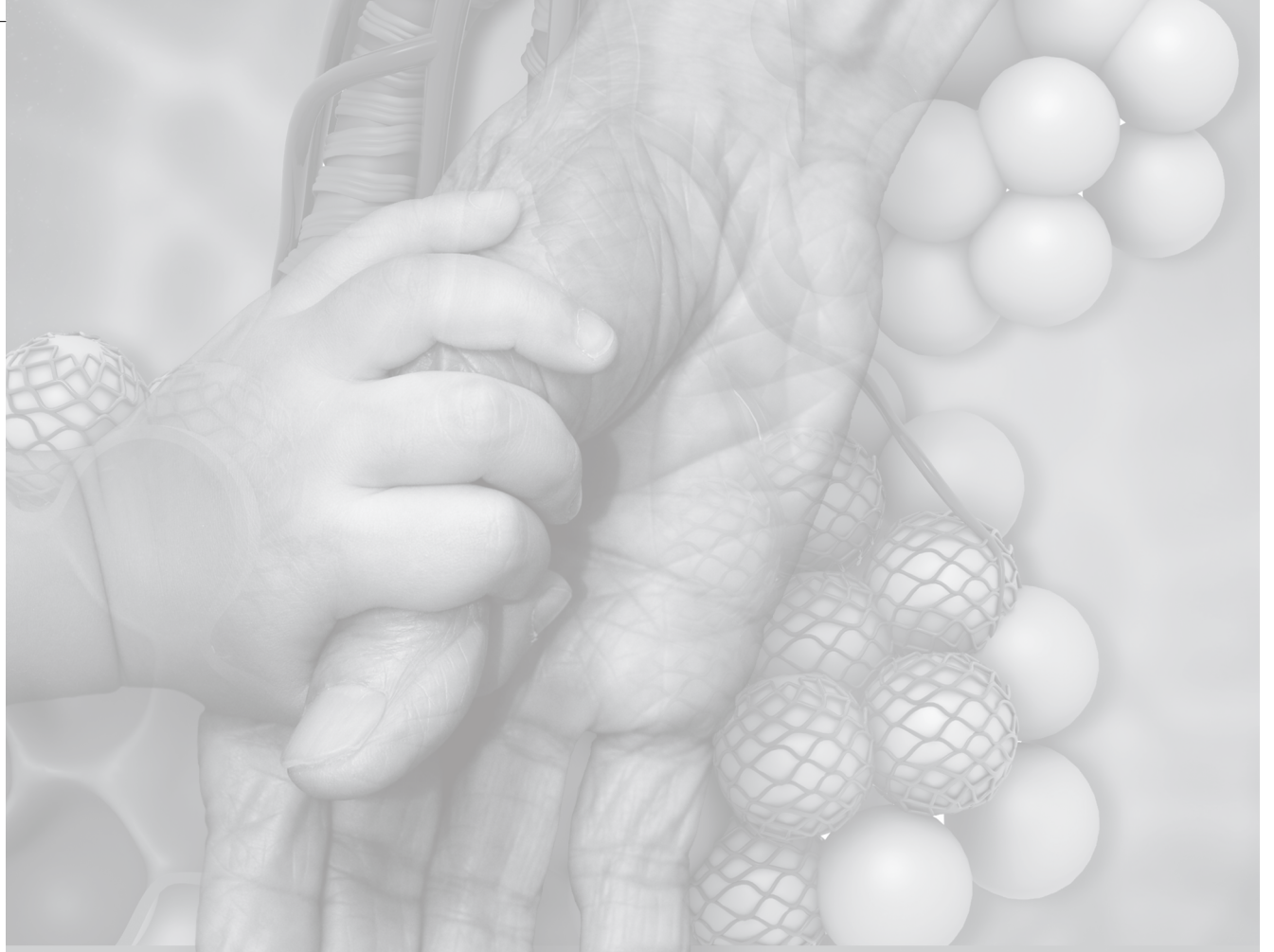
Part 1

Peri- and neonatal (mal)adaptation



Chapter 2

Oxidative injury of the pulmonary circulation in the perinatal period: short- and long-term consequences for the human cardiopulmonary system

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ABSTRACT

Development of the pulmonary circulation is a complex process with a spatial pattern that is tightly controlled. This process is vulnerable for disruption by various events in the prenatal and early postnatal periods. Disruption of normal pulmonary vascular development leads to abnormal structure and function of the lung vasculature, causing neonatal pulmonary vascular diseases. Premature babies are especially at risk of the development of these diseases, including persistent pulmonary hypertension and bronchopulmonary dysplasia. Reactive oxygen species play a key role in the pathogenesis of neonatal pulmonary vascular diseases and can be caused by hyperoxia, mechanical ventilation, hypoxia, and inflammation. Besides the well-established short-term consequences, exposure of the developing lung to injurious stimuli in the perinatal period, including oxidative stress, may also contribute to the development of pulmonary vascular diseases later in life, through so-called "fetal or perinatal programming." Because of these long-term consequences, it is important to develop a follow-up program tailored to adolescent survivors of neonatal pulmonary vascular diseases, aimed at early detection of adult pulmonary vascular diseases, and thereby opening the possibility of early intervention and interfering with disease progression. This review focuses on pathophysiologic events in the perinatal period that have been shown to disrupt human normal pulmonary vascular development, leading to neonatal pulmonary vascular diseases that can extend even into adulthood. This knowledge may be particularly important for ex-premature adults who are at risk of the long-term consequences of pulmonary vascular diseases, thereby contributing disproportionately to the burden of adult cardiovascular disease in the future.

INTRODUCTION

The development of the pulmonary vasculature is a highly complex process, in which temporal and spatial expression of multiple morphogens, transcription factors, and growth factors regulates the different stages of development.[1-3] This complex process of normal lung development, with its tight regulation of the expression of numerous regulators, can be disrupted at multiple levels and in various stages of development. Such disruption of normal development of the pulmonary vasculature plays a pivotal role in the pathogenesis of several neonatal pulmonary vascular diseases, including persistent pulmonary hypertension of the newborn (PPHN) and bronchopulmonary dysplasia (BPD). Understanding the response of the developing lung to injury, and its repair mechanisms, is of great importance for elucidating pathogenic processes.

The development of the lung starts with the appearance of a primitive lung bud, which splits to form the left and the right lung. In the human embryo, at day 34 of gestation, each lung bud is already supplied by a pulmonary artery extending from the outflow tract of the heart, which is connected to a primary capillary plexus and traced back via a vein to the prospective left atrium.[2,4,5] Lung development continues in 5 different stages (embryonic, pseudoglandular, canalicular, saccular and alveolar). The alveolar stage, when the gas-exchanging surface area develops, starts at week 36 of gestation and continues after birth, even up to the third year of life (Figure 1).[2,5,6]

The development of the pulmonary vasculature is closely related to that of the airways, as they develop at the same time and follow the same spatial pattern (Figure 1). Growing

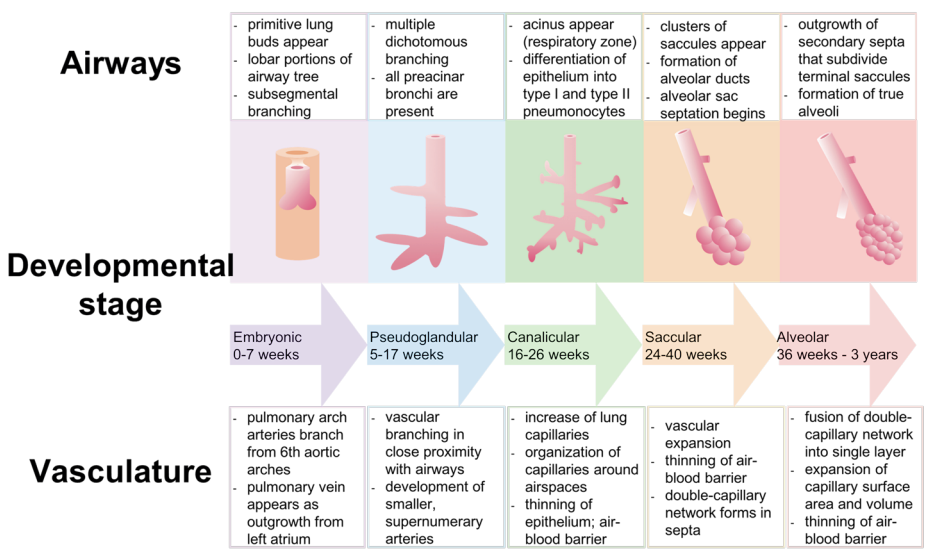

Figure 1. Diagram illustrating normal lung development: airway structure and pulmonary vasculature.

evidence exists that tissue interactions of lung mesenchyme, epithelium, and endothelium are critical for both branching of the airways and growth and differentiation of the pulmonary vasculature.[4,5,7,8]

The formation of the pulmonary vasculature is governed by two principal mechanisms: vasculogenesis and angiogenesis. Vasculogenesis is the process by which angioblasts or endothelial progenitor cells differentiate to vascular endothelial cells and form blood vessels de novo. In angiogenesis, new blood vessels arise by direct extension of pre-existing vessels.[8-10] The relative contribution of vasculogenesis and angiogenesis to vessel formation in the developing lung is still incompletely understood, but three hypotheses have been forwarded.[10] One hypothesis is that the proximal vasculature develops through angiogenesis, while the distal vessels are formed by vasculogenesis. In the pseudoglandular phase, these two structures then fuse through a lytic process.[11] The second hypothesis proposes that new arteries are derived from a continuous expansion and coalescence of the primary capillary plexus around the terminal airways, and thus principally from vasculogenesis.[4] The third hypothesis proposes that lung vascular development occurs through distal angiogenesis, through the formation of new capillaries from pre-existing vessels at the periphery of the lung as the lung bud grows.[12]

Pulmonary vascular development is regulated by interplay between many different factors. Although a lot of knowledge about the pulmonary vasculature development is obtained through experiments in rodents, we will focus on vascular endothelial growth factor (VEGF) and transforming growth factor-beta (TGF-β), because their roles have also been confirmed in human samples. Among all different regulators of lung vascular development, VEGF is a frequently studied and crucial regulator of normal pulmonary vascular development. VEGF induces angiogenesis and is a key player in the regulation of vasculogenesis. [13] VEGF exerts its effect by binding to two trans-membrane tyrosine-kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1), which are strongly expressed in endothelial cells. In addition to its mandatory role in the development of the pulmonary vasculature, VEGF also plays an important role in epithelial branching morphogenesis and alveolar development.[14,15] VEGF expression is regulated by hypoxia-inducible factors (HIF)-1 and 2, which are transcriptional complexes responding to changes in oxygen levels. Normal lung development takes place in the relatively hypoxic environment of the uterus. This “Everest in utero” stabilizes the HIF-complex, leading to transcription of hypoxic responsive target genes, such as VEGF, thereby stimulating epithelial branching and vascular development.[16-18]

Another important growth factor in normal pulmonary vascular development is transforming growth factor-beta (TGF-β). The exact role of TGF-β signaling in lung development is not known yet. However, it appears to play a key role in epithelial-mesenchymal as well as endothelial-mesenchymal interactions. Thus, tightly regulated temporal and spatial TGF-β signaling is necessary for both normal branching morphogenesis and pulmonary vascular development.[19-21] A role for TGF-β signaling in pulmonary vascular development is further

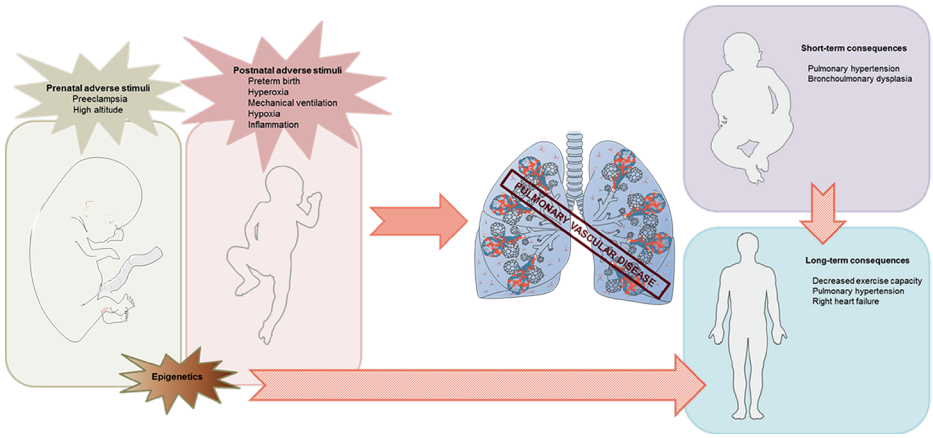

Figure 2. Schematic illustrating perinatal adverse stimuli contributing to pulmonary vascular disease that can extend even into adulthood.

underlined by the observation that mutations in the bone-morphogenetic protein receptor II (BMP-RII), which lead to a disturbed balance between TGF-β -and BMP-signaling, as well as in endoglin, a co-receptor of the TGF-β receptors, are associated with pulmonary arterial hypertension.[22,23]

In this review, we will focus on several pathophysiologic insults in the prenatal and early postnatal period, which can disrupt normal pulmonary vascular development and, consequently, lead to a variety of neonatal pulmonary vascular diseases that can extend even into adulthood (Figure 2). Most of these insults cause oxidative injury of the pulmonary vasculature, which will therefore be discussed in more detail. Babies that are born prematurely are particularly vulnerable to disruptions in the pulmonary vascular development. As improved neonatal care has dramatically improved the survival of these babies, there is a growing cohort of young adults that were born preterm. Knowledge about pathophysiological processes in the developing lung may be particularly important for these ex-premature adults who are at higher risk for the long-term consequences of pulmonary vascular diseases, thereby contributing disproportionately to the future burden of adult cardiovascular disease.[24,25]

OXIDATIVE INJURY IN EARLY LIFE

Reactive oxygen species (ROS) are oxygen-derived metabolites and can be subdivided into free radicals and oxidants. Examples of free radicals, defined as atoms or molecules that contain unpaired electrons, are superoxide, nitric oxide and hydroxyl- and peroxyl-radical. Oxidants, such as hydrogen peroxide, peroxynitrite, and lipid peroxide, do not contain an unpaired electron and are therefore not free radicals, but are highly reactive oxygenated mol-

ecules that can easily lead to free radical reactions.[26-30] Because of their highly reactive nature, ROS can react with various intracellular proteins and alter their structure and function.

Small amounts of ROS are continuously produced in the human body, mainly during ATP production in the mitochondria, and have been shown to play a role in many signaling processes.[29-34] However, in order to prevent deleterious effects of ROS and to maintain proper cellular function, the amount of ROS needs to be carefully controlled. Protection of the cells against oxidative damage is ensured by cellular antioxidants, which include endogenous antioxidants such as superoxide dismutase, catalase, glutathione peroxidase, and several metal-binding proteins (transferrin, ferritin and albumin). Furthermore, there are exogenous antioxidants present in food or dietary supplements like vitamin C and E.[29-34]

Oxidative stress occurs when antioxidant defense mechanisms are insufficient to cope with ROS production, either through increased production of ROS or through insufficient presence of antioxidants. Oxidative stress causes degradation of lipids, protein damage, and even DNA damage.[27,28,30,35-38] At birth, all newborns are exposed to a sudden increase in oxygen tension compared to the hypoxic environment in utero, leading to an increased ROS production. Also, several perinatal adverse events induce a high exposure to ROS, as described below in more detail. Neonates, and especially those who are born premature, are particularly vulnerable to oxygen toxicity, as their levels of antioxidant enzymes are inadequate and unable to protect the rapidly growing tissues, including the developing lung, from oxidative injury.[28,29,38-40] The endothelial cells and the alveolar type II cells especially are extremely susceptible to oxidative injury. Activation of transcription factors and pathways by oxidative stress lead to inactivation of surfactant, cellular dysfunction, and impaired cell survival.[3,39-42] Thus oxidative injury plays a critical role in the pathogenesis and pathophysiology of neonatal pulmonary vascular disease such as BPD.[43,44]

PRENATAL ADVERSE STIMULI

Placental hypoxia

Normal perfusion and function of the placenta is essential for the fetus, as the placenta is ultimately responsible for oxygen and nutrient supply to the fetus. A reduction in the placental perfusion, and consequently placental hypoxia, is associated with pre-eclampsia and intrauterine growth retardation (IUGR).[45,46]

Pre-eclampsia is the most common maternal complication of pregnancy, characterized by hypertension, edema, and proteinuria. It is a frequent cause of IUGR and premature birth, which are both risk factors for neonatal pulmonary vascular diseases.[47,48] Central to the pathogenesis of pre-eclampsia is placental hypoperfusion and/or inflammation, resulting in oxidative stress and the release of vasoactive factors by the diseased and hypoxic placenta.

It has been shown that ROS are elevated and antioxidant levels are decreased in the maternal circulation in pre-eclampsia.[27,49-51] Studies on the oxidative stress in infants born to mothers with pre-eclampsia have reported conflicting outcomes. Some papers show an increased antioxidant status, probably protecting the fetus against the maternal oxidative stress.[52-54] Others report elevated levels of ROS and decreased levels of antioxidants in neonates of pre-eclamptic mothers, contributing to 'oxygen radical disease of neonatology', including BPD.[42,55,56] The increased maternal oxidative stress and ensuing endothelial dysfunction are thought to aggravate to placental hypoperfusion and impact on the release of vasoactive factors by the diseased and hypoxic placenta.[57,58]

Placental hypoxia induces an imbalance between pro-angiogenic and anti-angiogenic factors. Thus, there is an increased placental production of soluble fms-like tyrosine kinase-1 (sFlt-1, or soluble VEGF-receptor 1) and soluble endoglin (sEng).[46,59,60] Elevated levels of sFlt-1 scavenge free VEGF, thereby inhibiting VEGF signaling.[46,59-62] Similarly, elevated levels of sEng interfere with the TGF-β pathway. sFlt-1 and sEng are thought to act synergistically in the development of the maternal complications of pre-eclampsia. [59] However, both sFlt-1 and sEng are also present in amniotic fluid and may thereby affect the developing fetus. Indeed, elevated levels of sEng in amniotic fluid during preterm labor were associated with development of BPD in the infants.[63] In contrast, amnionic sFlt-1 levels during mid-term gestation in humans were not predictive for the development of pulmonary vascular disease in their infants,[62] although it has recently been shown that elevated levels of intra-amniotic sFlt-1 lead to reduced VEGF signaling in the developing rat lung, resulting in impaired pulmonary vascular growth and alveolarization in newborn rat-pups.[61] Altogether, this suggests that prenatal exposure to high sEng and sFlt-1 levels may compromise normal fetal lung development and may be responsible for the increased risk of pulmonary vascular disease in preterm born neonates of pre-eclamptic mothers.[14,61]

High altitude

It is well-known that living at high altitude poses a major challenge to the human body. In adults, residing at high altitude can cause altitude-specific disorders, such as acute and chronic mountain sickness, high-altitude pulmonary edema, and symptomatic high-altitude pulmonary hypertension. These conditions can be considered a direct result of exposure to hypobaric hypoxia.[64,65]

In pregnant high-altitude residents, maternal exposure to hypoxia can negatively influence the oxygen delivery to the fetus and thereby hamper the development of the fetus. For example, it has been shown that pre-eclampsia is more common at high altitudes than at low altitudes. Also, hypoxia is a key factor responsible for lower birth weights and IUGR in newborns at high altitudes, independently of the presence of pre-eclampsia.[64,66,67] This may, at least in part, be due to altered function of the placenta due to hypoxia. Indeed, it has been shown that HIF-1 expression is increased in placentas from high-altitude residents and that

these placentas contain more TGF-β3 as well as VEGF and sFlt-1,[68,69] which may spillover into the fetal circulation and may impact on the developing pulmonary vasculature.

Because oxygen plays a crucial role in the perinatal period, the perinatal cardiopulmonary transition proceeds more slowly in babies that are born under conditions of high-altitude hypoxia. It has been shown that such infants have lower arterial oxygen saturations and that the physiologically rapid fall in pulmonary artery pressure after birth does not occur. This can ultimately lead in adult life to symptomatic high-altitude pulmonary hypertension, which is the condition of pulmonary hypertension accompanied by muscularization of the pulmonary arteries due to hypoxic vasoconstriction and vascular remodeling. In terminal stages of the disease, this results in right heart failure.[70,71] Another sign of disrupted perinatal transition in these infants is the persistence of the fetal vascular connections (ductus arteriosus and foramen ovale).[71]

An important observation is that the incidence of BPD is also increased at higher altitudes, the mechanism of which is currently unknown. It has been hypothesized that the exposure of the preterm infant to the hypoxic environment causes injury to the lung in a critical stage of development. Moreover, IUGR can play a role in the higher risk of BPD as well.[72]

In summary, although it is well-established that neonatal pulmonary vascular disease is more common in high-altitude pregnancies, the effect of high-altitude residence during gestation on the prenatal pulmonary vascular development and the exact underlying molecular and/or biochemical mechanisms are still incompletely understood in humans. Animals studies, however, have implicated a higher vasoconstrictor reactivity of the pulmonary small arteries.[73-75]

POSTNATAL ADVERSE STIMULI

Hyperoxia

The development and maturation of the fetal organs normally takes place under hypoxic conditions in the uterus. Preterm birth leads to premature transition of the pulmonary circulation from the hypoxic fetal environment to a relative hyperoxic postnatal environment (air). For adequate functioning of the body's tissues and organs, in particular the brain, intestines, and kidneys, sufficient oxygenation of these tissues is required. Although the optimal systemic oxygen saturation in preterm infants is currently unknown, the consensus is that systemically circulating oxygen saturation levels need to be targeted above 85% to fulfill the oxygen demands of the body. Because of the incomplete lung development in these infants, with simplified alveolar structure and thick alveolar septae, oxygen diffusion is hampered. To compensate for these diffusion abnormalities, often high levels of supplemental oxygen are required to increase alveolar oxygen tension and the diffusion gradient in

order to achieve the targeted intravascular oxygen saturation levels. This high level of oxygen supplementation further augments the already existing (relative) hyperoxic state postnatally. The rapid alteration in oxygen concentration at birth results in changes in oxygen sensitive molecular mechanisms.[3,41,76,77] Under normoxic and/or hyperoxic conditions rapid proteasomal degradation of the HIF-1α subunit occurs and thus binding to the promotor regions of target genes is hampered.[16-18,41] This leads to impaired VEGF expression, resulting in disrupted angiogenesis and alveolarization. So, (relative) hyperoxia induces vascular arrest, leading to pulmonary vascular diseases.

Relative hyperoxia also increases generation of ROS and induces oxidative stress, an important contributor to the development of neonatal pulmonary vascular disease. As outlined above, preterm infants are more prone to oxidative injury, due to lower levels of antioxidants including vitamin E, transferrin, and superoxide dismutase, and higher levels of free iron leading to the production of hydroxyl radical.[28,29,39,78,79]

Mechanical ventilation

Mechanical ventilation is essential and life-saving in the treatment of severely premature infants. Yet mechanical ventilation can also provoke ventilator-induced lung injury (VILI). The main mechanism resulting in VILI is over-distension of the lung, thereby over-stretching of the distal epithelium and capillary endothelium, which increases microvascular permeability, inhibits surfactant production and leads to the release of cytokines into the alveolar space and the systemic circulation.[3,80-83] The type and duration of mechanical ventilation as well as the volume and pressure that is used are contributing factors to the development of VILI.[81] Furthermore, the developmental stage of the lung, and thus gestational age at birth, is an important determinant of VILI. The lung in the alveolar stage can expand extensively without any stretch injury, while the more immature saccular lung has less surface area to expand and is more injury prone to stretch.[83]

Mechanical ventilation does not only directly injure the neonatal lung through overdistension, it also results in alterations in angiogenesis-related factors. Thus, VEGF-1 and its receptor flt-1 as well as angiopoietin 1 and its receptor Tie2 are downregulated while the TGF-β co-receptor endoglin is upregulated, in lungs of infants that were mechanically ventilated.[84,85] The imbalance in angiogenic factors likely contributed to dysmorphic angiogenesis and altered alveolarization observed in mechanically ventilated lungs. [84,85]

Hypoxia

In addition to periods of hyperoxia, due to premature perinatal transition (relative hyperoxia) and the need of supplemental oxygen, premature babies are exposed to chronic or intermittent hypoxia. Hypoxia can be caused by different mechanisms. Often, hypoxia is the result of immature lungs or occurs in the setting of apnea of prematurity. It can also be caused by inadequate ventilation of the preterm infant.[41,77] In infants born extremely pre-

mature, studies suggest the persistence of intrapulmonary arteriovenous shunts, which are physiologically present in the fetus and normally regress in the early neonatal phase. Beside the immaturity of the lung in premature born babies, a high pulmonary vascular resistance can also prevent the regression of intrapulmonary arteriovenous shunts. These shunts bypass the alveolar capillary gas exchange units and therefore cause hypoxemia in the neonate.[14,86,87]

In contrast to the vessels of the systemic circulation that dilate in response to hypoxia, the pulmonary vasculature constricts. This so called hypoxic pulmonary vasoconstriction (HPV) is an important physiologic mechanism to ensure ventilation-perfusion matching by preventing blood flow to areas of the lung that are not well-ventilated, thereby optimizing systemic oxygenation. The exact mechanisms underlying HPV remain incompletely understood, but animal studies show a critical role of ROS in HPV.[88-91] The "redox theory" states that precapillary pulmonary arterial smooth muscle (PASM) cells are the oxygen-sensing cells as well as the effector cells.[91-95] Mitochondria in the PASM cells senses a drop in alveolar O_2 and respond by creating a signal that alters opening of redox-sensitive potassium and calcium channels, thereby increasing vascular tone.[91-95] However, it is not yet resolved if increased or reduced levels of ROS during hypoxia underlie the signal transduction of HPV.[92-95] During general hypoxia, as seen in premature infants, generalized pulmonary vasoconstriction occurs, which results in an increase in pulmonary vascular resistance and hence pulmonary hypertension. When sustained, hypoxic vasoconstriction produces vascular remodeling of the pulmonary vascular bed and, ultimately, leads to right heart failure.[92,93]

Besides hypoxic vasoconstriction, it is well-known from animal studies that hypoxia interferes with the physiological process of alveolarization. In healthy newborns, a large part of this process takes place after birth in a normoxic environment (ambient air, 21% oxygen).[2,5,6] Postnatal exposure to hypoxia has been shown to impair alveolarization, resulting in alveolar simplification with fewer and larger alveoli. Since the airway and vascular development and maturation are closely related, impaired alveolarization also impairs the vascular maturation in the alveolar wall. Both perturbed signaling of HIF-1α, VEGF, as well as TGF-β, mediate these disruptions.[41,77,96]

Inflammation

Postnatal exposure to intermittent hypoxia and hyperoxia induces oxidative stress, which – in premature infants – is insufficiently reduced due to immature anti-oxidant mechanisms. As a result of direct cellular injury, oxidation of DNA, induction of cytokines, and recruitment of neutrophils and macrophages to the lung, oxidative stress induces pulmonary inflammation. In addition to oxygen-free radicals, mechanical ventilation also triggers pulmonary inflammation. Vice versa, oxygen radicals are rapidly released by immune cells with the oxidative burst, a crucial reaction in the immune system.[42] Infiltration of inflammatory cells in the immature lung, and the release of ROS, results in endothelial and epithelial cell injury. Interestingly, the pro-inflammatory cytokine IL-8 is increased and the anti-inflammatory cy-

tokine IL-10 is decreased in serum of preterm infants that subsequently developed BPD,[97-99] suggesting that indeed a balance between pro-inflammatory and anti-inflammatory cytokines is required for normal lung development. The exact role of oxygen tension in perinatal inflammation is currently unknown and should be the topic of future investigation.[41,100-102]

CONSEQUENCES

Short-term consequences

Both premature birth – with incomplete vascular growth, immature vascular function and decreased host defenses – as well as exposure to injurious stimuli after birth, contribute to an abnormal development of the lung circulation. Failure of normal lung development will lead to neonatal pulmonary vascular disease due to an altered function of the pulmonary vessels (with an increased vasomotor tone), as well as an altered structure of the pulmonary vasculature, i.e. vascular remodeling(including smooth muscle cell proliferation).[10,41,103,104]

Both these functional and structural changes elevate pulmonary vascular resistance by narrowing vessel diameter and by decreasing vascular compliance, leading to pulmonary hypertension.[10,14,41,103-106] Furthermore, disruption of normal pulmonary vascular development consequently leads to an arrest in the development of the airways. BPD, a common complication of preterm birth, is recognized a consequence of disrupted lung development. It is characterized by an arrest in vascular and alveolar growth, which leads to decreased and enlarged alveoli and a decrease in number of capillaries as compared to a normal lung.[15,107-110] Besides morbidity and mortality in the neonatal period, BPD is associated with a variety of long-term health problems including reduced lung function, cognitive impairments, cardiovascular dysfunction, and exercise intolerance.[111]

Long-term consequences

The "developmental origins of health and disease" (DOHaD) concept[112] has gained a great deal of attention in recent years, especially in pediatrics because of the dramatically increased survival of premature babies. Since approximately 10% of births are preterm, a growing cohort of prematurely born survivors reaches adolescence.[113-115] While the majority of research in this field has focused on the developmental origins of metabolic disease, now there is growing evidence that disruption of normal pulmonary vascular development in the perinatal period contributes to the development of (pulmonary) vascular disease in adulthood. In the late 1990s, it was shown that a transient perinatal insult to the pulmonary circulation increases the risk of developing pulmonary hypertension.[116] It also has been shown that pulmonary artery pressure is elevated in offspring of mothers with pre-eclampsia, demonstrating that placental hypoxia causes pulmonary vascular dysfunction.[117] Underlying mechanisms of this so-called "fetal or perinatal programming" are currently unknown,

although oxidative stress has been proposed to play a key role. As described above, many perinatal insults are associated with oxidative injury. Reactive molecules can cause epigenetic changes by inducing DNA methylation and histone modification. Modulation of epigenetic modifications during this sensitive developmental period will alter organogenesis and organ function, thereby producing long-term programmed consequences.[118-121] In view of the growing awareness of the long-term consequences of neonatal pulmonary vascular disease, it will become clinically more important to routinely screen high-risk (ex-premature) patients.

An important diagnostic tool in this patient population is exercise testing. By placing the cardiopulmonary system under stress with exercise testing, subtle dynamic abnormalities that are not apparent during conventional static tests may be revealed. Studies evaluating exercise capacity in long-term survivors of prematurity have reported highly variable results, with some research groups reporting no evidence of exercise limitation,[122-127] while other investigators demonstrated significantly impaired exercise performance in former preterms.[128-138]

Exercise capacity is a resultant of pulmonary function and cardiovascular performance.[139,140] Until now, most studies concerning long-term health outcomes of (extremely) premature infants have focused on respiratory outcomes. Indeed it is well-known that pulmonary function in childhood and adolescence is impaired in these patients, and this is even more pronounced in survivors of neonatal pulmonary vascular diseases like BPD.[110,113,141-144]

Less is known about cardiovascular function in survivors of neonatal pulmonary vascular disease. Exposure of the immature pulmonary vasculature to injurious stimuli after birth can potentially result in remodelling of the pulmonary vascular bed, in endothelial dysfunction, pulmonary hypertension, and, finally, right ventricular failure. A recent cardiac magnetic resonance imaging (MRI) study demonstrated that young adults, born preterm, have smaller right ventricular lumen size and greater mass, resulting in right ventricular dysfunction.[145] Although less pronounced, adverse changes have also been shown in the left ventricle.[146] These alteration in cardiac function and structure may increase the risk for cardiovascular events later in life, thereby contributing disproportionately to the burden of adult cardiovascular disease in the future.[115,141,145,146]

CLINICAL IMPLICATIONS

Oxygen therapy is a cornerstone in the treatment of premature infants and is crucial for their survival. However, as outlined above, too much oxygen, or hyperoxia, causes injury and damage to several tissues including the lung. Paradoxically, hypoxia also interrupts normal lung vascular development. Therefore, both hyperoxia and hypoxia, together or independently, can lead to pulmonary vascular disease. Consequently, there is uncertainty about the optimal target of oxygen saturation in (prematurely born) neonates. A recently published systematic review and meta-analysis concluded that infants (born <28 weeks of

pregnancy) cared for with a liberal saturation target (SpO_2 91-95%) had significantly lower mortality before hospital discharge than infants cared for with a restricted oxygen target (SpO_2 85-89%), although the quality of evidence for this estimate of effect was low. No significant difference was found for the incidence of BPD.[147-149]

In view of the growing cohort of adult survivors of prematurity and/or neonatal vascular disease, more research into the long-term consequence of perinatal pulmonary vascular events is imperative. Since this is a relatively new patient population, there is a lack of consensus for the follow-up of high-risk (formerly premature) patients. The American Heart Association and American Thoracic Society have made a guideline for diagnosis, evaluation, and monitoring of pediatric patients with pulmonary hypertension.[150] They recommend monitoring of children with pulmonary hypertension (or neonatal pulmonary vascular disease) provided by comprehensive, multidisciplinary team of pulmonologists, cardiologists, neonatologists, anesthesiologists, and experienced nurses. Children with chronic diffuse lung disease should be evaluated for concomitant cardiovascular disease or pulmonary hypertension by echocardiogram every 3 to 6 months. The 6-min walk distance (6MWD) test or cardiopulmonary exercise test (CPET) can be useful to monitor exercise tolerance. MRI can be performed to assess right ventricular function and structure.[150] These diagnostic tools could be very useful in the development of a follow-up program that might facilitate an optimal transition of the patient from the pediatric to the adult setting to ensure strong continuity of care to optimize clinical outcome. It is a future challenge to evolve a follow-up program for (neonatal) pulmonary vascular disease that ensures early detection of health problems in these patients, thereby diminishing the cardiovascular morbidity and mortality and improving the quality of life. Furthermore, the development of an exercise training program tailored for prematurely born adolescents, who may be at higher risk for early-onset adult diseases, should be considered. Increasing the level of regular physical activity has beneficial effects on overall health and plays an important role in the prevention of diseases in the long term. Establishing early, adequate levels of fitness and activity should therefore be a cornerstone in the follow-up of formerly premature adults.

CONCLUSIONS

Both antenatal and postnatal injurious stimuli can disrupt the normal lung vascular development, potentially leading to neonatal pulmonary vascular diseases entities such as BPD and pulmonary hypertension. These diseases not only contribute to morbidity and mortality in the neonatal period, but have also been shown to significantly increase the risk for a variety of health problems later in life. Pulmonary vascular disease can lead to endothelial dysfunction, vascular remodeling, and ultimately to cardiac dysfunction (right ventricular hypertrophy and failure).

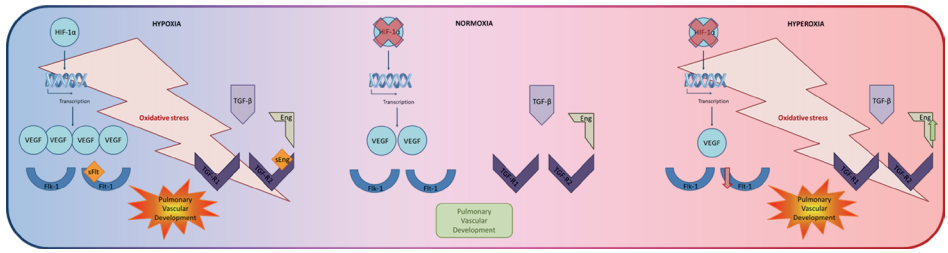

Figure 3. Summary figure depicting the receptors, ligands, and signaling pathways of relevance for pulmonary vascular development during perinatal hypoxia and hyperoxia as compared to normoxia.

Oxygen tension is a key player in the pathogenesis of neonatal pulmonary vascular diseases. Both antenatal and postnatal exposure to either hypoxia and/or hyperoxia contributes to disruption of the normal development of the pulmonary vascular bed (Figure 3). Despite decades of research focusing on the role of oxygen in pulmonary vascular development, the exact pathophysiologic mechanisms remain incompletely understood. Furthermore, it is important to study the optimal targeted oxygen saturation limits in much more detail, in order to improve clinical practice at the neonatal intensive care unit. Future endeavors should also include the development of a follow-up program tailored towards prematurely born adolescents survivors and/or survivors of (transient) neonatal pulmonary vascular diseases. Such a program may prove key step in the early diagnosis and treatment of long-term vascular diseases to reduce morbidity and mortality in adult life, which is necessary in order to improve the health of the growing cohort of prematurely born survivors of neonatal pulmonary vascular disease and to reduce the burden of adult cardiopulmonary morbidity and mortality.

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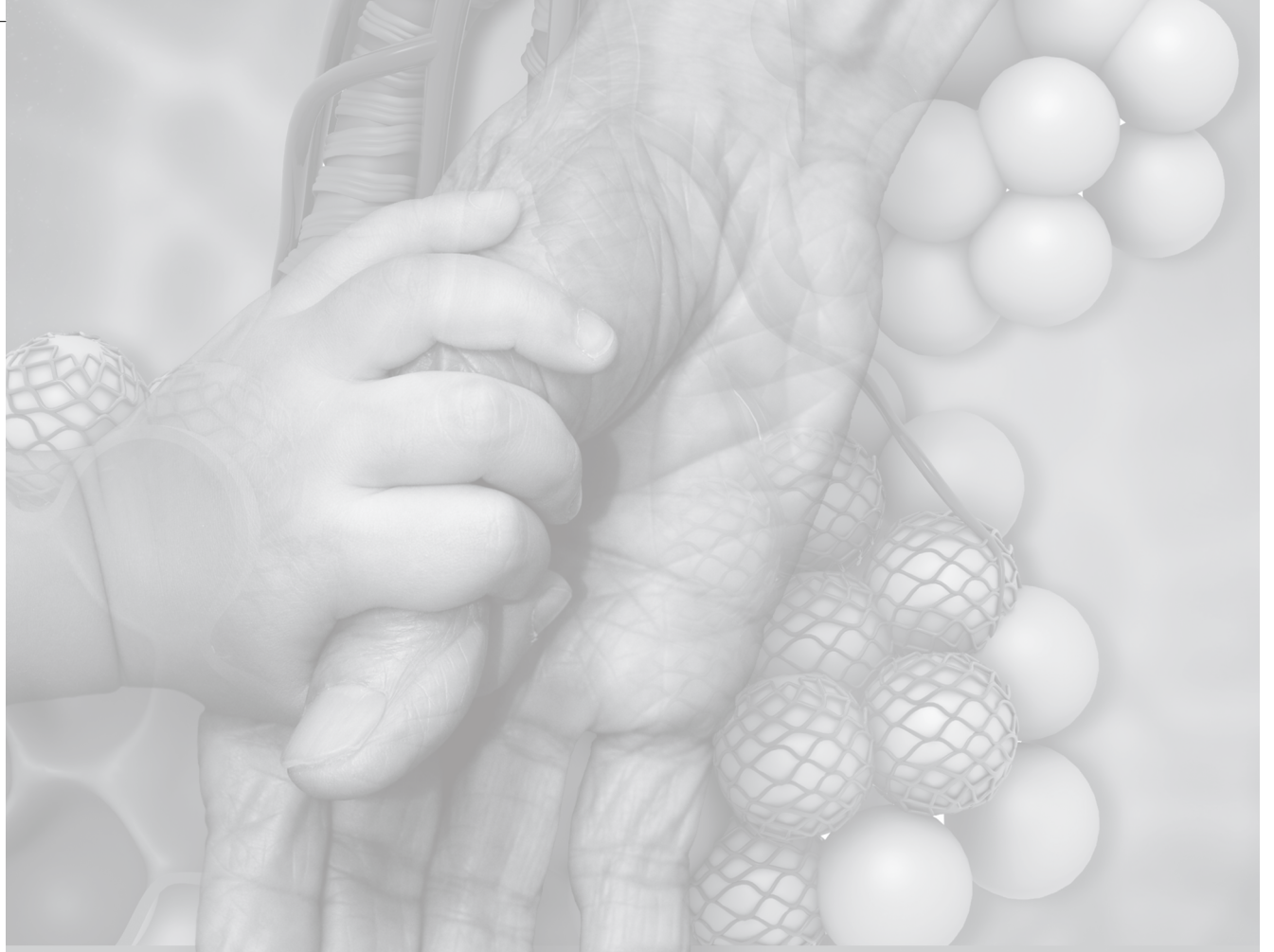

Chapter 3

Surgical placement of catheters for long-term cardiovascular exercise testing in swine

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ABSTRACT

This protocol describes the surgical procedure to chronically instrument swine and the procedure to exercise swine on a motor-driven treadmill. Early cardiopulmonary dysfunction is difficult to diagnose, particularly in animal models, as cardiopulmonary function is often measured invasively, requiring anesthesia. As many anesthetic agents are cardiodepressive, subtle changes in cardiovascular function may be masked. In contrast, chronic instrumentation allows for measurement of cardiopulmonary function in the awake state, so that measurements can be obtained under quiet resting conditions, without the effects of anesthesia and acute surgical trauma. Furthermore, when animals are properly trained, measurements can also be obtained during graded treadmill exercise. Flow probes are placed around the aorta or pulmonary artery for measurement of cardiac output and around the left anterior descending coronary artery for measurement of coronary blood flow. Fluid-filled catheters are implanted in the aorta, pulmonary artery, left atrium, left ventricle and right ventricle for pressure measurement and blood sampling. In addition, a 20 G catheter is positioned in the anterior interventricular vein to allow coronary venous blood sampling. After a week of recovery, swine are placed on a motor-driven treadmill, the catheters are connected to pressure and flow meters, and swine are subjected to a five-stage progressive exercise protocol, with each stage lasting 3 min. Hemodynamic signals are continuously recorded and blood samples are taken during the last 30 sec of each exercise stage. The major advantage of studying chronically instrumented animals is that it allows serial assessment of cardiopulmonary function, not only at rest but also during physical stress such as exercise. Moreover, cardiopulmonary function can be assessed repeatedly during disease development and during chronic treatment, thereby increasing statistical power and hence limiting the number of animals required for a study.

INTRODUCTION

Adequate cardiopulmonary function is essential to supply the body with oxygen and nutrients, particularly during conditions of increased metabolic demand such as during exercise.[1] The cardiopulmonary response to exercise is characterized by a number of adaptations in cardiac function, i.e. an increase in heart rate, contractility and stroke volume, and microvascular function, i.e. vasodilation in the vascular beds supplying exercising muscles as well as in the pulmonary vasculature, and vasoconstriction in the vascular beds supplying the gastrointestinal system as well as inactive muscles.[1] Impaired exercise capacity is an early hallmark of cardiopulmonary dysfunction, and cardiopulmonary exercise testing is used as an effective method to delineate between cardiac dysfunction, vascular dysfunction and/ or pulmonary dysfunction in patients with impaired exercise capacity.[2] Early cardiopulmonary dysfunction is difficult to diagnose, particularly in animal models, as cardiopulmonary function is often measured invasively, requiring anesthesia, with many anesthetic agents possessing cardiodepressive properties.[3]

Chronic instrumentation allows for measurement of cardiopulmonary function in the awake state, and when the animals are fully adjusted to the laboratory conditions measurements can be obtained under quiet resting conditions without the effects of anesthesia and acute surgical trauma. Furthermore, when the animals are appropriately trained, measurements can also be obtained during graded treadmill exercise.[4,5] More specifically, left and right ventricular function can be assessed and related to myocardial perfusion, while regulation of vasomotor tone in the coronary, systemic and pulmonary microcirculation can be determined. The use of fluid-filled catheters allows measurement of pressure as well as taking blood samples without imposing additional stress on the animals. Another advantage of studying chronically instrumented animals is that cardiopulmonary exercise testing can be repeated allowing the use of an animal as its own control, either during disease development or during chronic treatment, thereby increasing statistical power and hence limiting the number of animals required for a study.

Cardiopulmonary anatomy of swine closely resembles that of humans and it is possible to induce various forms of cardiopulmonary disease, such as diabetes[6], myocardial infarction[7], pulmonary hypertension[8,9] and pacing-induced heart failure.[10,11] Moreover, the size of swine allows chronic instrumentation, and repeated blood sampling of sufficient quantity to analyze not only blood gases, but also to perform neurohumoral measurements and/or to search for biomarkers of disease.

This protocol describes the surgery used to chronically instrument swine as well as the protocol for exercising the swine on a motor-driven treadmill.

PROTOCOL

Procedures involving animal subjects have been approved by the Animal Care Committee at Erasmus Medical Center Rotterdam (NL). Swine with weights between 6 and 80 kg have been successfully instrumented using this protocol.

1. Adaptation of the animals to human handling

1.1) After arrival in the facility, house the animals solitarily but enable them to interact with each other.

1.2) Accustomize swine to human handling and transportation from the animal facility to the experimental laboratory, by handling the animal at least once a day for one week.

1.3) Train the animals appropriately for exercise experiments on a motor-driven treadmill by exercising them on the treadmill for a minimum of three times before surgery.

1.4) Animals should be fasted overnight before surgery to prevent nausea, vomiting and thereby potential aspiration of stomach fluids.

2. Preparation for surgery

2.1) Sedation

2.1.1) Prepare medication for sedation in a 10 mL syringe. Premedication consists of tiletamine/zolazepam (5 mg/kg), xylazine, (2.25 mg/kg) and atropine (1 mg).

2.1.2) Inject the medication intramuscularly in the trapezius muscle with a 19G 1.5" needle to sedate the pig.

2.1.3) Wait for approximately 10 minutes and check for muscle relaxation and unconsciousness to confirm appropriate and stable level of sedation.

2.1.4) Place a 20G peripheral safety catheter in an ear vein for subsequent intravenous administration of anesthesia and/or fluids.

2.2) Intubation and ventilation

2.2.1) Place the animal on a table and/or trolley in supine position.

2.2.2) Open the mouth of the animal with an oral spreader.

2.2.3) In case of insufficient relaxation of the jaws or presence of swallowing reflexes, which hinder intubation, administer thiopental (10 mg/kg) intravenously via the ear vein catheter. Alternatively, the pig could be masked with isoflurane to induce sedation

2.2.4) Use a conventional laryngoscope with a light and a Miller blade to allow the laryngoscopist to directly view the larynx. If there is laryngospasm, apply 2% lidocaine to the cords and larynx to reduce the spasm and allow intubation.

2.2.5) Insert an intubating stylet into the endotracheal tube to make the tube conform better to the upper airway anatomy and pass the tube through the mouth and between the vocal cords into the trachea.

2.2.6) Inflate the balloon cuff with a 10 mL syringe to help secure it in place, to prevent leakage of respiratory gases, and to protect the airways from possible aspiration of stomach fluid.

2.2.7) Connect the tube to a breathing filter (heat and moisture exchanger) and to the mechanical ventilator.

2.2.8) Place the animal on its right side on the surgical table.

2.2.9) To achieve pO_2 levels of 100-120 mmHg, ventilate the animal with a mixture of oxygen and nitrogen (1:2 v/v), using the following ventilator settings: Pressure control mode: positive end-expiratory pressure (PEEP) 4 cmH_2O; peak inspiratory pressure 16-18 cmH_2O; breathing frequency depending on the size of the animal, (20 bpm for a 20 kg animal, decrease frequency with increasing body weight) this should result in a tidal volume of ~ 10 ml/kg, monitor ventilation with capnography.

2.2.10) Monitor temperature using a rectal thermometer and maintain temperature between 37-39°C using a heat lamp or heat mat. Moreover, monitor heart rate with electrocardiography.

2.3) Anesthesia

2.3.1) Induce and maintain anesthesia preferably by adding 2.0% of isoflurane (v/v) to the ventilation gas-mixture or alternatively by intravenous administration of fentanyl (10 µg/kg/h) via the ear vein catheter.

2.3.2) Check adequate depth of anesthesia by testing pain reflexes with a hind leg toe pinch before starting surgery. When necessary, add additional anesthesia or wait for a few minutes. Check pain reflexes regularly throughout the surgery.

2.4) Fluids and antibiotics

2.4.1) Administer the first dose of amoxicilline (25 mg/kg) intravenously via the ear vein catheter.

2.4.2) Connect a transfusion system to the ear vein catheter to enable slow infusion of glucose 10% (500 mL) during surgery.

2.5) Sterilization of surgical site

2.5.1) Shave and clean the skin of the animal over an area of approximately 25 cm width from the vertebral column all the way to the left axilla.

2.5.2) Scrub the moisturized skin with povidone-iodine scrub (75 mg/mL) for approximately 5 minutes.

2.5.3) Remove the povidone-iodine soap from the skin with sterile gauzes, before sterilizing the skin with povidone-iodine lotion (100 mg/mL).

2.5.4) Cover the animal with sterile surgical drapes to reduce bacterial transfer and subsequent contamination of the surgical site.

3. Surgery

3.1) Opening the thorax (thoracotomy)

3.1.1) Make an incision in the skin, starting 1 cm caudal to the left inferior angle of the scapula down to the left axilla (Figure 1). Use diathermy to cauterize blood vessels in the skin to prevent excessive bleeding.

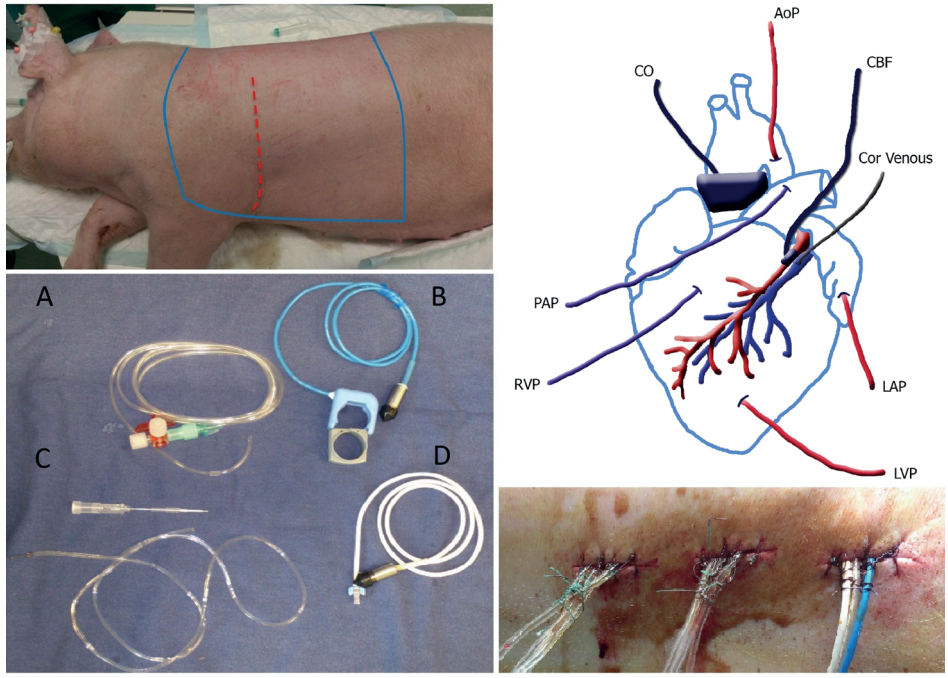

Figure 1. Overview of the surgery. Top left panel: The sterile area of the animal, which should be shaved and sterilized lies between the bleu lines. The incision site is depicted as the red dotted line. Bottom left panel: Picture of catheters and flow probes: fluid-filled catheter (A), aorta/ pulmonary flow probe including rubber band (B), coronary venous catheter including 20G needle (C) and the coronary flow probe (D). Top right panel: Schematic overview of placement of the catheters and flow probes. MAP, mean arterial pressure; Cor venous, coronary venous catheter; LAP, left atrial pressure; LVP left ventricular pressure; RVP, right ventricular pressure; PAP, pulmonary artery pressure; CO, cardiac output; CBF, coronary blood flow. Bottom right panel: Tunneled catheters exiting the back secured with a stitch and a knot at approximately 1 cm distance along the suture.

3.1.2) Cut through the serratus muscle and pectoralis major muscle, using the cutting modality of the diathermy. Also use diathermy to cauterize blood vessels in the muscle layer to prevent excessive bleeding.

3.1.3) Use blunt dissection to carefully divide the intercostal muscle of the fourth left intercostal space with a mosquito clamp. Now the costal surface of the left lung covered with visceral and parietal pleura should be exposed.

3.1.4) To enter the pleural cavity, carefully pierce both layers of the pleura and tear them open.

3.1.5) Use a thoracic retractor to separate the edges of the wound and the ribs and to forcefully drive tissues apart to obtain good exposure of the pleural cavity.

3.1.6) Push away the left lung in the caudal direction and keep it in place with a wet gauze. Now the heart and great vessels should be clearly exposed.

3.2) Placement of catheters and flow probes (Figure 1)

3.2.1) Use blunt dissection to remove ~2 cm^2 of the surrounding connective tissue of the descending thoracic aorta.

3.2.2) Perform a purse-string suture, consisting of three stitches, in the aortic wall with a non-absorbable USP3-0 braided silk suture (∅0.2mm).

3.2.3) Penetrate the aortic vessel wall with a stainless steel 16G needle in the middle of the purse-string suture.

3.2.4) Insert the tip of the fluid-filled catheter (until the ring) into the aorta, pull the purse-string suture firmly together and tie the two strings of the suture.

3.2.5) To keep the catheter in place, wind the suture 3 times around the catheter above the ring and again tie the two strings of the suture. Further secure the catheter with a new stitch approximately 1 cm cranial from the insertion place.

3.2.6) Connect the fluid-filled catheter to the calibrated pressure transducer, which is connected to the computer, to monitor the mean arterial pressure during the surgery. Obtain an arterial blood gas to verify or adjust for correct ventilation settings.

3.2.7) Open the pericardium with a crossed cut. Be aware to keep the phrenic nerve that runs over the pericardium intact.

3.2.8) Identify the pulmonary artery and pull it slightly in the caudal direction with a Farabeuf retractor. Now the ascending aorta and aortic arch should be exposed. Monitor mean arterial pressure while retracting the pulmonary artery.

3.2.9) Make a small cut (~1 cm) in the connective tissue between the ascending aorta and the pulmonary artery using Metzenbaum scissors, to be able to dissect either the ascending aorta or the pulmonary artery with a large curved mosquito clamp to place the flow probe.

3.2.10) Place the rubber band of the flow probe around the vessel. To make this easier, place a suture through one end of the rubber band, place this suture around the vessel and pull it until the rubber band surrounds the vessel.

3.2.11) Fix the flow probe measurement device on the rubber band. Connect the flow probe to the computer and check the cardiac output signal on the computer to confirm a correct placement of the flow probe.

3.2.12) Place fluid-filled catheters in the pulmonary artery, right ventricle, left ventricle and left atrium at the same manner as described for the aortic fluid-filled catheter (3.2.2 – 3.2.5). Note that it is not necessary to remove connective tissue before performing a purse-string suture in these structures.

3.2.13) Expose and dissect the proximal part of the left anterior descending coronary artery by first lifting the tissue with a forceps and making a small (2-3 mm) cut with Metzenbaum scissors, followed by carefully teasing the tissue away from the artery with a cotton swab. Ensure complete dissection of the coronary artery by passing a small straight angled mosquito clamp underneath.

3.2.14) Make a stitch parallel to the anterior interventricular coronary vein with a suture, which is connected to the coronary venous catheter.

3.2.15) Puncture the coronary vein with the 20G needle of the coronary venous catheter and insert the cannula of the catheter intravenously.

3.2.16) Remove the needle and secure the catheter with the already performed stitch (3.2.14). Further secure the catheter with a new stitch approximately 1cm from the place of initial puncture.

3.2.17) Place the coronary flow probe around the previously dissected left anterior descending coronary artery. When the artery is constricted and is hardly visible, use lidocaine 10% spray to relax the vessel to get a better exposure of the vessel. Check the signal of the coronary flow on the computer to confirm a correct placement of the flow probe (Figure 2).

3.3) Tunneling

3.3.1) Tunnel the flow probes individually through the third left intercostal space beneath the muscle and above the rib by using a large curved mosquito clamp.

3.3.2) Tunnel the fluid-filled catheters through either the third or the fifth left intercostal space by piercing the intercostal muscle. Clamp off the fluid-filled catheters and remove the three-way stopcock to minimize the piercing area and prevent leakage of the fluid-filled catheters during the tunneling.

3.3.3) Fix the flow probes and the fluid-filled catheters with non-absorbable USP2-0 braided silk (∅0.3mm) by means of a purse string suture on the intercostal muscle. This suture also serves to prevent air leakage after re-instating negative intrathoracic pressure.

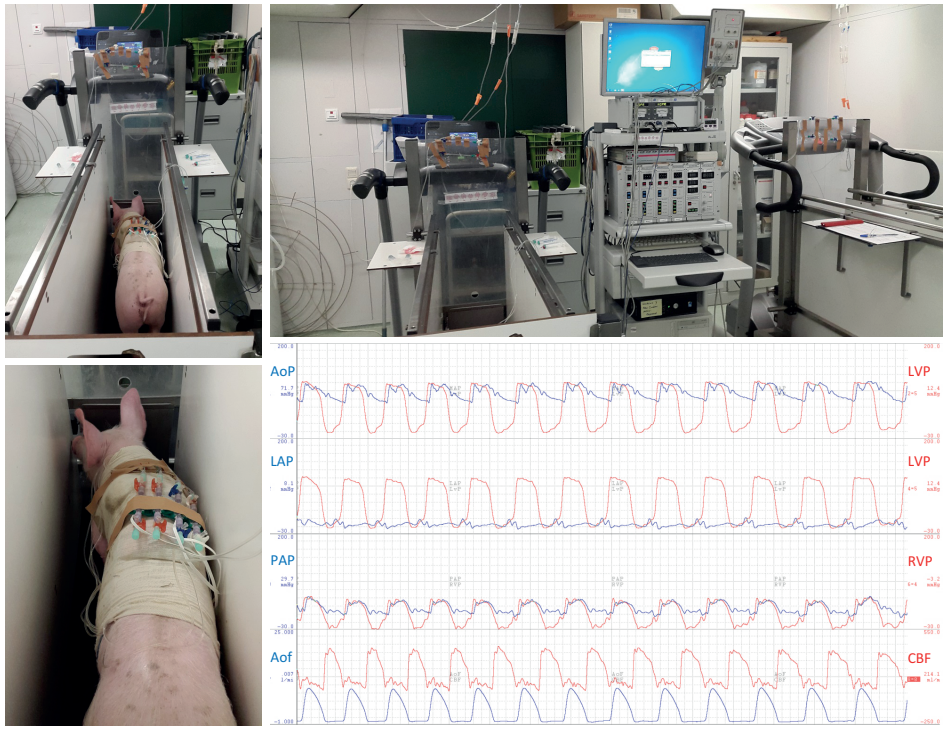

Figure 2. Treadmill Experiment. Left panels: Instrumented swine on the treadmill. Fluid-filled catheters are connected to the pressure transducers, placed on the back of the swine. Top right panel: Overview of the total experimental set-up, including treadmill, amplifier and recording computer. Bottom right panel: Typical example of recorded hemodynamic data. From top to bottom; aortic pressure (AoP, blue) and left ventricular pressure (LVP, red); left atrial pressure (LAP,blue) and left ventricular pressure (red); pulmonary artery pressure (PAP, blue) and right ventricular pressure (RVP, red); aortic flow/cardiac output (AoF, blue); coronary blood flow (CBF, red).

3.3.4) Make three incisions in the skin approximately 2 cm sinister and parallel to the vertebral column, approximately 3 cm in length 3 cm apart of each other.

3.3.5) Pierce a trochar beneath the left latissimus dorsi muscle from rostral incision site to the incisions on the back. Tunnel the flow probes and fluid catheters to the back within this trochar.

3.3.6) Place the stopcocks on the fluid-filled catheters and remove the clamp. Withdraw blood to remove clots and air bubbles and fill the fluid-filled catheters with 1000 IU/mL heparin. Coronary venous catheters should be filled with 5000IU/mL heparin.

3.4) Closing the thorax

3.4.1) Make an incision with a length of approximately 1.5 cm, 8 cm caudal and parallel to the first incision.

3.4.2) Lead the drain from the pleural cavity through the sixth intercostal muscles subcutaneously to this incision with a large curved mosquito clamp. Connect the drain to the suction device to remove any remaining fluid and reinstate negative pressure in the pleural cavity during the closing of the thorax.

3.4.3) Relieve and inflate the lung with an end-inspiratory hold. Ensure adequate filling of the lung by visual monitoring.

3.4.4) Close the thorax by pulling the ribs of the fourth intercostal space together at two separate sites with non-absorbable USP6 braided polyester (∅0.8mm).

3.4.5) Close the serratus muscle and pectoralis major muscle with a running stitch and the skin with a running subcuticular suture using non-absorbable USP2-0 braided silk (∅0.3mm).

3.4.6) Suture the incisions on the dorsal side with non-absorbable USP2-0 braided polyester (∅0.3mm) between the catheters. First tie a knot directly onto the skin to close the incision, then fixate the catheters to the suture with a knot 1 cm from the skin. For the flow probes, use an absorbable USP2-0 braided polyglactin (∅0.3mm) suture to prevent cutting of the suture in the flow probe wire (Figure 1).

3.4.7) Carefully remove the drain while applying pressure on the cranial side of the incision to maintain negative pressure in the pleural cavity. Close the incision with a purse string suture using non-absorbable USP2-0 braided polyester (∅0.3mm) and seal the wound with petroleum jelly.

3.5) Termination of anesthesia and recovery from surgery

3.5.1) Stop anesthesia when all incision sites are closed.

3.5.2) Provide analgesia by administering buprenorphine (0.015 mg/kg) i.m. in the gracilis muscle.

3.5.3) Stop the ventilation when the animal is breathing independently and disconnect the tracheal tube from the ventilator. Check regularly if the animal is breathing sufficiently.

3.5.4) Place gauze pads between exteriorization sites of the catheters to absorb wound fluid.

3.5.5) To protect the external segments of the catheters, give the animal an elastic vest and package the catheters between two pieces of artificial sheepskin.

3.5.6) Deflate the balloon of the tracheal tube and extubate when the animal regains its swallowing reflex.

3.5.7) Provide long-term analgesia by means of a Fentanyl slow-release patch (12 µg/h for a 20 kg pig; adjust strength according to bodyweight). Place the patch on a

thin part of the skin (such as the lower abdomen) to ensure adequate delivery of analgesia.

3.5.8) House the animal separately for the entire post-operative period. Provide a heating lamp for the first week after surgery to keep the animal warm.

3.5.9) Supply enough fluid i.v. if the animal is not drinking independently.

3.5.10) Flush the fluid-filled catheters daily, by first withdrawing blood to remove clots, then refilling with saline and finally with heparinized saline (1000-5000 IU/mL) to prevent blood clot formation. Take care not to infuse any air bubbles while flushing the catheters.

3.5.11) Administer amoxicillin (25 mg/kg) i.v. daily for 6 days after surgery to prevent post-surgical infections.

3.5.12) Allow the animal to recover for one week before starting the treadmill experiments.

4. Treadmill experiment (Figure 2)

4.1) Flush the fluid-filled catheters as described (3.5.10) and attach the flushed catheters to the pressure transducers. Measure the rectal temperature to be able to obtain temperature corrected blood gas values.

4.2) Flush the pressure transducers with saline to prevent damping of the signals due to air bubbles. Attach the pressure transducers to the elastic vest on the dorsal side.

4.3) Connect the pressure transducers and flow probes to the amplifier. Start measuring in the computer program and calibrate the pressure transducers and flow probes with 0 mmHg being open to the air (and closed to animal) and 100 mmHg using a manometer.

4.4) Switch the three-way stopcock in a way that the fluid catheters have an open connection with the pressure transducers. Note that the blood pressures can now be obtained. Check signals for shape and amplitude (Figure 2).

4.5) If required, connect an extension line to either of the fluid catheters for sampling of mixed venous and arterial blood.

4.6) Measure hemodynamics when the animal is lying as well as standing quietly on the treadmill. Average blood pressures are measured over a timeframe of 10 sec.

4.7) Obtain arterial and mixed venous blood samples by first withdrawing 5 mL of blood using a 10 mL syringe so that 1 ml of pure blood can be obtained using a heparinized 1 mL syringe. For the coronary venous blood samples, a 2 mL syringe is used instead of the 10 mL syringe and withdrawal of 1 mL is sufficient to obtain pure blood.

4.8) Keep the sealed 1 mL syringes on ice before processing the blood samples with a blood gas analyzer to determine the metabolic and ventilatory condition of the animal.

4.9) Subject the swine to a five-stage exercise protocol on the treadmill, 3 minutes per speed, 1-5 km/h (~85% of maximal heart rate). Obtain hemodynamics and blood gases after 1.5-2 min per speed on each speed as in the resting position.

4.10) After the exercise protocol close the stopcocks and check if drift has occurred in the 0 mmHg calibration, make a note of this calibration. Remove the pressure transducers of the fluid-filled catheters and disconnect the flow probes.

4.11) Flush the fluid-filled catheters with saline and heparin (1000-5000 IU/mL). Protect the catheters and flow probes by putting them beneath the elastic vest between two pieces of artificial sheepskin. The animal can now be returned to its cage.

REPRESENTATIVE RESULTS

Exercise up to 5 km/h resulted in a doubling of cardiac output from 4.3±0.3 to 8.5±0.7 L/min which was principally accomplished by an increase in heart rate from 137±7 to 256±8 beats per minute in combination with a small increase in stroke volume from 32±2 to 36±3 mL (Figure 3). The increase in stroke volume was facilitated by an increase in left ventricular contractility, as evidenced by an increase in the maximum of the first derivative of left ventricular pressure dP/dtmax together with an increased rate of relaxation of the left ventricle and an increase in left atrial pressure, being the filling pressure of the left ventricle (Figure 3). The increase in cardiac output together with an increase in hemoglobin concentration (from 8.5±0.4 to 9.2±0.4 g/dl) and an increase in body oxygen extraction from 45±1 to 71±1% allowed a tripling of body oxygen consumption (Figure 3). Systemic vasodilation occurred as evidenced by an increase in systemic vascular conductance and a decrease in systemic vascular resistance, which accommodated the increase in cardiac output almost completely, so that mean aortic pressure increased only slightly (Figure 3). Exercise also resulted in modest vasodilation in the pulmonary circulation, as evidenced by a 33±8% increase in pulmonary vascular conductance. However, the 101±8% increase in cardiac output, together with the increase in left atrial pressure (from 3±1 to 10 ±1 mmHg), resulted in an increase in pulmonary artery pressure and thereby in an increase in right ventricular afterload (Figure 3).

The increase in heart rate, together with the slight increase in arterial pressure resulted in an increase in left ventricular myocardial oxygen consumption, which was principally met by an increase in coronary blood flow which, in combination with the increase in hemoglobin concentration resulted in an increase in myocardial oxygen delivery (from 310±37 to 738±68 µmol/min). The increase in myocardial oxygen demand was commensurate with the increase in myocardial oxygen supply, as myocardial oxygen extraction (79.8±1.9% at rest 81.6±1.9% during maximal exercise) was essentially maintained constant, resulting in an unchanged coronary venous oxygen saturation and coronary venous oxygen tension (Figure 3).

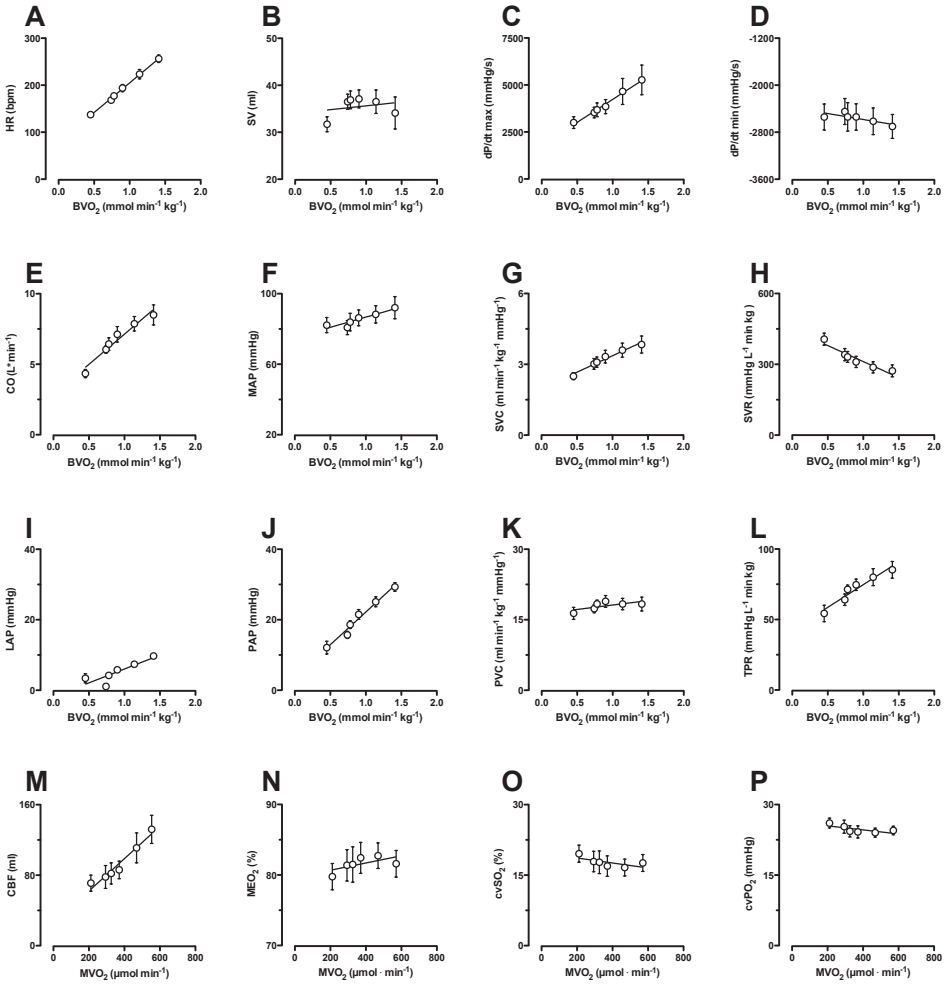

Figure 3. Typical hemodynamic response to exercise. Body oxygen consumption (BVO2) was used as an index for exercise intensity (x-axes of panel A-L). Shown are the responses of heart rate (HR, panel A), stroke volume (SV, panel B), maximum and minimum of the first derivative of left ventricular pressure (dP/dtmax, panel C and dP/dtmin, panel D resp) as indices of contractility and rate of relaxation, cardiac output (CO, panel E), mean arterial pressure (MAP, panel F), systemic vascular conductance (SVC, panel G), systemic vascular resistance (SVR, panel H), Pulmonary artery pressure (PAP, panel J), left atrial pressure (LAP, panel I), pulmonary vascular conductance (PVC, panel K). Total pulmonary resistance (TPR index for right ventricular afterload increased during exercise, Panel L).
The increase in heart rate, together with the slight increase in arterial pressure resulted in an increase in left ventricular myocardial oxygen consumption (x-axes of panels M-P), which was principally met by an increase in coronary blood flow (CBF, panel M), as myocardial oxygen extraction (MEO2, panel N), coronary venous oxygen saturation (CVSO2, panel O) and coronary venous oxygen tension (cvPO2, panel P) were minimally affected. All data are presented as mean with standard error of the mean (SEM).

DISCUSSION

The present study describes the surgery for chronical instrumentation of swine as well as the protocol for exercising the instrumented swine on a motor-driven treadmill while measuring hemodynamics and taking blood samples for measurement of oxygen content in arterial, mixed venous and coronary venous blood.

Critical steps within the protocol

There are several critical steps within the protocol that start already during the intubation procedure. Thiopental (2.1.5) is a respiratory depressive agent, therefore requiring swift intubation upon administration. Also, it is important to carefully monitor ventilator settings during the procedure. Thus, when the thoracic cavity is opened (step 3.1.4), this results in a loss of the negative intrathoracic pressure. To compensate for this loss and to prevent alveolar collapse, ventilation requires positive end expiratory pressure (PEEP). Moreover, ventilator settings (peak inspiratory pressure) should be adjusted to maintain a tidal volume of ~10 mL/kg. Also note that when the left lung is pushed away (3.1.6.) tidal volume is likely to be decreased because only part of the left lung is ventilated. Ventilator settings should be adjusted based on blood gasses.

Another important note with respect to hemodynamic measurements with fluid filled catheters is that there is a hydrostatic pressure difference between the pressure transducer and the insertion site of the fluid-filled catheter into the cardiovascular system. The height difference between the level of the pressure transducer pressure on the elastic vest (4.2), and the insertion point of the catheter should be estimated during surgery and at sacrifice of the animal and corrected for by interpolation either pre- or post- processing of the data.

Another important point to consider when using this technique is that blood loss, either during surgery or during repeated blood sampling should be minimized, despite the fact that swine are relatively large and consequently have a large blood volume (65 mL/kg). During surgery, blood loss during insertion of the catheters can be minimized by simply applying compression on the puncture wounds. According to animal experimentation guidelines, up to 10% of the circulating blood volume can be taken on a single occasion from normal, healthy animals with minimal adverse effects, but it will take an animal about 14 days to replenish this amount of blood.[15] This means that the recovery from surgery is prolonged when a significant amount of blood is lost.

During the repeated blood sampling during the exercise experiments, a maximum of 1.0% of an animal's circulating blood volume, or 0.6 mL/kg can be removed every 24 hours.[15] This also means that the amount of blood that is sampled during treadmill exercise, should be well-planned and that, after removal of the initial clots that are invariably present in the lumen of the catheter near the tip at the interface with the blood, the remaining blood withdrawn to flush the lines should be given back to the animals.

Modifications and troubleshooting

Implanted fluid-filled catheters should be flushed daily to prevent malfunctioning because of blood clot formation. Depending on the amount of blood clots in the fluid filled catheters, the amount of heparin in each line can be varied from 1000IU/mL to 5000IU/mL. The amount of heparin should be kept to a minimum in the first week after surgery to prevent bleeding from surgical incision wounds due to the presence of the anti-coagulant heparin.

However, even when flushed daily, some fluid-filled catheters will get clogged. When this happens, try withdrawing blood with a smaller 2 mL syringe by applying minimal and/or pulsatile suction. It can take several minutes before the catheter will be unclogged. When this does not work, carefully flush a small amount of saline into the catheter and immediately try to withdraw blood. Be aware that infusion can result in a release of thrombus into the circulation and embolism of distal organs, depending on the site of the catheter. When careful flushing does not work, connect the clogged line to a pressure-transducer to check if there is still a hemodynamic signal. If there is no signal, the fluid filled line should be sealed by several knots and cut off.

Interpretation and limitations

When all points as mentioned above are taken into account, the combination of hemodynamic measurements and blood samples allows for interpretation of the exercise response in terms of whole body and myocardial oxygen consumption, which are better measures for exercise intensity than treadmill speed alone.[7,12-14]

In order to meet the increased metabolic requirements of the body, exercise requires changes in cardiac function as well as changes in local perfusion. Tissue perfusion is regulated by changes in diameter of the small arteries and arterioles of the vascular bed supplying the tissue. Myriad vasoactive factors, derived from neurohumoral systems, the endothelium and local metabolites interact to determine vascular tone and ensure adequate tissue perfusion.[1,5,12,16] Changes in systemic and pulmonary vascular resistance or the inverse, vascular conductance, can be calculated from the blood pressure and flow signals and interpreted in terms of changes in vasomotor tone in the systemic and pulmonary vasculature. Intuitively, vascular resistance is often used to assess changes in vascular tone. However, in our research group, we advocate the use of conductance although conductance and resistance are mathematically related, with conductance being flow normalized for pressure, and resistance equaling pressure divided by flow. Although conductance and resistance are interchangeable if one investigates the effect of only a single stimulus (i.e. exercise)[7,17], interpretation of the two parameters can differ when combining exercise with pharmacological interventions, to investigate the contributions of various vasoactive systems to regulation of vascular tone.[4,5,7,14,18]

During exercise, the systemic circulation transforms from a system at rest that is characterized by a low flow and a high resistance (i.e. low conductance) into a system with high flow

and low resistance, (high conductance). As such, pharmacological vasodilation has different consequences for conductance and resistance during rest versus exercise. The decrease in resistance that is produced by a pharmacological vasodilator at rest is large while the increase in conductance is only small. In contrast, during exercise the same degree of vasodilation translates into a large increase in conductance, but only a small decrease in resistance. Thus, when conductance is used, a greater vasodilation seems to occur during exercise, while when looking at resistance vasodilation appears to be larger at rest. Interpretation of the data thus differs when using resistance or conductance. Although the choice between resistance and conductance may seem rather arbitrary, in physics the variable that undergoes the primary change is designated as the numerator of the index for a response.[7,17,18] Since during exercise aortic blood pressure remains fairly constant whereas cardiac output increases markedly, the most appropriate parameter to describe the systemic vascular response to exercise would appear to be systemic vascular conductance (cardiac output / aortic blood pressure), rather than resistance. Moreover, the systemic circulation consists of a multitude of vascular beds from a variety of organs that are principally perfused in a parallel manner. Since parallel resistors add up reciprocally, while parallel conductors add up in a linear manner, any change in conductance of a particular regional vascular bed translates into an identical (absolute) change of the total systemic vascular conductance. This consideration lends further support to the use of vascular conductance to describe the systemic vascular responses to exercise and pharmacological interventions.

The choice for either resistance or conductance to describe the vascular responses to exercise in the pulmonary bed appears to be less obvious, because exercise produced increases in cardiac output as well as pulmonary artery pressure.[7,17] A choice for either resistance or conductance is also less critical, in view of the relatively minor exercise-induced changes in PVR and PVC as compared to the degree of vasodilation produced by, for example, ET-receptor blockade.[7] As a result, the use of either resistance or conductance to characterize the vascular effects of a pharmacological vasodilator in the pulmonary circulation will yield similar conclusions.

In the coronary circulation, interpretation of the data is even more complex as systemic administration of pharmacological antagonists of endogenous vasoactive substances results not only in alterations in coronary resistance vessel tone, but often also produce pronounced changes in systemic hemodynamic variables.[7,14,17,19] These altered hemodynamics influence cardiac work, and thereby cause changes in coronary blood flow resulting from changes in metabolic requirements of the heart or from autoregulation, rather than as a direct effect of the intervention on coronary vascular tone. For example, blockade of an endogenous vasoconstrictor system decreases mean aortic pressure, as a consequence of systemic vasodilation, and elicits autoregulatory adjustments in coronary microvasclar tone. Moreover, baroreceptor reflex activation acts to increase heart rate and myocardial contractility. Such changes in heart rate and/or blood pressure subsequently will result in alterations in myocardial

metabolism, requiring an adjustment in myocardial oxygen supply and hence in coronary blood flow.

To take into account the effects of such drug-induced alterations in myocardial oxygen consumption, investigators examine the relation between coronary venous oxygen levels and myocardial oxygen consumption (MVO_2)[4,5], as this approach allows assessment of regulation of coronary resistance vessel tone independently of changes in myocardial oxygen demand. Administration of a vasodilator will increase myocardial oxygen delivery at a given level of MVO_2. As this increase in oxygen delivery occurs without a change in oxygen consumption, myocardial oxygen extraction will decrease, thereby leading to increases in coronary venous oxygen content and hence in an upward shift of the relation between MVO_2 and coronary venous oxygen levels. It is therefore imperative to measure both myocardial oxygen demand as well as myocardial oxygen supply in order to correctly study the regulation coronary resistance vessel tone.[4,5]

Notwithstanding its elegance and usefulness, some investigators have pointed out the limitations of this approach.[20] Thus, plotting MVO_2 versus coronary venous PO_2 or coronary venous SO_2 could be considered to be inappropriate because these variables are actually part of the equation to compute MVO_2. Consequently, MVO_2 is not a variable that is independent of coronary venous PO_2 or SO_2. Alternatively, investigators should consider using another index of myocardial work, the rate-pressure product (RPP), which is the product of heart rate and left ventricular systolic pressure. However, as RPP and MVO_2 are almost linearly related, substituting RPP for MVO_2 yields virtually identical results [14], and the relation between MVO_2 and coronary venous oxygen levels is considered a sensitive way of studying alterations in coronary vasomotor tone.

Significance with respect to existing methods

Another method commonly used to assess changes in regulation of vascular tone is the use of isolated coronary and pulmonary small arteries or arterioles in a pressure or wire myograph.[6,14,21] The advantage of myograph studies is that vessels can be studied independent of surrounding tissue and without potentially confounding effect from circulating factors. These in vitro techniques are therefore complementary to the in vivo measurements. However, in vivo and in vitro techniques sometimes give opposing results. For example, the response to the potent vasoconstrictor endothelin was reduced in the intact coronary circulation after myocardial infarction, but was augmented in isolated coronary small arteries from swine with myocardial infarction as compared to healthy control swine.[21] This difference between the in vivo and in vitro data was due to an increased suppression of the vasoconstrictor influence of endothelin by prostanoids in vivo.[21]

Future applications

Given the proposed role of changes in coronary microvascular function in both left and right ventricular dysfunction, assessment of these changes in relevant models of cardiovascular disease is required. The use of chronically instrumented animals allows correlations of the severity of the disease with microvascular (dys)function. Moreover, both coronary and pulmonary microvascular function may appear normal under basal resting conditions, while microvascular dysfunction may be revealed under cardiovascular stress, such as during exercise.

Several swine models of cardiopulmonary disease, such as diabetes[6], myocardial infarction[22], pulmonary hypertension[8,9] and pacing induced heart failure[10] are available and could be combined with chronic instrumentation. A potential drawback is that, when commercially available swine breeds such as Yorkshire, Landrace, Large White etc., are used, adult swine are very large and may therefore be difficult to handle. Therefore, juvenile swine are often used. However, as juvenile swine grow rapidly, positioning and function of flow probes and pressure catheters and patency of fluid-filled catheters may become compromised, limiting the duration of serial measurements within individual animals to approximately 10 weeks. An alternative is the use of adult miniature swine, such as Yucatan or Gottingen swine, of which the adult weight is 40-60 kg.[23]

In conclusion, the use of chronically instrumented animals allows serial assessment of cardiopulmonary function either during development of disease or evaluation of treatment, thereby increasing statistical power and limiting the number of animals required for a study.

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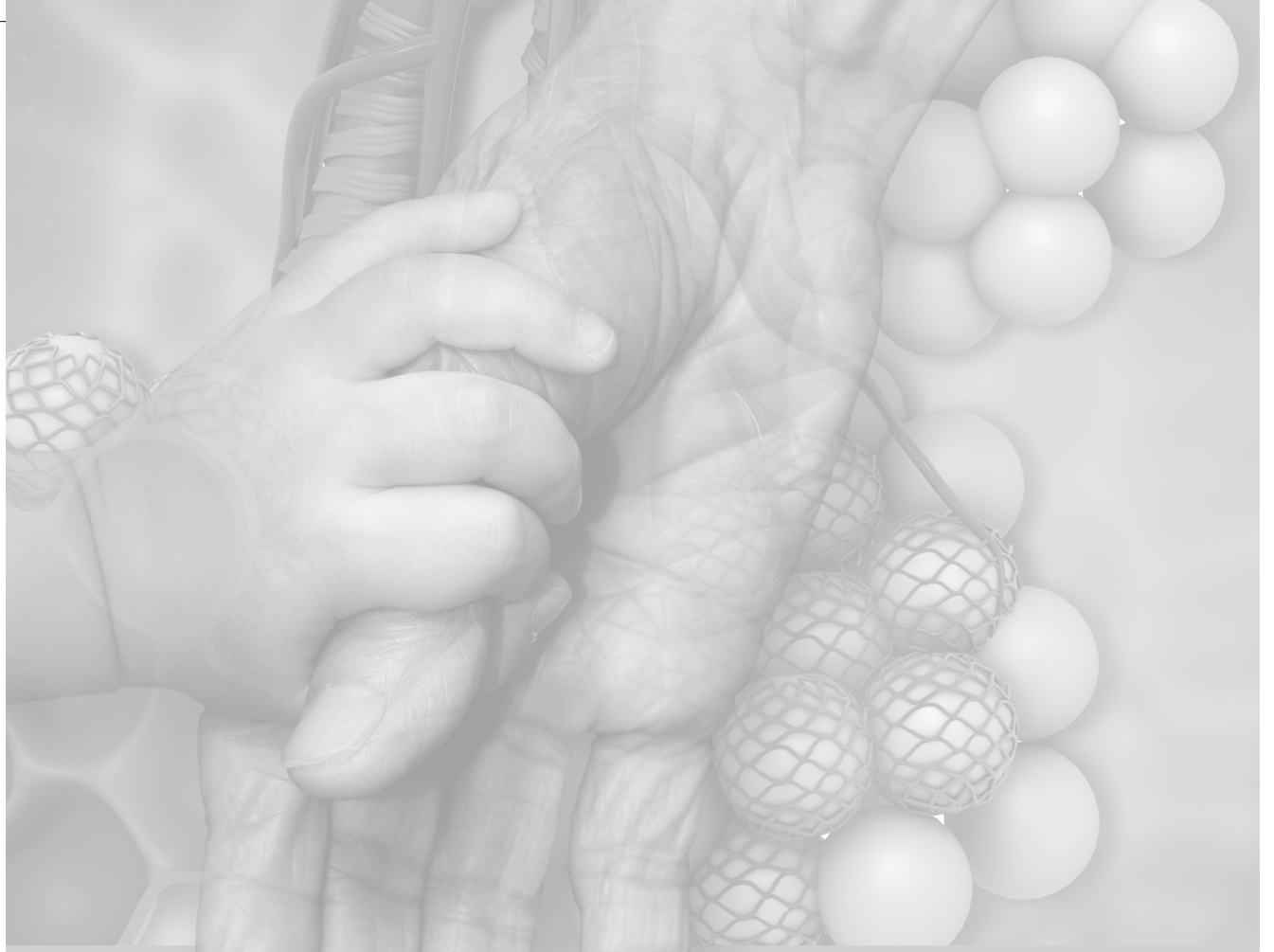

Chapter 4

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

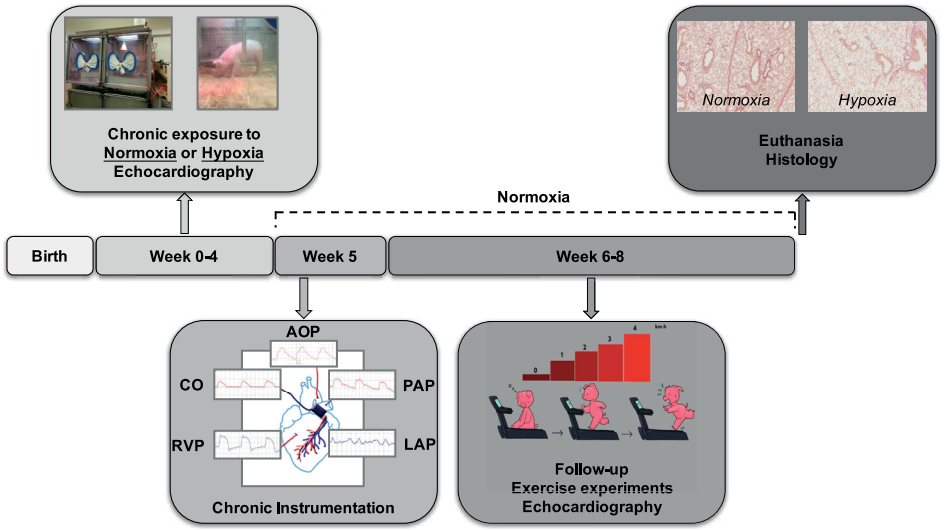

Figure 1. Schematic representation of the experimental design of the study. AOP, aortic pressure; CO, cardiac output; LAP, left atrial pressure; PAP, pulmonary arterial pressure; RVP, right ventricular pressure.

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

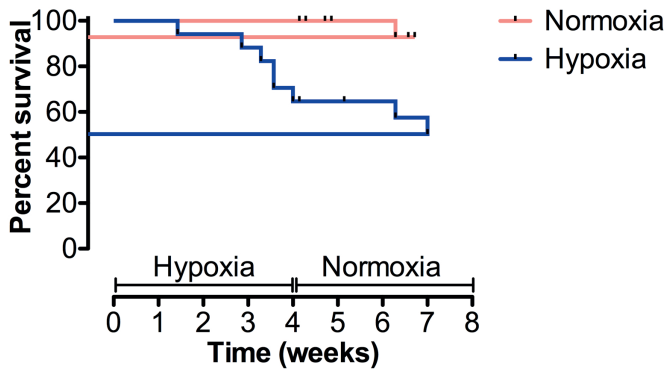

Figure 2. Kaplan Meier survival curve showing the risk of death caused by hypoxia-induced pulmonary hypertension-related disease. Causes of death, other than hypoxia-induced pulmonary hypertension-related disease, are marked as "lost to follow-up" (black ticks without decrease in survival).

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

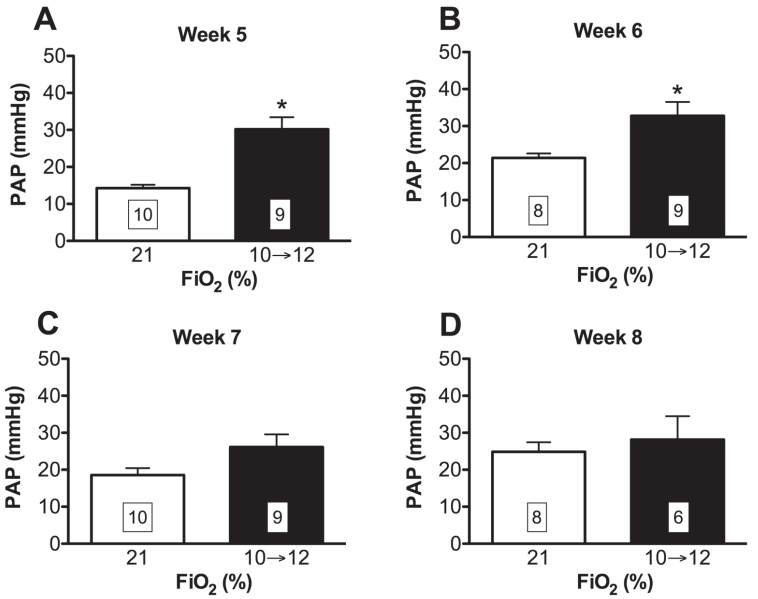

Figure 3. Mean pulmonary artery pressure (PAP) at rest in the different study groups over time. A: week 5 (postoperative day 1). B: week 6. C: week 7. D: week 8. Numbers per study group are presented in the boxes in the bars. Values are mean ± SE. * P ≤ 0.05 vs normoxia.

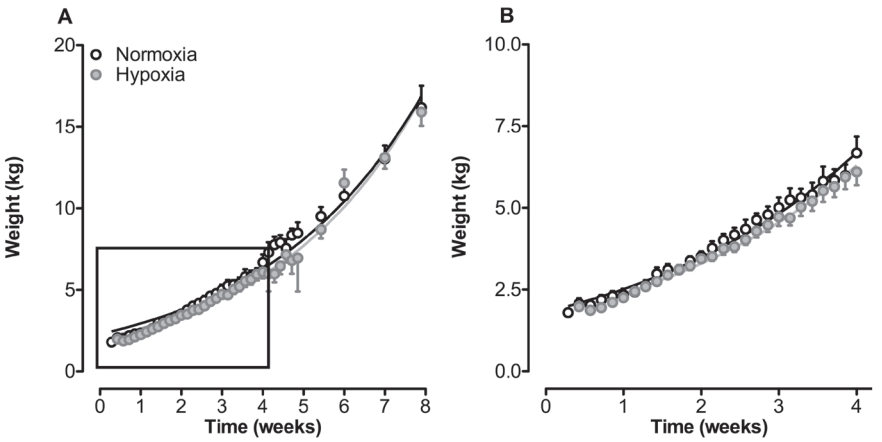

Figure 4. Growth curves of animals raised in normoxia and hypoxia. A: growth curves of the entire study period (weeks 0 – 8). B: growth curves of the period in the incubator (weeks 0 – 4). There were no significant differences in growth between the study groups.

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.

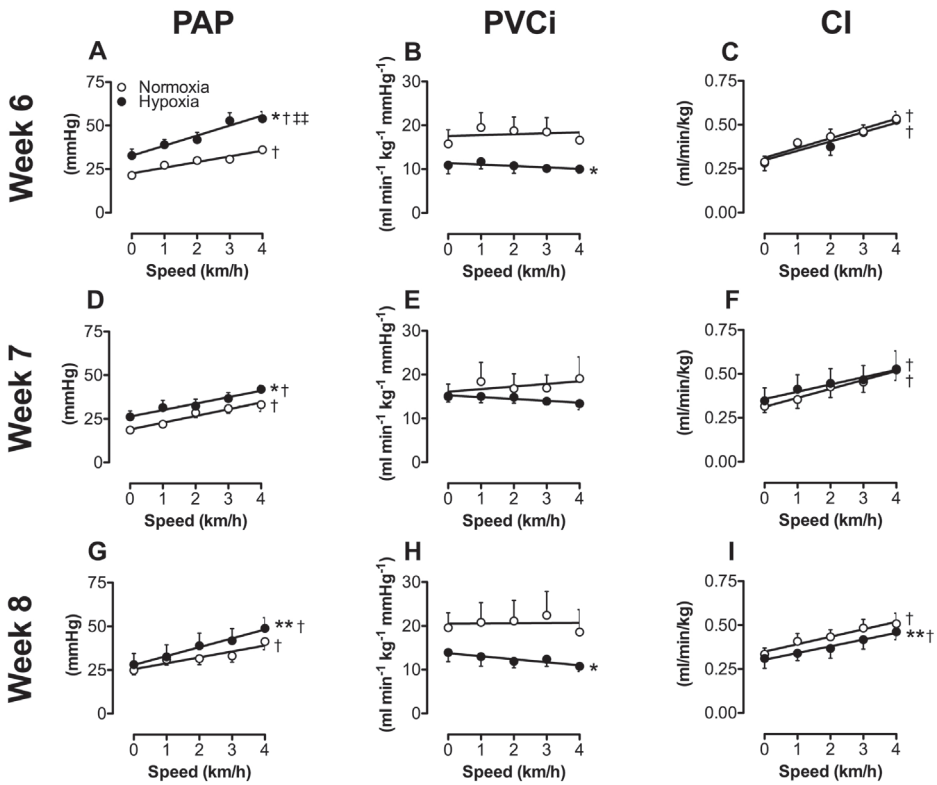

Figure 5. Changes in pulmonary hemodynamics during graded treadmill exercise in normoxic and hypoxic piglets. Relation between treadmill speed and mean pulmonary arterial pressure (PAP; A, D, and G), pulmonary vascular conductance index (PVCi; B, E and H), and cardiac index (CI; C, F, and I). Week 6: 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 ≤ 0.05 vs. normoxia; ** P ≤ 0.1 vs. normoxia; † P ≤ 0.05 effect of exercise; ‡‡ P ≤ 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.

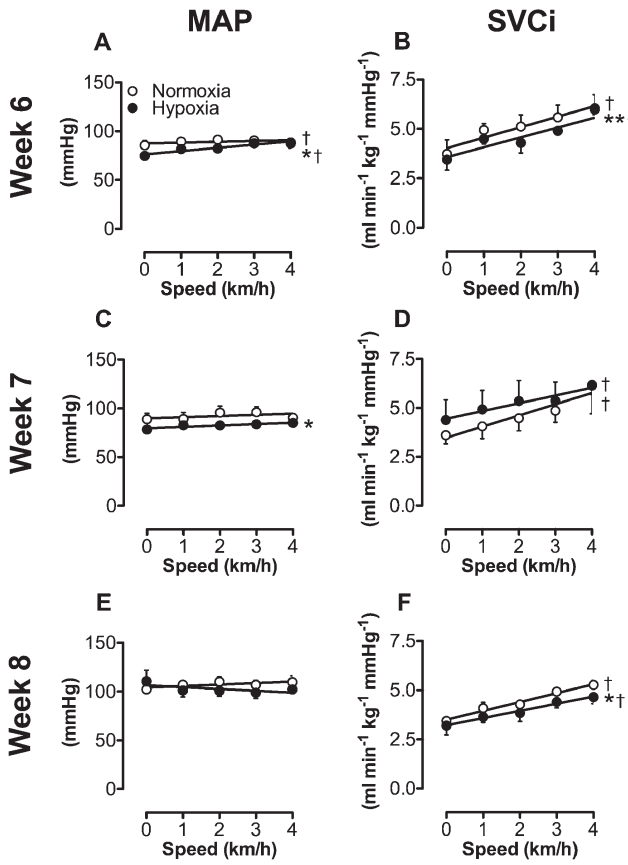

Figure 6. Changes in systemic hemodynamics during progressive levels of exercise in normoxic and hypoxic piglets. A, C, and E; relation between treadmill speed and mean aortic pressure (MAP). B, D, and F: relation between tredsmill speed and systemic vascular conductance index (SVCi). Week 6: n=8 normoxia-exposed 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.

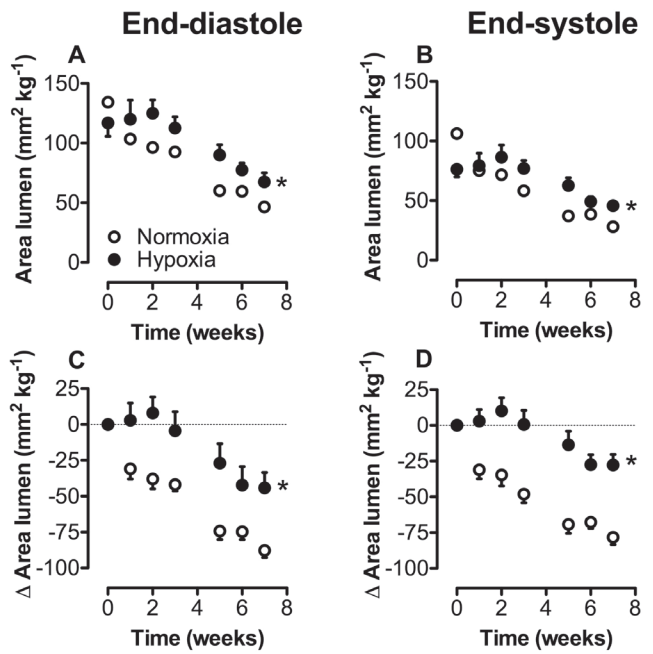

Figure 7. Size (area) of the right ventricular lumen, obtained by echocardiography, corrected for bodyweight over time. A and B: end-diastolic area (A) and end-systolic area (B) of the right ventricular lumen. C and D: chance in right ventricular lumen area compared with baseline (week 1) at end-diastole 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).

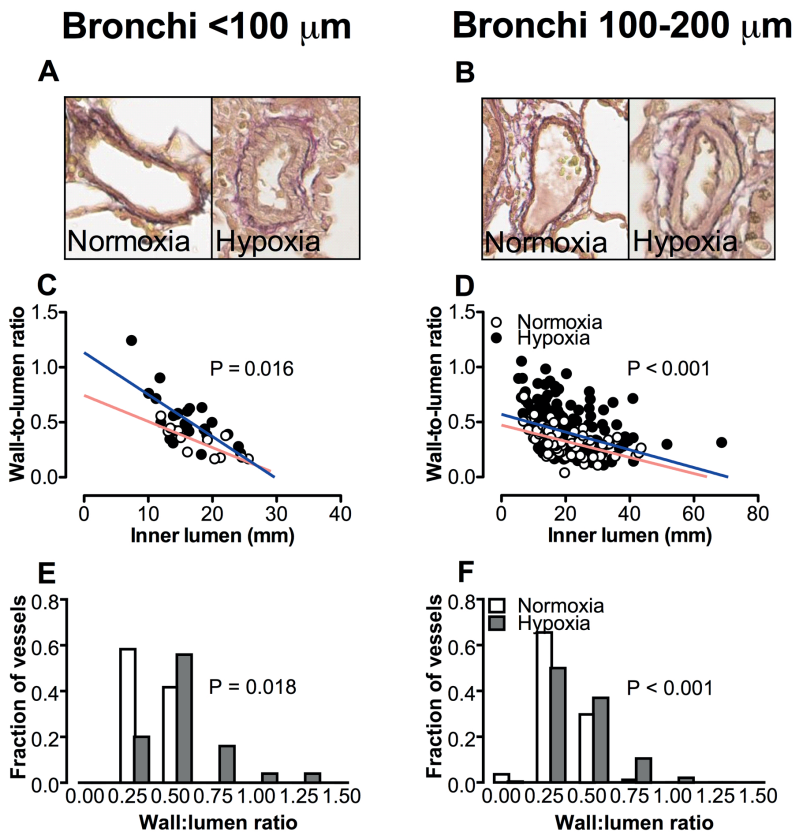

Figure 8. Histological analysis of the lung vasculature. A and B: typical examples of pulmonary arterioles of normoxia-exposed and hypoxia-exposed animals (magnification: x20). C and D: there was a higher wall-to-lumen ratio in hypoxia-exposed animals (blue lines, n=8) compared with normoxia-exposed 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

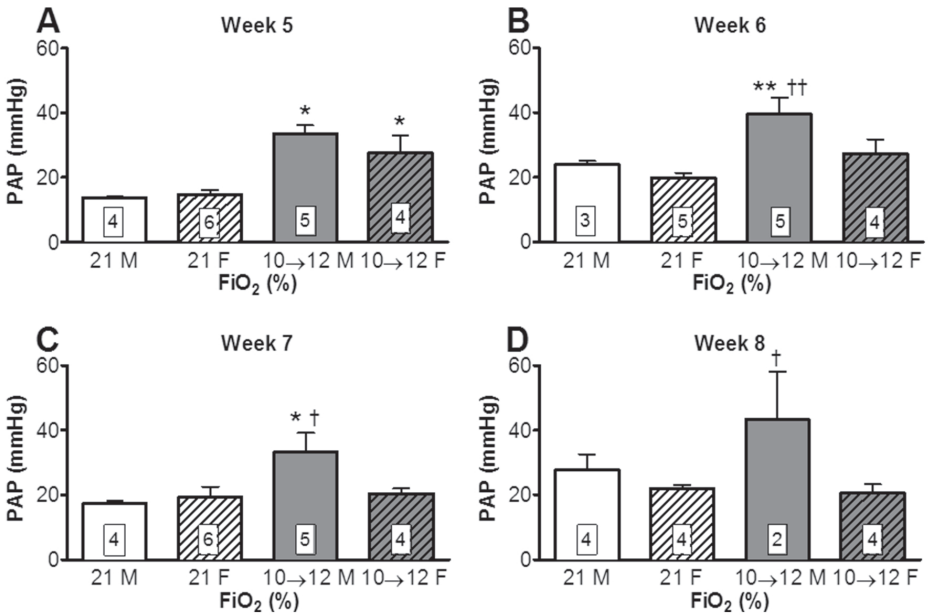

Figure 9. Mean pulmonary artery pressure (PAP) at rest in male and female piglets raised in normoxia or hypoxia (fraction of inspired O_2 (FiO_2): 10-12%) over time. A: week 5 (postoperative day 1). B: week 6. C: week 7. D: week 8. Numbers per study group are presented in boxes in the bars. Values are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

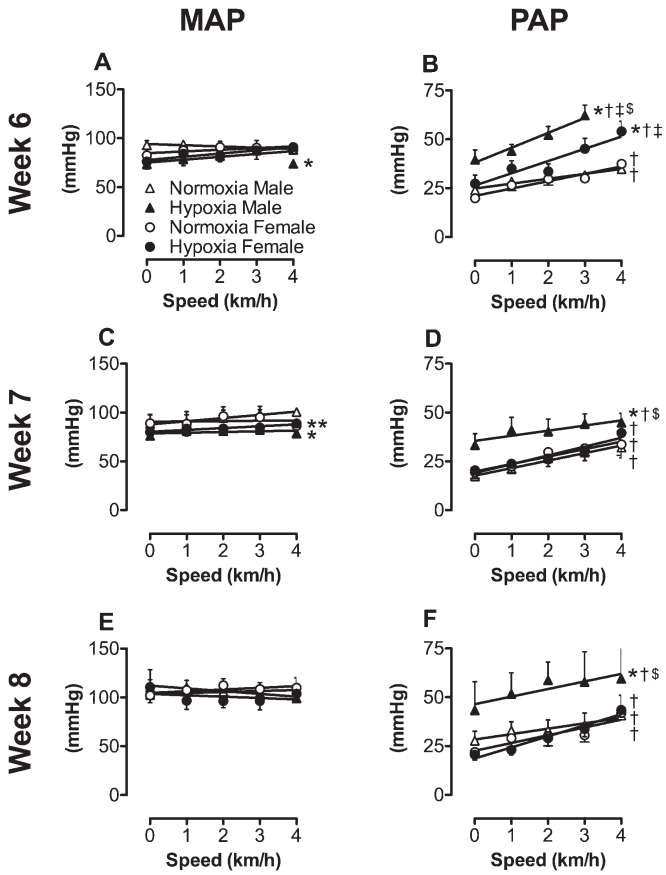

Figure 10. Changes in pulmonary and systemic hemodynamics during progressive levels of exercise in male and female piglets raised in normoxia or hypoxia. A, C, and E: relation between treadmill speed and mean aortic pressure (MAP). B, D, and F: relation between treadmill speed and mean pulmonary arterial 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.

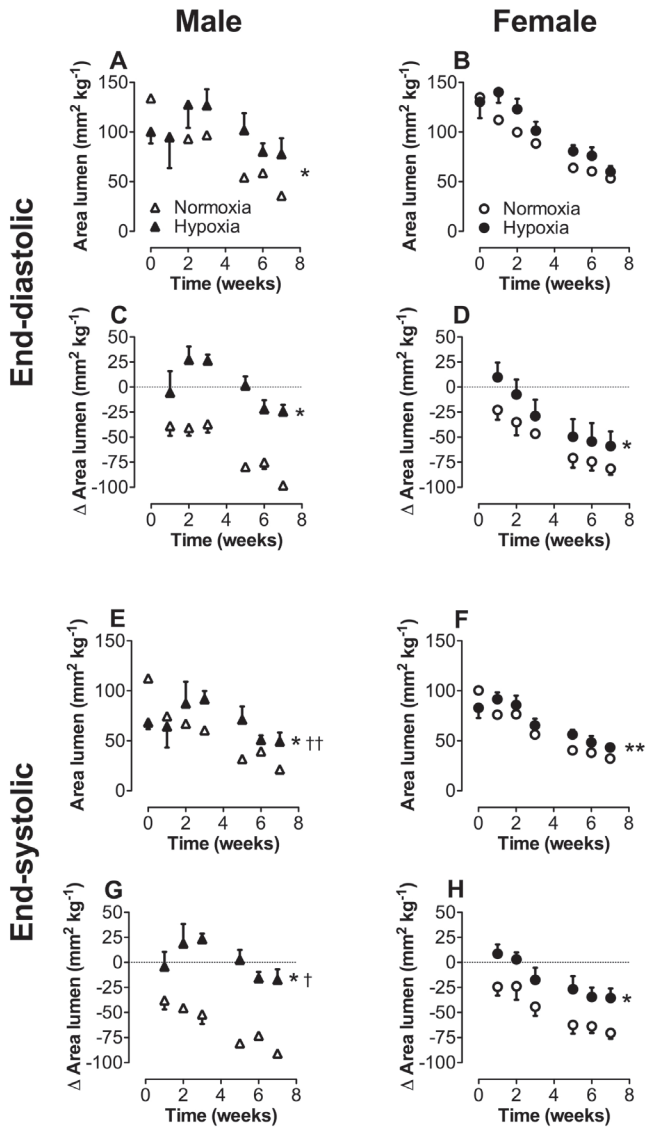

Figure 11. Size (area) of the right ventricular lumen in male and female piglets raised in normoxia or hypoxia (fraction of inspired O_2 (FiO_2): 10-12%), obtained by echocardiography, corrected for body-weight over time. A-H: end-diastolic size (A-D) and end-systolic size (E-H) of the right ventricular 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.

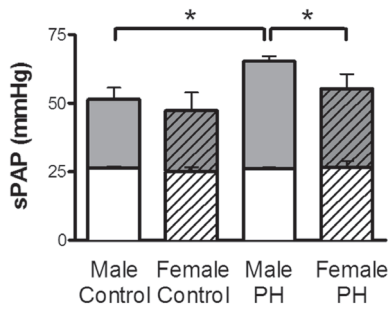

Figure 12. Systolic pulmonary artery pressure (sPAP) in control subjects (6 men and 4 women) and subjects with transient perinatal pulmonary hypertension (PH; 7 men and 3 women). White bars represent sPAP (as measured with echocardiography) baseline values at sea levels; grey bars represent sPAP values 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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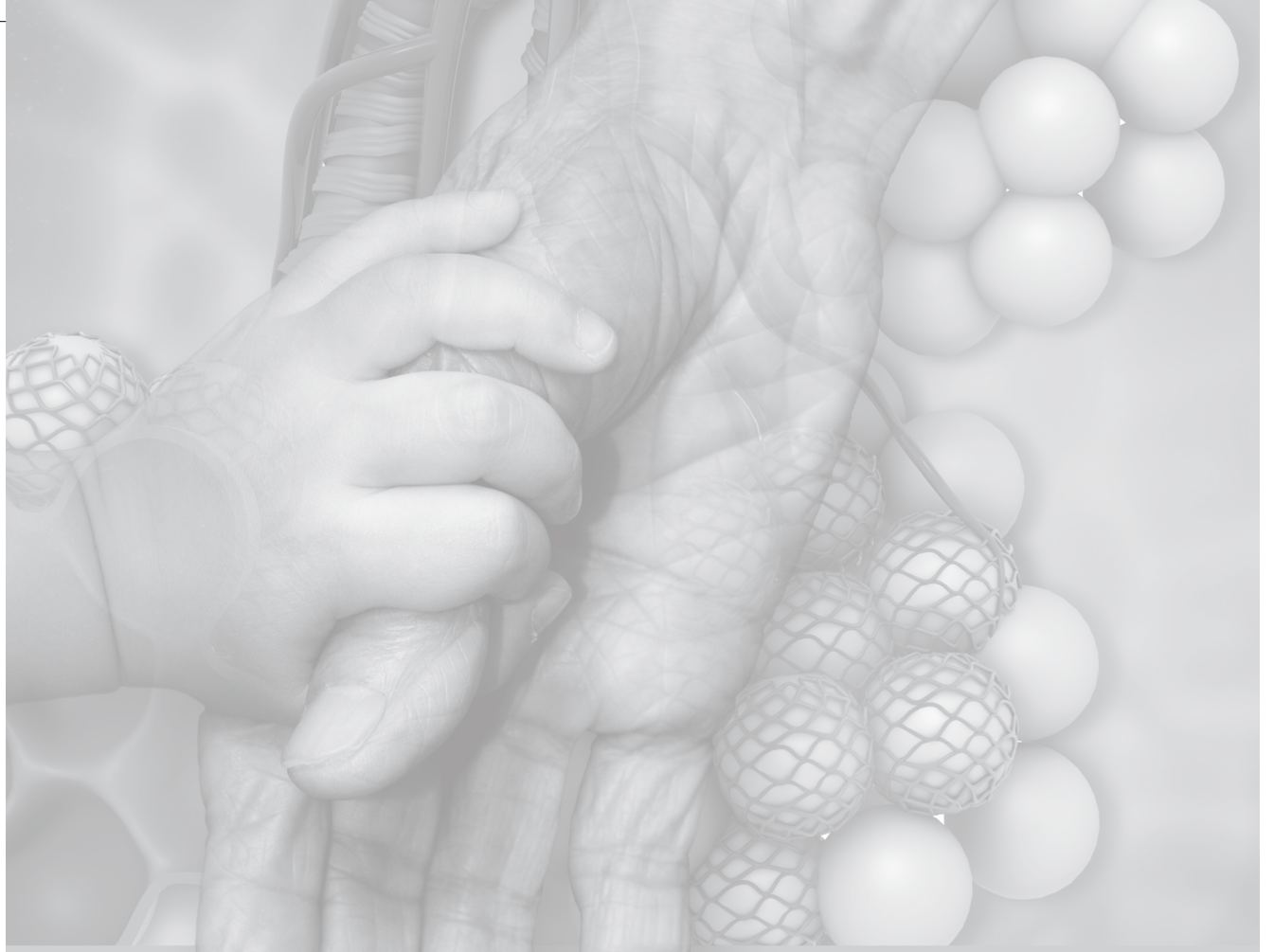

Chapter 5

Changes in the nitric oxide pathway of the pulmonary vasculature after exposure to hypoxia in swine model of neonatal pulmonary vascular disease

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ABSTRACT

Neonatal pulmonary vascular disease (PVD) is increasingly recognized as a disease that complicates the cardiopulmonary adaptations after birth and predisposes to long-term cardiopulmonary disease. There is growing evidence that PVD is associated with disruptions in the nitric oxide (NO)-cGMP-phosphodiesterase5 (PDE5) pathway. Examination of the functionality of different parts of this pathway is required for better understanding of the pathogenesis of neonatal PVD. For this purpose, the role of the NO-cGMP-PDE5 pathway in regulation of pulmonary vascular function was investigated in vivo, both at rest and during exercise, and in isolated small pulmonary arteries in vitro, in a neonatal swine model with hypoxia-induced PVD. Endothelium-dependent vasodilation was impaired in piglets with hypoxia-induced PVD both in vivo at rest and in vitro. Moreover, the responsiveness to the NO-donor SNP was reduced in hypoxia-exposed piglets in vivo, while the relaxation to SNP and 8-bromo-cyclicGMP in vitro were unaltered. Finally, PDE5 inhibition-induced pulmonary vasodilation was impaired in hypoxia-exposed piglets both in vitro and in vivo at rest. During exercise, however, the pulmonary vasodilator effect of PDE5 inhibition was significantly larger in hypoxia-exposed as compared to normoxia-exposed piglets. In conclusion, the impaired endothelium-dependent vasodilation in piglets with hypoxia-induced PVD was accompanied by reduced responsiveness to NO, potentially caused by altered sensitivity and/or activity of souluble guanylyl cyclase (sGC), resulting in an impaired cGMP production. Our findings in a newborn animal model for neonatal PVD suggests that sGC stimulators/activators may be a novel treatment strategy to alleviate neonatal PVD.

INTRODUCTION

Dysmorphic pulmonary vascular growth and impaired alveolarization are hallmarks of the disruption of normal lung development that characterizes bronchopulmonary dysplasia (BPD).[1-3] Pulmonary vascular disease (PVD) encompasses decreased angiogenesis, abnormal vascular function with increased vasomotor tone and altered vasoreactivity, and abnormal vascular structure (vascular remodeling) with smooth muscle cell proliferation. These changes result in an increased pulmonary vascular resistance (PVR), thereby increasing the risk of pulmonary hypertension (PH) in BPD patients.[1-4] Retrospective studies have determined the incidence of BPD-associated PH to be approximately 20-40%.[5-8] Although most studies concerning long-term outcome of BPD focus on respiratory function, there is increasing awareness that PVD (including PH) imposes additional morbidity, including prolonged oxygen requirements and exercise intolerance, and mortality in the already vulnerable BPD infants.[6,8] Therapies currently used in clinical practice for infants with PVD are supplemental oxygen therapy to avoid hypoxic pulmonary vasoconstriction[4,9], pulmonary vasodilators such as sildenafil (phosphodiesterase 5 inhibitor) and inhaled nitric oxide (iNO)[4,9], that may also limit oxidative stress caused by hyperoxia.[10,11] However, the knowledge regarding treatment efficacy and safety of BPD-associated PH is limited and needs to be further investigated.

Previous studies in swine showed an important role for the nitric oxide (NO)-cGMP signaling pathway in the pathogenesis of chronic PH. Nitric oxide (NO) is produced in the vascular endothelial cells from L-arginine by the enzyme endothelial nitric oxide synthase (eNOS), and then diffuses to the vascular smooth muscle cell. There, NO stimulates soluble guanylyl cyclase (sGC), resulting in the production of cGMP, which activates cGMP-dependent protein kinases. Ultimately, this leads to opening of the large conductance K (BK_{Ca}) channel, smooth muscle cell relaxation and thus vasodilation.[12] There is growing evidence that neonatal pulmonary hypertension is associated with multiple disruptions in this signaling cascade. A decreased eNOS activity and reduced vasodilator response to NO were found in different animal models for neonatal PH.[13-18] There are, however, only a few studies regarding the role of more downstream disruptions in the NO-cGMP signaling pathway, including sGC -and cGMP-dependent mechanisms.[13,16,19] Furthermore, no studies were performed to assess the long-term consequences of disruptions in the NO-cGMP signaling pathway. Therefore, examination of the functionality of different parts of this pathway is required for better understanding of the pathogenesis of BPD-associated PH, not only in the neonatal period but also later in life.

We previously developed a neonatal swine model with hypoxia-induced PH showing the clinical features resembling those found in patient with neonatal PVD and/or PH, in terms of pulmonary hemodynamics, abnormalities in the structure of the right ventricle and disruptions in normal lung development (vascular remodeling).[20] This model allows 3-weeks of follow-up after re-exposure 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 helps to assess the severity of the disease. Consequently, the main purpose of this study was to determine the functionality of different parts of the NO-cGMP signaling pathway in the long-term, in vivo (at rest and during incremental exercise) and in vitro, to achieve greater understanding of the pathogenesis of neonatal PH associated with BPD (or other chronic cardiopulmonary disorders associated with hypoxia) and the long-term sequelae of damage to the developing neonatal lung.

METHODS

Ethical approval

Studies were performed in accordance with European Directive 2010/63/EU as well as with the "Guiding Princi- ples in the Care and Use of Laboratory Animals" as approved by the Council of the American Physiological Society, and with approval of the Animal Care Committee of the Erasmus MC Rotterdam (Protocolnumber 109-12- 11, EMC 2702).

In vivo animal experiments

Thirty-four crossbred Landrace x Yorkshire piglets of either sex entered the study when they were 48 h old. Shortly after arrival, all piglets received a single dose of artificial colostrum (Colo-active) and were placed in an incubator, in which the fraction of inspired oxygen (FiO_2) can be regulated, for 4 weeks. Piglets assigned to the control group were exposed to a normobaric normoxic environment (FiO_2 21%; N=16), whereas piglets assigned to the intervention group were exposed to a normobaric hypoxic environment (N=18). Piglets in the Hypoxia group were exposed to FiO_2 10% for at least 1 week, and FiO_2 was adjusted to higher levels (max. FiO_2 12%) based on clinical signs of severe pulmonary hypertension (severe dyspnea, growth retardation, septal shift on echocardiography). Piglets were fed age appropriate (Lactowean Extra, Babywean, Topwean; Denkavit, Voorthuizen, The Netherlands) and received a supplementary feed for piglets, based on egg-yolk (MS Pig Pusher Oral, Schippers BV, Bladel, The Netherlands) from day 1-3, to support the immune system and to increases the vitality of the newborn piglets, especially in case of insufficient colostrum. They were weighed daily.

After 4 weeks 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.

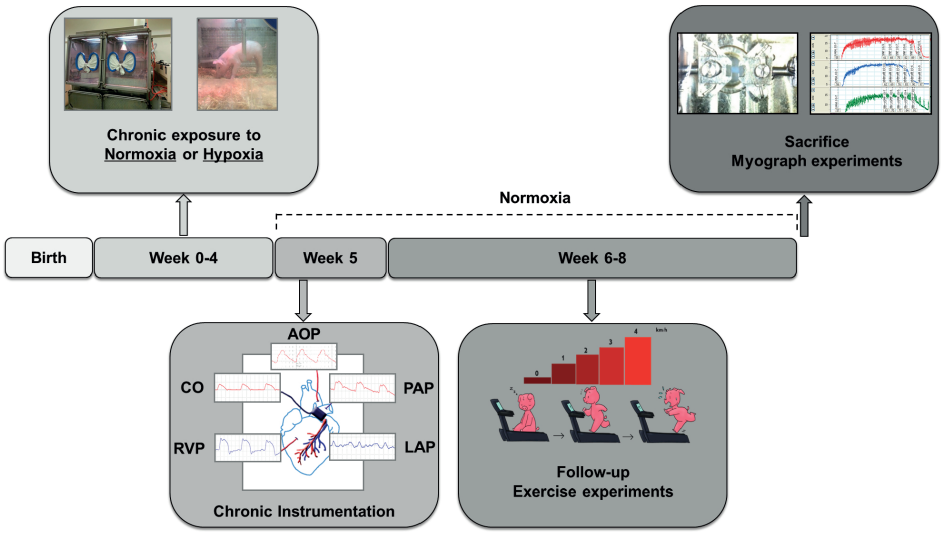

Figure 1. Schematic representation of the experimental design of the study.

Surgical procedures

Piglets were sedated with tiletamine/zolazepam (3 mg kg^{-1} i.v.), xylazine, (1.75 mg kg^{-1} i.v.), and atropine (0.5 mg), intubated and ventilated with a mixture of O_2 and N_2 (1:2) to which 2.0% (v/v) isoflurane was added for adequate anesthesia. The depth of anesthesia was checked regularly using a pain stimulus (toe-pinch). Due to premature death, caused by health problems related to exposure to hypoxia (N=6), only 28 piglets (Normoxia, N=16;, Hypoxia N=12) were instrumented under sterile conditions as previously described.[21] 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 for pressure measurements. Furthermore, these catheters were used for blood sampling as well as for the infusion of drugs. In a subset of animals (N=13), a flow probe (14-16 mm, Transonic Systems) was positioned around the pulmonary artery for measurement of cardiac output. Catheters and electrical wires were tunneled subcutaneously to the back and the chest was closed in layers. All animals were placed in a normoxic environment and were allowed to recover, receiving analgesia (Buprenorphine 0.015 mg kg^{-1} i.m., Fentanyl slow-release patch 6 µg hr^{-1}) and antibiotic prophylaxis (Augmentin 25/5 mg kg^{-1} i.v.) for 7 days. The catheters were flushed daily with heparinized saline (1000-5000 IE mL^{-1}). Five piglets died during or shortly after surgery, and four piglets were not able to perform exercise experiments due to limb infection. Finally, 19 piglets (Normoxia, N=10, 6 female piglets, 4 male piglets; Hypoxia, N=9, 5 female piglets, 4 male piglets) were included for analysis.

Experimental protocols; agonist-induced vasodilation

Studies were performed 10±1 days after surgery in both groups. Fluid-filled pressure transducers were positioned on the back of the animals and calibrated at midchest level. Baseline hemodynamic measurements were obtained in resting piglets (Normoxia, N=8, 6 female piglets, 2 male piglets; Hypoxia, N=8, 4 female piglets, 4 male piglets), consisting of heart rate, cardiac output (CO), aortic pressure (MAP), pulmonary artery pressure (PAP), left atrial pressure (LAP), and right ventricular pressure (RVP). To study the responsiveness of the vascular beds to nitric oxide (NO), we determined the hemodynamic responses to the endothelium-independent exogenous NO-donor sodium nitroprusside (SNP; 0.5-5 μg kg^{-1} min^{-1}, i.v.) in resting swine.

Experimental protocols; exercise experiments

Controle exercise trials were performed 9±1 days after surgery in both groups with piglets exercising on a motor-driven treadmill. Fluid-filled pressure transducers were positioned on the back of the animals and calibrated at midchest level. With piglets (Normoxia, N=10, 6 gemale piglets, 4 male piglets; Hypoxia, N=9, 5 female piglets, 4 male piglets) standing on the treadmill, resting hemodynamic measurements, consisting of heart rate, cardiac output (CO), MAP, PAP, LAP, and 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^{-1}); each exercise stage lasted 3 min. Hemodynamic parameters were continuously recorded and blood samples were collected during the last 45 sec of each exercise stage, at a time when hemodynamics had reached a steady state.[21] After completing the exercise protocol, animals were allowed to rest on the treadmill. After 90 min of rest, two different protocols (see below) were performed in a subset of swine, on different days and in random order. The number of swine in each protocol, as well as overlap between protocols, are shown in Table 1. Excellent reproducibility of consecutive exercise trials has been reported previously.[22]

Ninety minutes after piglets had undergone a control exercise trial (as described above) the NO-synthase inhibitor N^{ω}-nitro-L-Arginine (LNNA, Sigma) was administered at a dose of 20 mg kg^{-1} i.v. in nine normoxia-exposed (6 female piglets, 3 male piglets, 19±1 days after

Table 1. Schematic representation of the overlap of swine used in the different protocols.

	Control	LNNA	EMD360527	Total
Control	**10N/9H**	9N/8H	6N/5H	
LNNA		**9N/8H**	6N/5H	
EMD360527		-	**6N/5H**	
Total				**10N/9H**

Bold font indicates the total number of swine in each experimental protocol.
N: normoxia, H: hypoxia

surgery) and eight hypoxia-exposed (5 female piglets, 3 male piglets, 18±1 days after surgery and re-exposure to normoxia) piglets. Ten minutes after completion of the infusion, resting measurements were obtained and the four-stage exercise protocol was repeated.[23]

On a different day (16±2 days after surgery for normoxia animals and 14±1 days after surgery and re-exposure to normoxia for the hypoxia-exposed animals), the exercise protocol was repeated, but during the second exercise protocol the phosphodiesterase-5 (PDE5) inhibitor EMD360527 (a gift from Merck, Darmstadt, Germany) was infused continuously in a dose of 300 μg kg^{-1} min^{-1} i.v. in six normoxia-exposed (2 female piglets, 4 male piglets) and five hypoxia-exposed (3 female piglets, 2 male piglets) piglets. Ten minutes after starting the infusion, resting measurements were obtained and the four-stage exercise protocol was performed.[24,25]

In vitro myograph experiments

At the end of the study (4-6 weeks after re-exposure to normoxia), all piglets (Normoxia, N=10; Hypoxia, N=9) were reanesthetized and killed by inducing cardiac arrest using electromechanical dissociation of the heart. Right lungs were immediately excised and pulmonary small arteries (diameter ≈ 300 μm) were dissected out from the lower lung lobe and stored overnight placed in cold, oxygenated Krebs bicarbonate solution of the following composition (in mmol/L): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 8.3; pH 7.4. The next day, pulmonary small arteries were cut into segments of ~2mm length and mounted in microvascular myographs (Danish MyoTechnology) with separated 6mL organ baths containing Krebs bicarbonate solution aerated with 95% O2-5% CO2 and maintained at 37°C. Changes in contractile force were recorded with a Harvard isometric transducer. Following a 30-minute stabilization period, a length-tension curve was constructed and the internal diameter was set to a tension equivalent to 0.9 times the estimated diameter at 20 mmHg effective transmural pressure. Vessels were then exposed to 30 mmol/L KCl twice. Endothelial integrity of pulmonary arteries was verified by observing dilation to 10 nmol/L substance P after precontraction with 100 nmol/L of the stable thromboxane A_2 analogue pyridoxalphophate-6-azophenyl-2'4'-disulfonic acid (U46619). If no vasorelaxation to substance P was observed, the vessel segment was excluded from further study. Then vessels were subjected to 100 mmol/L KCl to determine the maximal vascular contraction. Thereafter, pulmonary arteries were allowed to equilibrate in fresh Krebs solution for 30 min, before initiating different experimental protocol.[25]

Response to vasoactive substances in vitro

After 30 minutes of equilibration in fresh Krebs, pulmonary small arteries were precontracted with 100 nmol/L U46619 before starting with one of five different experimental protocols.[25] Only one protocol was executed per vessel and within one protocol, all vessels were obtained from different animals.

In a first set of segments (Normoxia, N=5, 3 female, 2 male; Hypoxia, N=8, 4 female, 4 male), endothelium-dependent vasodilation to bradykinin (BK; 10^{-10} – $10^{-6.5}$ mol/L; Sigma-Aldrich, Zwijndrecht, The Netherlands) was recorded. Concentration-response curves (CRC to the PDE-5 inhibitor sildenafil (10^{-10} – 10^{-5} mol/L; Sigma-Aldrich) were constructed from a second set of segments (Normoxia, N=6, 4 female, 2 male; Hypoxia, N=8, 4 female, 4 male). To study whether responses to endothelium-independent but NO-mediated vasodilation were altered by transient exposure to chronic hypoxia, CRCs to exogenous NO-donor sodium nitroprusside (SNP; 10^{-9} – 10^{-6} mol/L; Sigma-Adrich) were examined (Normoxia, N=9, 6 female, 3 male; Hypoxia, N=8, 4 female, 4 male). Finally, separate vessel segments were studied to determine whether impairments in smooth muscle cell relaxation to the NO second messenger, cyclic GMP, were involved in altered responses to SNP. For these studies, CRCs to 8-bromo-cyclic GMP (10^{-7} – $10^{-3.5}$ mol/L) were measured (Normoxia, N=8, 5 female, 3 male; Hypoxia, N=8, 4 female, 4 male).

Data analysis and statistical analysis

Digital recording and off-line analysis of hemodynamics have been described previously.[26] Pulmonary vascular conductance (PVC) was defined as CO divided by PAP minus LAP.[27] Systemic vascular conductance (SVC) was calculated as the ratio of CO and MAP. To accommodate for the varying weights between animals and groups, CO, PVC, and SVC were indexed to body weight. Statistical analysis was performed using SPSS version 21.0 (IBM, Armonk, NY). To test whether the effect of drug intervention (change vs. control; Δ) on exercise response was different in hypoxia vs. normoxia, regression analysis was performed with FiO_2 and treadmill speed, as well as their interaction as independent variables and animal number as case label. To test for the effect of hypoxia (vs. normoxia) on endothelium-independent but NO-mediated vasodilation (SNP), General Linear Model (GLM) – Repeated Measures were used.

Vascular relaxation responses to the different vasoactive agents were expressed as percentage of contraction to U46619. Statistical analysis was performed using SPSS version 21.0 (IBM) and Prism version 5.0 (Graphpad Software, Inc., La Jolla, CA). The maximal relaxation (E_{max}) and half maximal effective concentration (EC50) in each experiment was calculated using the GraphPad Prism version 5 for Windows (Graphpad Software, San Diego, CA). Statistical analysis of maximal relaxation and EC50 was performed using nonlinear regression (log (agonist) vs. respons). Statistical analysis of maximal relaxation and EC50 was performed nonlinear regression (log (agonist) vs. respons).

Statistical significance was accepted at $P \leq 0.05$. Grouped data are presented as mean ± SEM.

RESULTS

Effect of chronic exposure to hypoxia on hemodynamics at rest and during exercise

Exercise up to 4 km h^{-1} produced a significant increase in heart rate (Figure 2A) and cardiac index (Figure 2B) both in animals raised in normoxia and hypoxia. Although MAP was slightly, but significantly, lower in hypoxia-raised piglets as compared to normoxia-raised piglets, the exercise-induced systemic vasodilatation, as measured by the decrease in SVCi, was comparable between the two groups, and minimally affected MAP in either the normoxic or the hypoxic group (Figure 2C and 2D).

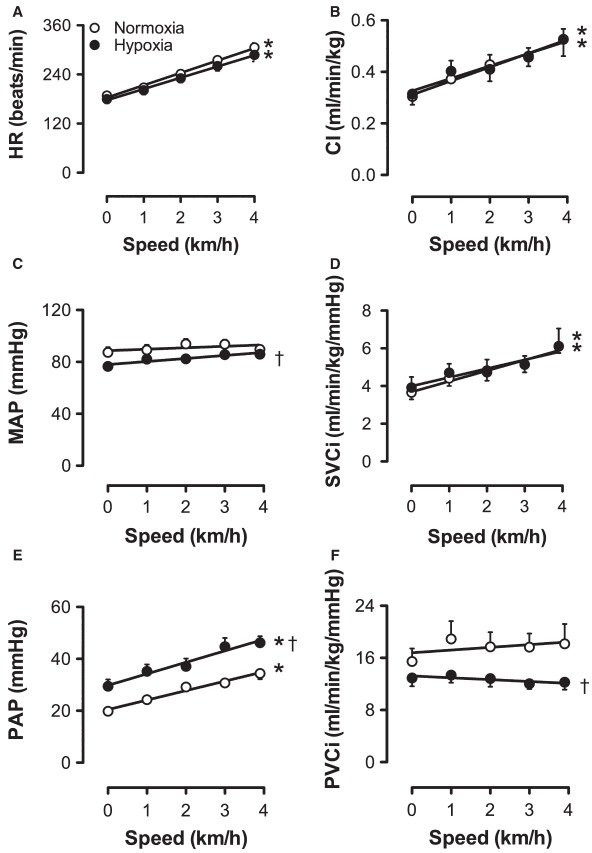

Figure 2. Changes in systemic and pulmonary hemodynamics during graded treadmill exercise in normoxia (N=10, 9±1 day postsurgery) -and hypoxia (N=9, 9±1 day postsurgery and re-exposure to normoxia) exposed piglets. Relation between treadmill speed and (A) heart rate (HR), (B) cardiac index (CI), (C) mean arterial pressure (MAP), (D) systemic vascular conductance index (SVCi), (E) mean pulmonary arterial pressure (PAP) and (F) pulmonary vascular conductance index (PVCi). Values are mean ± SEM. * $P \leq 0.05$ effect of exercise; † $P \leq 0.05$ versus controls vs.normoxia.

In the pulmonary circulation, PVCi remained constant during exercise due to the small vasodilator capacity of the lung vasculature (Figure 2F; normoxia, 16 ± 10%; hypoxia, −3 ± 6). The increase in LAP during exercise, in combination with the marked increase in cardiac index, which caused an increase in the pressure drop across the pulmonary vasculature, in the face of a constant PVCi, resulted in a progressive increase in PAP with incremental levels of exercise. This exercise-induced increase in PAP was similar in both groups (Figure 2E).

Both at rest, and during incremental exercise, PAP was significantly higher in hypoxia-exposed piglets as compared to normoxia-exposed piglets (Figure 2E). PVCi was significantly lower in animals raised in hypoxia, indicative for pulmonary vasoconstriction and/or vascular remodeling (Figure 2F). Thus, exposure to chronic hypoxia in early life leads to pulmonary hypertension at rest and during exercise, even following re-exposure to normoxia.

Effect of chronic exposure to hypoxia on the NO-pathway in the pulmonary vasculature in vivo

In the systemic circulation in vivo, administration of incremental dosages of the exogenous NO-donor SNP resulted in a similar decrease in mean aortic pressure in hypoxia- and normoxia-exposed animals (Figure 3A), while heart rate, cardiac index (CI) and SVCi did not significantly change in either group (data not shown). In contrast to the systemic hemodynamic response, SNP caused a dose-dependent decrease in PAP only in normoxia-exposed piglets (Figure 3B). In hypoxia-exposed piglets, the pulmonary vasodilator response to SNP was abolished (Figure 3B), indicative for a reduced responsiveness of the pulmonary vascular bed to NO.

Administration of the NO-synthase inhibitor LNNA in vivo increased MAP and decreased SVCi to a similar extent in normoxia-exposed and hypoxia-exposed piglets, both at rest and during incremental levels of exercise (Table 2). This increase in aortic pressure

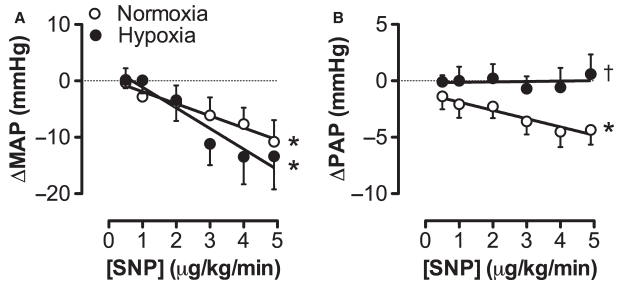

Figure 3. Effect of exogenous NO-donor sodium nitroprusside (SNP) on mean arterial pressure (MAP) and mean pulmonary artery pressure (PAP) during graded treadmill exercise in normoxia- and hypoxia-exposed piglets. Relation between SNP-dosage and (A) change in mean arterial pressure (ΔMAP; Normoxia, N = 9; Hypoxia, N = 8), and (B) change in mean pulmonary artery pressure (ΔPAP; Normoxia, N = 10; Hypoxia,N = 8). Experiments were performed 10±1 days postsurgery in both groups. Values are mean±SEM. *P ≤ 0.05 effect of SNP-dosage; †P ≤ 0.05 versus normoxia.

Table 2. Hemodynamics in normoxia-exposed and hypoxia-exposed piglets.

		Normoxia				Hypoxia			
		Rest	Maximum Exercise	Δ Rest	Δ Maximum Exercise	Rest	Maximum exercise	Δ Rest	Δ Maximum Exercise
HR	Control	171 ± 6	289 ± 13 *	−24 ± 6 §	−26 ± 22	174 ± 8	290 ± 13 *	−8 ± 9	−23 ± 14
(Beats min^{-1})	LNNA	147 ± 8 ‡	263 ± 19 *			166 ± 10	267 ± 17 *		
	Control	166 ± 21	221 ± 71	10 ± 10	−4 ± 10	174 ± 42	253 ± 32 *	9 ± 35	123 ± 104
	EMD360527	176 ± 26	218 ± 69			183 ± 18	376 ± 78 **		
MAP	Control	92 ± 4	96 ± 8	36 ± 4 §	26 ± 11 §§	82 ± 5	85 ± 3	30 ± 7 §	36 ± 2 §
(mmHg)	LNNA	127 ± 5 ‡	122 ± 15 ‡‡			111 ± 4 †‡	120 ± 3 *‡		
	Control	102 ± 11	96 ± 8	−8 ± 2 §	−5 ± 7	83 ± 3	91 ± 2 *	−8 ± 4 §§	−19 ± 1 *†§
	EMD360527	95 ± 11 ‡	91 ± 1			75 ± 5 ‡‡	72 ± 2 †‡		
LAP	Control	4 ± 2	8 ± 3	4 ± 2 §	−1 ± 3	4 ± 1	7 ± 1 *	4 ± 2 §§	2 ± 2
(mmHg)	LNNA	8 ± 3 ‡	7 ± 4			8 ± 2 ‡‡	9 ± 2		
	Control	4 ± 2	6 ± 4	2 ± 1	3 ± 1 *§§	2 ± 1	9 ± 2 *	0 ± 1	−2 ± 1 **†
	EMD360527	5 ± 2	9 ± 3 **‡‡			3 ± 1	7 ± 2 *		
CI	Control	0.39 ± 0.03	0.46 ± 0.07 *	−0.11 ± 0.02 §	−0.19 ± 0.04 §	0.34 ± 0.06	0.51 ± 0.07 *	−0.06 ± 0.03	−0.14 ± 0.03 **§
(L min^{-1} kg^{-1})	LNNA	0.28 ± 0.04 ‡	0.27 ± 0.06 *‡			0.28 ± 0.05	0.37 ± 0.05 *‡		
	Control	0.31 ± 0.04	0.44 ± 0.07 *	0.00 ± 0.02	0.04 ± 0.02	0.26 ± 0.03	0.45 ± 0.04 *	0.12 ± 0.12	0.15 ± 0.11
	EMD360527	0.31 ± 0.03	0.48 ± 0.09 *			0.29 ± 0.02	0.60 ± 0.14 **		
SVCi (ml min^{-1}	Control	4.5 ± 0.4	5.8 ± 0.5 *	−2.1 ± 0.2 §	−2.8 ± 0.1 §	3.3 ± 0.5 ††	5.1 ± 0.2	−0.9 ± 1.1	−2.5 ± 0.2 §
kg^{-1}mmHg^{-1})	LNNA	2.4 ± 0.3 ‡	2.9 ± 0.4 **‡			2.4 ± 0.5	2.7 ± 0.1 ‡		
	Control	3.1 ± 0.1	5.3 ± 0.5 **	0.3 ± 0.4	0.9 ± 0.2	3.2 ± 0.6	5.0 ± 0.5 *	0.8 ± 0.1 §	3.3 ± 1.5
	EMD360527	3.5 ± 0.5	6.2 ± 0.4			4.0 ± 0.6 ‡	8.3 ± 2.0 **		

Table 2. Hemodynamics in normoxia-exposed and hypoxia-exposed piglets. (*continued*)

		Normoxia				Hypoxia			
		Rest	Maximum Exercise	Δ Rest	Δ Maximum Exercise	Rest	Maximum exercise	Δ Rest	Δ Maximum Exercise
PVCi (ml min^{-1}	Control	26.5 ± 3.6	22.0 ± 7.3	−12.8 ± 2.2 §	−13.1 ± 4.2 §	14.6 ± 1.0 †	14.9 ± 1.4	−2.9 ± 5.3 ††	−8.7 ± 1.1 §
kg^{-1} mmHg^{-1})	LNNA	13.7 ± 4.5 ‡	8.9 ± 3.4 ‡			11.8 ± 4.3	6.2 ± 0.4 ‡		
	Control	15.9 ± 2.5	15.1 ± 3.3	10.6 ± 5.6	7.3 ± 2.3 §§	16.6 ± 3.0	16.6 ± 4.6	4.5 ± 1.7 §	10.3 ± 2.3 *§
	EMD360527	26.5 ± 5.3	22.4 ± 4.1 ‡‡			21.1 ± 2.5 ‡	22.4 ± 3.5 ‡		

Values are means ± SEM; N=9 normoxia-exposed and N=8 hypoxia-exposed piglets in the control/LNNA group; N=6 normoxia-exposed and N=5 hypoxia-exposed piglets in the control/EMD360527 group. Maximum exercise is 4 km h^{-1}. HR, heart rate; MAP, mean arterial pressure; LAP, left atrium pressure; CI, cardiac index; SVCi, systemic vascular conductance indexed for bodyweight; PVCi, pulmonary vascular conductance indexed for bodyweight. * P ≤ 0.05 effect of exercise; ** P ≤ 0.10 effect of exercise; † P ≤ 0.05 versus normoxia; †† P ≤ 0.10 versus normoxia; ‡ P ≤ 0.05 versus hemodynamic value during control treadmill experiment (without vasoreactive agent); ‡‡ P ≤ 0.10 versus hemodynamic value during control treadmill experiment (without vasoreactive agent); § P ≤ 0.05 versus no difference (vs. Δ=0); §§ P ≤ 0.10 versus no difference (vs. Δ=0).

was accompanied by a decrease in CI (Table 2). Similar to the systemic hemodynamic response, PAP markedly increased after LNNA administration in both groups (Figure 4A). This increase in PAP was the result of extensive pulmonary vasoconstriction, as evidenced by a marked decrease in PVCi (Table 2). At rest, the LNNA-induced decrease in PVCi tended to be smaller in hypoxia-exposed piglets (Table 2, ΔPVCi normoxia vs. hypoxia p=0.09), resulting in a trend toward a smaller increase in PAP in hypoxia-exposed piglets as compared to normoxia-exposed piglets (Figure 3A; ΔPAP normoxia, +17 ± 3 mmHg; hypoxia, +12 ± 3 mmHg; p=0.06). In contrast, the effect of LNNA on PAP during exercise increased significantly more in hypoxia-exposed piglets as compared to controls (Figure 4A; FiO2*exercise p=0.05).

At rest, PDE5 inhibition with EMD360527 resulted in a decrease in MAP in normoxia-exposed and hypoxia-exposed piglets (Table 2). This decrease in MAP was accompanied by an EMD360527-induced increase in SVCi, reaching statistical significance in hypoxia-exposed piglets only. The effect of PDE5 inhibition during exercise on MAP was significantly larger in hypoxia-exposed piglets as compared to normoxia-exposed pigets, resulting in a significantly lower MAP at maximal exercise in hypoxia-exposed piglets (Table 2). In the pulmonary circulation of normoxia-exposed piglets, PDE5 inhibition increased PVCi and decreased PAP to a similar extent at rest and during exercise (Table 2, Figure 4B). However, in hypoxia-exposed piglets the EMD360527-induced increase in PVCi was significantly augmented during graded treadmill exercise (Table 2; ΔPVCi rest vs maximal exercise, p=0.05). Consequently, while the decrease in PAP at rest tended to be smaller, the effect of PDE5 inhibition during exercise was significantly larger in hypoxia-exposed

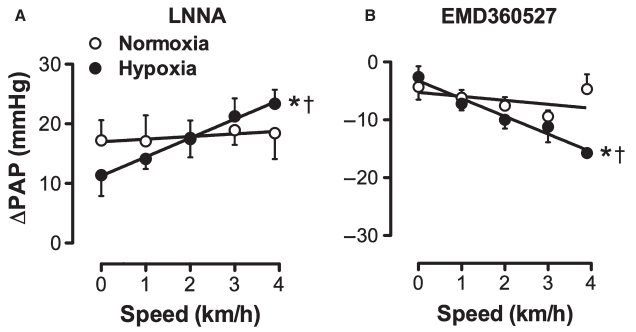

Figure 4. Effect of drug intervention on mean pulmonary artery pressure (PAP) during graded treadmill exercise in normoxia- and hypoxia- exposed piglets. Relation between treadmill speed and (A) change in mean pulmonary arterial pressure (ΔPAP) after administration of NO- synthase inhibitor LNNA (Normoxia, N = 9; Hypoxia, N = 8), and (B) change in mean pulmonary artery pressure (ΔPAP) after administration of PDE5-inhibitor EMD360527 (normoxia, N = 6; Hypoxia, N = 5). LNNA-experiments were performed 18±1 (normoxia) and 19±1 (hypoxia) days postsurgery, whereas EMD360527 experiments were performed 16±2 (Normoxia) and 14±1 (Hypoxia) days postsurgery. Values are mean±SEM. *P ≤ 0.05 effect of exercise; †P ≤ 0.05 versus Normoxia.

as compared to normoxia-exposed piglets (Figure 4B; FiO2*exercise p=0.01). These data suggest an impaired cGMP production or PDE5 activity in hypoxia-exposed piglets at rest, which recovers during exercise.

Effect of chronic exposure to hypoxia on the NO-pathway in isolated pulmonary small arteries

Six weeks following re-exposure to normoxia and chronic instrumentation, responses of pulmonary small arteries were determined in vitro. There were no significant differences in concentration-dependent vasodilation to SNP in precontracted isolated porcine pulmonary small arteries from either normoxia-exposed or hypoxia-exposed piglets (Figure 5A). Also, no differences in relaxation to 8-bromo-cyclic GMP were found in pulmonary small arteries isolated from hypoxia-exposed piglets as compared to controls (Figure 5B).

Cumulative concentrations of bradykinin produced a concentration-dependent vasodilation up to 100% in precontracted isolated porcine pulmonary small arteries from both normoxia-exposed and hypoxia-exposed piglets (Figure 5C). This vasodilator response to bradykinin was significantly shifted to the right in pulmonary small arteries from hypoxia-exposed piglets as compared to normoxia-exposed controls (logEC50 normoxia −8.32±0.09M; hypoxia −7.82±0.09M; P<0.05), indicative for impaired endothelium-dependent vasodilation in hypoxia-exposed piglets (Figure 5C). These findings are in agree-

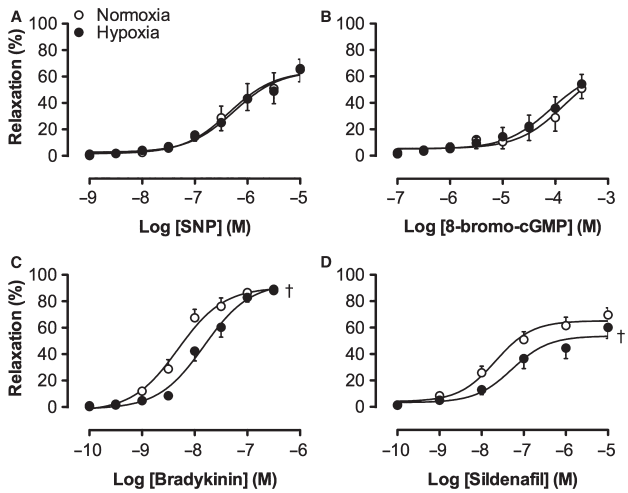

Figure 5. Vasodilator effects of different vasoactive agents in isolated pulmonary small arteries pre-constricted with U46619 (100 nmol/L) from normoxia- and hypoxia-exposed piglets. Shown are the concentration-response curves to (A) exogenous NO-donor sodium nitroprusside (SNP; normoxia, N = 9; hypoxia, N = 8), (B) 8-bromo-cyclic GMP (Normoxia, N = 8; Hypoxia, N = 8), (C) bradykinin (Normoxia, N = 5; Hypoxia, N = 8), and (D) PDE5-inhibitor sildenafil (Normoxia, N = 6; Hypoxia, N = 8). Values are mean±SEM. †P ≤ 0.05 versus controls (vs. normoxia).

ment with the reduced vasoconstriction in the pulmonary vasculature in response to LNNA at rest in hypoxia-exposed piglets in vivo.

In accordance with to the smaller pulmonary vasodilator response in vivo in resting hypoxia-exposed piglets, the CRC to the PDE5 inhibitor sildenafil was significantly different in precontracted isolated porcine pulmonary small arteries from hypoxia-exposed piglets as compared to those from normoxia-exposed piglets (Figure 5D, logEC50 normoxia –7.67±0.17 M; hypoxia –7.28±0.24 M)). These data are consistent with an impaired basal cGMP production or lower PDE5 activity in pulmonary small arteries from hypoxia-exposed piglets.

DISCUSSION

The present study compared the pulmonary vascular response of normoxia-exposed control piglets and piglets with hypoxia-induced pulmonary vascular disease, both in vivo and in vitro, to determine the functionality of different parts of the NO-cGMP signaling pathway. The main findings of the present study were that (1) exposure to chronic hypoxia in early life leads to pulmonary hypertension at rest and during exercise, even following re-exposure to normoxia. (2) The exogenous NO-donor SNP produced a dose-dependent decrease in PAP in normoxia-exposed but not hypoxia-exposed piglets, indicative for a reduces responsiveness of the pulmonary vascular bed to NO in this groups. (3) eNOS inhibition with LNNA resulted in an increase in PAP that tended to be smaller at rest, and larger during exercise in hypoxia-exposed compared to normoxia-exposed piglets. (4) In vivo, the PDE5 inhibition-induced decrease in PAP tended to be smaller at rest, while the effect of PDE5 inhibition during exercise was significantly larger in the pulmonary vasculature of hypoxia-exposed as compared to normoxia-exposed piglets. (5) In vitro, the vasodilator response to BK was significantly shifted to the right in hypoxia-exposed piglets and (6) the vasorelaxant response to the PDE5 inhibitor sildenafil was significantly blunted in precontracted isolated porcine pulmonary small arteries from hypoxia-exposed piglets as compared to normoxia-exposed piglets. However, (7) no impairments in vasorelaxation to SNP or the NO second messenger, 8-bromo-cyclic GMP, were found in pulmonary small arteries isolated from hypoxia-exposed piglets as compared to controls. The implications of the present findings are discussed below.

Animal model

Infants suffering from cardiopulmonary disorders associated with persistent or episodic hypoxia, such as BPD, are at risk for the development of PVD (including PH). Unfortunately, these patients often have a poor responsiveness to inhaled NO and alternative effective treatments for chronic PH in these patients remain largely limited. To better understand the pathophysiological mechanisms and to develop new therapies, several animal models for

neonatal PVD and pulmonary hypertension, including a neonatal swine model with hypoxia-induced pulmonary hypertension, have already been established.[28-35] Swine lungs share many anatomical, histological, biochemical, and physiological features with human lungs[36] and the relevance of the developing pulmonary circulation of neonatal piglets to human infants has been established already in the early '80s.[37,38] Although alveolar multiplication occurs faster in piglets (2-4 weeks compared to 3 years in human infants), the morphological development of pulmonary architecture in swine is comparable with humans.[36] We recently developed a neonatal swine model with hypoxia-induced PH, allowing long-term follow-up for several weeks after re-exposure to normoxia.[20] We showed that pulmonary hypertension induced by chronic hypoxia is transient, as pulmonary artery pressure was normalized 2-3 weeks after re-exposure to normoxia particularly in female swine. However, despite normalization of PAP, structural and functional changes in the right ventricle and the lung vasculature (vascular remodeling with smooth muscle cell proliferation) persisted throughout the 6-week study period.[20] In the present study, we elaborated on these findings and showed that the structural pulmonary microvascular changes were accompanied by altered regulation of pulmonary microvascular tone both in vivo and in vitro. Unfortunately, sample size in the different experimental protocols did not allow to investigate whether sex affected the vasoreactivity of the pulmonary vasculature.

A limitation of our model is that the flow probe, that was placed around the pulmonary artery in a subset of animals to measure cardiac output in vivo, caused a significant pulmonary artery stenosis that precluded pulmonary hemodynamic as well as right ventricular structural analyses beyond 3 weeks of re-exposure to normoxia.

The NO-cGMP pathway in neonatal pulmonary vascular disease at rest

The NO-cGMP signaling pathway is important for the adjustments in the pulmonary vasculature that accompany the transition from pre- to postnatal life following birth. There is increasing evidence that alterations in the NO-cGMP signaling pathway play an important role in the pathogenesis of neonatal PVD, including PH.[13-15,30] In neonatal intensive care units, iNO is used as rescue therapy for preterm infants with respiratory disease undergoing ventilation.[4,9,39] As perinatal hypoxia impacts the NO-cGMP pathway[14,15,40,41], and we have previously shown that neonatal hypoxia-induced pulmonary vascular alterations persist for several weeks following re-exposure to normoxia[20], we investigated the functionality of different parts of this pathway in our model of neonatal hypoxia-induced PH. Endothelium-dependent vasodilation was impaired in isolated pulmonary small arteries from hypoxia-exposed piglets, as evidenced by a significant rightward shift of the vasodilator response to bradykinin. These data are consistent with a study showing that perinatal hypoxia results in an impaired vasodilator response to bradykinin in isolated pulmonary small arteries of lambs.[41] We have previously shown that pulmonary vasodilation to bradykinin is largely NO-dependent[42], suggesting that the reduced response to bradykinin reflects impaired

NO signaling. In accordance with these findings in older swine, preliminary data show that eNOS inhibition reduced the vasodilator response to bradykinin in vessels from both hypoxia-exposed piglets (N = 8, logEC50 from 7.82±0.09 mol/L to 7.70±0.42 mol/L, maximum response from 94±4% to 3±7%, P<0.05) and control (N=2, logEC50 from 8.32±0.09 mol/L to 7.29±0.25 mol/L, maximum response from 91±3% to 32±6%, P < 0.05) and abrogated the difference in response to bradykinin between groups. These data are consistent with our in vivo findings that eNOS inhibition resulted in a smaller increase in PAP and PVCi at rest in hypoxia-exposed piglets as well as with findings of previous studies in neonatal piglets with hypoxia-induced PH form other groups. Both Fike et al. and Berkenbosch et al. found an impaired production of NO, through reduced eNOS protein expression and/ or activity [13,15] or a dysfunction/ uncoupling of eNOS [14,30,43] in swine following exposure to hypoxia in the early postnatal period. Timing of hypoxia and/or the animal model used may influence the effect of hypoxia on the NO-pathway. Thus, perinatal hypoxia in lambs did not affect the contribution of NO to bradykinin-induced dilatation.[41]

In addition to this evidence showing an impaired NO-production in neonatal PH, a recent study by Baczynski et al. showed that the positive response rate to iNO in preterm neonates with acute PH is only 46%[39], suggesting disruptions in the NO-cGMP pathway more downstream to eNOS/NO-production which result in an apparent reduction the responsiveness to NO. In agreement with findings of other studies[13,43], chronic postnatal hypoxia was associated with a diminished vasodilator responsiveness to the exogenous NO-donor SNP in vivo in the present study. In contrast, there were no significant differences in concentration-dependent vasodilation to SNP in precontracted isolated porcine pulmonary small arteries from either normoxia-exposed or hypoxia-exposed piglets. A limitation of our in vitro study is that the isolated pulmonary small arteries were used after overnight storage at 4°C, and hence it could be argued that the discrepancy between the in vivo and in vitro results may originate from this overnight storage. This is, however, unlikely as cellular processes that may affect vascular function will be decelerated at 4°C. Indeed, it has been shown that over- night storage of middle cerebral arteries does not affect vascular function to a wide variety of vasoconstrictors (prostaglandin F2a, UTP), endothelium-independent (SNP, papaverine) and (partially) endothelium-dependent, receptor-mediated (noradrenaline, histamine), and endothelium-dependent, receptor-independent eNOS-mediated (L-arginine) vasodilators.[44] Furthermore, overnight storage is a standard procedure in our laboratory, endothelial function as assessed with substance P was preserved in the vessel segments from both normoxia -and hypoxia-exposed animals, and we have previously shown clear differences between pulmonary small arteries from swine with pulmonary hypertension secondary to pulmonary vein banding and healthy controls that were performed within a week of killing, and that were consistent with in vivo observations.[45]

An alternative explanation for the discrepancy between our in vivo and in vitro results could be that the response of one segment of the pulmonary vasculature, that is the pulmo-

nary small arteries, in vitro does not completely reflect the response of the intact pulmonary vasculature with vessels from different sizes contributing to overall pulmonary vascular resistance responses. However, it is most likely that the discrepancy between in vivo and in vitro findings is explained by the length of re-exposure to normoxia. In vivo experiments were performed 1-3 weeks after re-exposure to normoxia, whereas piglets were killed after 4-6 weeks. Indeed, pulmonary small arteries from lambs exposed to prenatal hypoxia, that were maintained in normoxia for approximately 3 weeks following delivery showed a reduced responsiveness to SNP.[40] This reduced responsiveness to SNP was accompanied by upregulation of PDE5. Conversely, in our study, a reduced pulmonary vasodilator response to PDE5 inhibition in hypoxia-exposed piglets as compared to normoxia-exposed control piglets was present both in vivo and in vitro, which is consistent with a reduced cGMP production in piglets with hypoxia-induced pulmonary vascular disease. The effectors of cGMP-mediated vasodilation are the BK_{Ca} channels. These BK_{Ca} channels are upregulated in pulmonary vascular smooth muscle cells by exposure to hypoxia in vitro[46], and following prenatal hypoxia and postnatal normoxia in vivo.[40] However, their contribution to bradykinin-induced vasodilatation in vitro following perinatal hypoxia was reduced.[41] Altogether, evidence from literature as well as the present study suggests that the impaired endothelium-dependent vasodilation in piglets with hypoxia-induced PH in the first 3 weeks after re-exposure to normoxia is due to a reduced responsiveness to NO, probably caused by altered sensitivity and/or activity of sGC, resulting is an impaired cGMP production, which may (partially) be compensated by an increased expression of BK_{Ca} channels. Our findings are consistent with previous in vivo and in vitro studies which investigated the effect of sGC activators and stimulators in acute and chronic hypoxia. Lundgren et al. showed that sGC stimulation completely reversed the pulmonary vasoconstrictor response to acute hypoxia in pigs.[47] Weissmann et al. also found a dose-dependent attenuation of acute pulmonary hypoxic vasoconstriction in isolated perfused mouse lung upon sGC stimulation.[48] Furthermore, they found that administration of the sGC activator HMR1766 during exposure to chronic hypoxia reduced pulmonary hypertension, as well as right ventricular hypertrophy and structural remodeling of the lung vasculature.[48] In addition, sGC stimulation and/or activation have been shown to inhibit or reverse the development of chronic hypoxic pulmonary hypertension in neonatal[49] and adult rats[50] and adult mice[51]. Our study adds important information to these previous studies by showing that alterations in the NO-pathway in a neonatal porcine model are still present several weeks after re-exposure to normoxia, and we speculate that this is due to a decrease in sGC sensitivity/activity.

The NO-cGMP pathway in neonatal pulmonary vascular disease during exercise

Exercise resulted in an increase in pulmonary artery pressure in both normoxia and hypoxia-exposed swine. Such exercise-induced increase in pulmonary artery pressure is generally

observed in quadrupeds, and much less in humans.[27] The main difference between the lungs of quadrupeds and humans is that the lungs of quadrupeds are located for a large part above heart level and that the entire lung is already perfused under resting conditions, whereas the large lower lung lobes of humans are at heart level, and the upper lobes are generally minimally perfused at rest. This means that recruitment of the hypo-perfused lung lobes during exercise as occurs in humans is not possible in swine, resulting in an increase in pulmonary artery pressure with an increase in cardiac output.[27]

It is well known that the NO-cGMP signaling pathway plays an important role in exercise-induced pulmonary vasodilation.[27] Given the exercise intolerance in patients with pulmonary vascular disease, it is of interest to investigate the functionality of this pathway during exercise. In the present study, we are the first to investigate the effect of exercise on NO-cGMP signaling in a model for neonatal hypoxia-induced pulmonary vascular disease.

In contrast to the smaller vasoconstrictive response of the pulmonary vasculature to NO-synthase inhibition at rest, the effect of LNNA on PAP during exercise tended to be larger in hypoxia-exposed piglets as compared to controls.

Interestingly, the effect of PDE5 inhibition during exercise was significantly larger in the pulmonary vasculature of hypoxia-exposed as compared to normoxia-exposed piglets, whereas it tended to be smaller at rest. This apparent discrepancy between the findings at rest and during exercise suggests a normalization of cGMP production during exercise. Given the impaired sGC activity, this normalization may involve membrane-bound or particulate guanylyl cyclase (pGC), might be involved. The activity of pGC can be stimulated by natriuretic peptides (ANP and BNP).[52] It is possible that the significant increase in PAP during incremental exercise causes secretion of natriuretic peptides by cardiomyocytes in response to cardiac stretch[53], and thus pGC activation. In support of this hypothesis, PAP and the pulmonary vssodilator effect of PDE5 inhibition were highly correlated (r^2=0.82; P<0.05; Figure 6). In hypoxia-exposed piglets, PAP is significantly higher as compared to normoxia-exposed piglets, resulting in higher natriuretic peptide levels and, consequently,

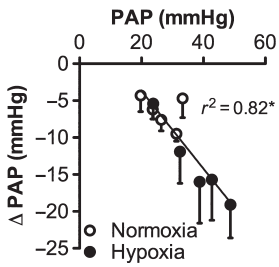

Figure 6. Effect of mean pulmonary arterial pressure (PA) on the pulmonary vasodilator effect of PDE5 inhibition with EMD360527. A significant correlation between PAP and the pulmonary vasodilator effect (ΔPAP) of PDE5 inhibition (r^2=0.82) is shown. Values are mean±SEM. * P ≤ 0.05 correlation between PAP and ΔPAP.

higher pGC activity. Whether higher natriuretic peptide levels are indeed present in this animal model and act to enhance cGMP production and thereby increase the vasodilator response to PDE5 inhibition, should be tested in future experiments.

Conclusion and Implications

In conclusion, hypoxia-induced PH is accompanied by impaired endothelium-dependent vasodilation in the pulmonary vasculature. In addition to evidence for an impaired NO-production in neonatal hypoxia-induced PH, through a reduced eNOS protein expression and/or activity[13,15] or dysfunction/uncoupling of eNOS[14,30,43], the present study provides evidence that there are disruptions in the NO-cGMP pathway more downstream to eNOS/NO. Thus, in our model for neonatal PH the impaired endothelium-dependent vasodilation was accompanied by a reduced responsiveness to NO in vivo, which may be caused by altered sensitivity and/or activity of sGC.

Our findings in a newborn animal model for neonatal pulmonary vascular disease suggests that sGC stimulators/activators could be of benefit as a novel treatment strategy to stop or even reverse neonatal pulmonary vascular disease and/or PH, especially since the use of iNO for preterm infants with respiratory failure is currently under debate.[54-57]

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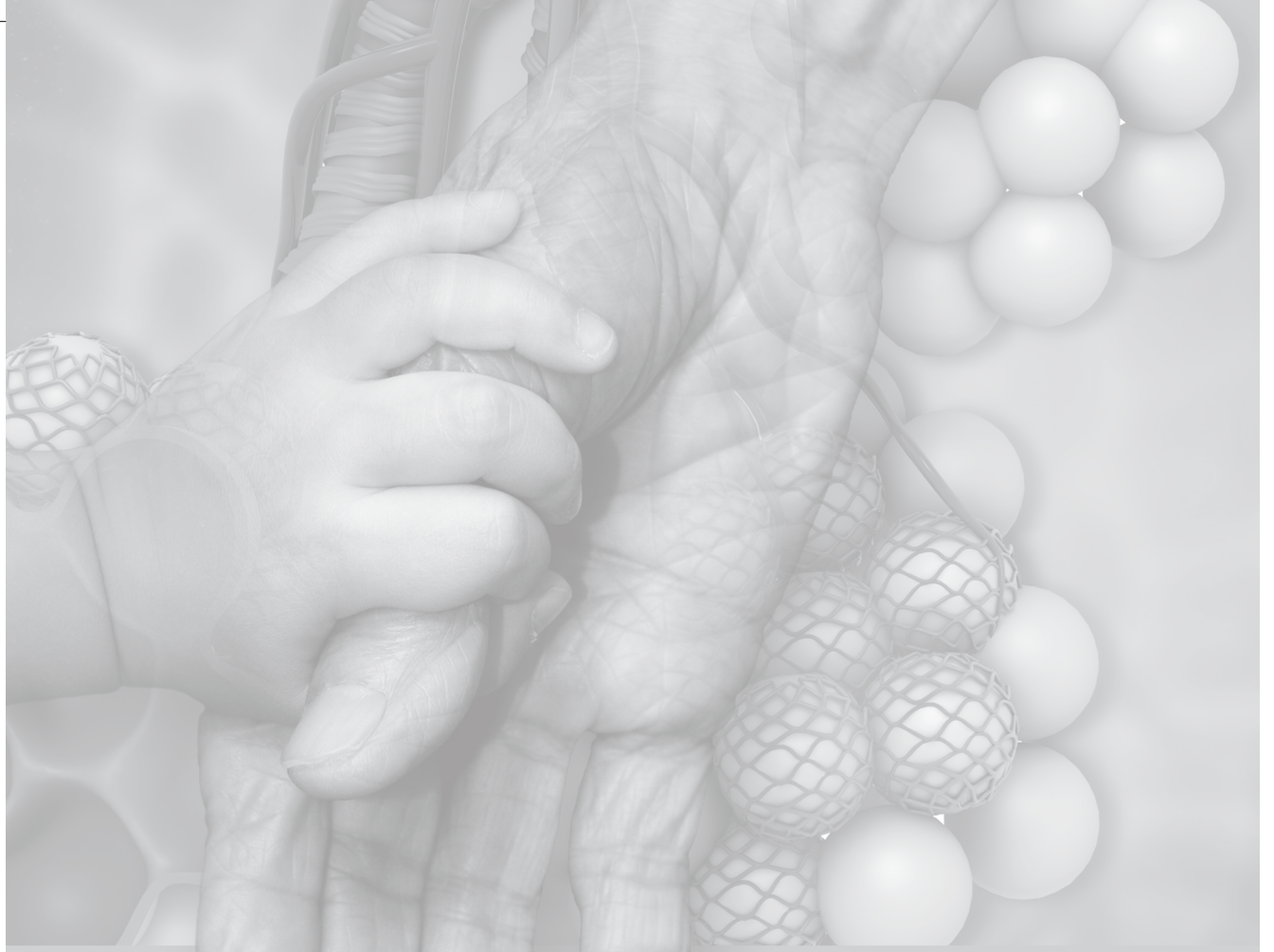
Part 2

Endothelial function in the adolescent pulmonary vasculature



Chapter 6

Sex differences in pulmonary vascular control: focus on the nitric oxide pathway

de Wijs-Meijler DPM, Danser AHJ,
Reiss IKM, Duncker DJ, Merkus D.

Physiol Rep. 2017 Jun;5(11). pii: e13200.

ABSTRACT

Although the incidence of pulmonary hypertension is higher in females, the severity and prognosis of pulmonary vascular disease in both neonates and adults have been shown to be worse in male subjects. Studies of sex differences in pulmonary hypertension have mainly focused on the role of sex hormones. However, the contribution of sex differences in terms of vascular signaling pathways regulating pulmonary vascular function remains incompletely understood. Consequently, we investigated pulmonary vascular function of male and female swine in vivo, both at rest and during exercise, and in isolated small pulmonary arteries in vitro, with a particular focus on the NO-cGMP-PDE5 pathway. Pulmonary hemodynamics at rest and during exercise were virtually identical in male and female swine. Moreover, NO synthase inhibition resulted in a similar degree of pulmonary vasoconstriction in both male and female swine. However, NO synthase inhibition blunted bradykinin-induced vasodilation in pulmonary small arteries to a greater extent in male than in female swine. PDE5 inhibition resulted in a similar degree of vasodilation in male and female swine at rest, while during exercise there was a trend toward a larger effect in male swine. In small pulmonary arteries, PDE5 inhibition failed to augment bradykinin-induced vasodilation in either sex. Finally, in the presence of NO synthase inhibition, the pulmonary vasodilator effect of PDE5 inhibition was significantly larger in female swine both in vivo and in vitro. In conclusion, the present study demonstrated significant sex differences in the regulation of pulmonary vascular tone, which may contribute to understanding sex differences in incidence, treatment response, and prognosis of pulmonary vascular disease.

INTRODUCTION

Endothelial function is a key factor in vascular development as well as in maintenance of vascular structure and function throughout life. In the pulmonary vasculature, a healthy endothelium is essential for the transition from intrauterine to extrauterine life after birth, and endothelial dysfunction is an important factor in neonatal pulmonary vascular diseases such as bronchopulmonary dysplasia and neonatal pulmonary hypertension. Also later in life, endothelial dysfunction plays a critical role in the pathogenesis of adult pulmonary vascular disease, including pulmonary hypertension (PH). The pathogenesis of PH encompasses a combination of endothelial dysfunction, vasoconstriction, inflammation, structural remodeling of the pulmonary vasculature with formation of plexiform lesion and a high incidence of in situ thrombosis.[1-4]

Although the incidence of PH is estimated to be 2-to-10-fold higher in females than in males[5,6], the severity and prognosis of pulmonary vascular disease in both neonates and adults have been shown to be worse in male as compared to female subjects.[7,8] However, the mechanisms behind these sex-differences are not completely understood. To date, research investigating sex differences in development and progression of pulmonary hypertension focused on the role of sex hormones, particularly female reproductive hormones. Although sex hormones are thought to play an important role in the pathophysiology of pulmonary hypertension, it remains unclear whether estrogens and other sex hormones have a protective or detrimental effect.[9-14] Moreover, protective effects of estrogen are unlikely to explain all the sex-differences in neonatal PH, at a time prior to full development of sex-hormonal systems.

It is well known that the nitric oxide (NO) pathway plays an important role in the pathogenesis of pulmonary hypertension. In patients with pulmonary hypertension, NO deficiency contributes to the increased pulmonary vascular tone and vascular remodeling.[2,15] Although estrogen administration enhances eNOS activity in rat pulmonary vessels[16], the contribution of intrinsic sex-related differences in the NO-pathway to regulation of pulmonary vascular function remains incompletely understood. Consequently, the aim of the present study is to determine whether sex influences pulmonary vascular function through alterations in the NO pathway even in healthy conditions. For this purpose, we investigated the pulmonary vascular function in chronically instrumented male and female swine at rest and during treadmill exercise. We first compared the pulmonary vasodilator response to exercise. Subsequently, we investigated sex differences in the response of pulmonary small arteries to different vasoactive agents, that modulate the NO pathway, in vivo and in vitro.

MATERIALS AND METHODS

In vivo animal experiments

Studies were performed in accordance with the "Guiding Principles in the Care and Use of Laboratory Animals" as approved by the Council of the American Physiological Society, and with approval of the Animal Care Committee of the Erasmus MC Rotterdam. Fifty-nine crossbred Landrace x Yorkshire swine of 2-3 months old (31 males, 28 females) entered the study. Daily adaptation of animals to laboratory conditions started 1 week before surgery and continued during the first week after surgery.

Surgical procedures

Swine were sedated with ketamine (20-30 mg kg^{-1} i.m.) and midazolam (1mg kg^{-1} i.m.), anesthetized with thiopental (10-15 mg kg^{-1} i.v.), intubated, and ventilated with a mixture of O_2 and N_2 (1:2) to which 0.2-1% (v/v) isoflurane was added. Anesthesia was maintained with midazolam (2 mg kg^{-1} + 1 mg kg^{-1} h^{-1} i.v.) and fentanyl (10 µg kg^{-1} h^{-1} i.v.) and the depth of anaesthesia was checked regularly using a pain stimulus (toe-pinch). Swine were instrumented under sterile conditions as previously described.[17] Briefly, the chest was opened via the fourth left intercostal space and fluid-filled polyvinylchloride catheters were inserted into the aortic arch, pulmonary artery and left atrium for pressure measurements. Furthermore, these catheters were used for blood sampling to determine the pO_2, pCO_2, pH, O_2 saturation (sO_2) and hemoglobin concentration (ABL 820, Radiometer) as well as for the infusion of drugs. A flow probe (14-16 mm, Skalar/Transonic) was positioned around the ascending aorta for measurement of cardiac output. Catheters and electrical wires were tunnelled subcutaneously to the back and the chest was closed in layers. Animals were allowed to recover, receiving analgesia (0.3 mg buprenorphine i.m.) for 2 days and antibiotic prophylaxis (25 mg kg^{-1} amoxicillin and 5 mg kg^{-1} gentamycin i.v.) for 5 days. The catheters were flushed daily with heparinized saline (1000-5000 IE ml^{-1}). After completing all experimental protocols, animals were killed by an intravenous overdose of pentobarbitone sodium.

Experimental protocols

Studies were performed 1-3 weeks after surgery with swine exercising on a motor-driven treadmill. Fluid-filled pressure transducers were positioned on the back of the animals and calibrated at mid-chest level. With swine (male, n=31 and female, n=28) resting on the treadmill, resting hemodynamic measurements, consisting of heart rate, cardiac output, mean aortic pressure (MAP), mean pulmonary artery pressure (PAP), and mean left atrial pressure (LAP), were obtained. Rectal temperature was measured, and arterial and mixed venous blood samples were collected. Subsequently, a five-stage treadmill exercise protocol was started (1-5 km h^{-1}); each exercise stage lasted 3 min. Hemodynamic variables were continuously recorded and blood samples were collected during the last 45 sec of each

Table 1. Schematic representation of the overlap of swine used in the different protocols

	LNNA	EMD360527	LNNA/EMD360527	Total
LNNA	**24M/19F**	5M/7F	5M/5F	
EMD360527	-	**7M/12F**	5M/4F	
LNNA/EMD360527	-	-	**5M/5F**	
Total				**31M/28F**

Bold values represent total number of animals per protocol
M: male, F: female

exercise stage, at a time when hemodynamics had reached a steady state.[17] After completing the exercise protocol, animals were allowed to rest on the treadmill. After 90 minutes of rest, three different protocols were performed on a subset of swine, on different days and in random order (see below). The number of swine in each protocol, as well as overlap between protocols, is shown in Table 1. Excellent reproducibility of consecutive exercise trials has been reported previously.[18,19]

Effects of sex on the response to NO synthase inhibition during treadmill exercise

Ninety minutes after swine had undergone a control exercise trial (as described above) the NO synthase inhibitor N^{ω}-nitro-L-Arginine (LNNA, Sigma) was administered at a dose of 20 mg kg^{-1} i.v. in 24 male and 19 female swine. Ten minutes after completion of the infusion, resting measurements were obtained and the five-stage exercise protocol was repeated.[20]

Effects of sex on the response to PDE5 inhibition during treadmill exercise

Ninety minutes after swine had undergone a control exercise trial (as described above) the phosphodiesterase-5 inhibitor EMD360527 (a gift from Merck, Darmstadt, Germany) was infused continuously in a dose of 300 µg kg^{-1} min^{-1} i.v. in 7 male and 12 female swine. Ten minutes after starting the infusion, resting measurements were obtained and the five-stage exercise protocol was repeated.[21,22]

Effects of sex on the response to PDE5 inhibition in the presence of NO synthase inhibition

Ninety minutes after swine had undergone a control exercise trial (as described above) the NO synthase inhibitor N^{ω}-nitro-L-Arginine (LNNA, Sigma) was administered at a dose of 20 mg kg^{-1} i.v. in 5 male and 5 female swine. Ten minutes after completion of the infusion, resting measurements were obtained and the five-stage exercise protocol was repeated. Ninety minutes later, animals received the phosphodiesterase-5 inhibitor EMD360527 (300 µg kg^{-1} min^{-1} i.v.). Ten minutes after starting the infusion, resting measurements were obtained and swine underwent a third exercise trial.[20-22]

Blood gas measurements

Blood samples were maintained in iced syringes until the conclusion of each exercise trial. Measurements of paO_2 (mmHg), $paCO_2$ (mmHg), and pH, sO_2 and hemoglobin (g/100mL) were then immediately performed with a blood gas analyzer (ABL 820, Radiometer), and corrected for body temperature. Blood O_2 content (μmol/mL) was computed as follows: (Hb x 0.621 x sO_2) + (0.00131 x pO_2). Body O_2 consumption (BVO_2) was calculated as the product of cardiac output and the difference in O_2 content between arterial and mixed venous blood.

Data analysis and statistical analysis

Digital recording and off-line analysis of hemodynamics have been described previously.[18,19] Pulmonary vascular conductance (PVC) was defined as cardiac output divided by mean PAP minus mean LAP. Systemic vascular conductance (SVC) was calculated as the ratio of cardiac output and MAP. To accommodate for the varying weights between animals and groups, cardiac output, PVC, SVC, and BVO_2 were indexed to body weight.[22]

Statistical analysis was performed using SPSS version 21.0 (IBM, Armonk, NY). Statistical significance was accepted at $P \leq 0.05$. Data are presented as mean ± SEM.

To test for the effects of sex and drug intervention on the relation between BVO_2 and PVC, regression analysis was performed with sex, drug treatment, and BVO_2, as well as their interactions as independent variables and animal as case label (SPSS version 21.0, IBM) Statistical analysis of the effect of drug intervention (vs. control) on PVC at rest and at maximal exercise was performed using unpaired t-test to compare data from male and female swine (SPSS version 21.0).

In vitro myograph experiments

Swine lungs (male, n=14 and female, n=13) were obtained at a local slaughterhouse. Pulmonary small arteries (diameter ≈ 300 μm) were dissected out from the lower lung lobe and stored overnight in cold, oxygenated Krebs bicarbonate solution of the following composition (in mmol/L): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 8.3; pH 7.4. The next day, pulmonary small arteries were cut into segments of ~2mm length and mounted in microvascular myographs (Danish MyoTechnology) with separated 6mL organ baths containing Krebs bicarbonate solution aerated with 95% O_2 and 5% CO_2, and maintained at 37°C. Changes in contractile force were recorded with a Harvard isometric transducer. Following a 30min stabilization period, the internal diameter was set to a tension equivalent to 0.9 times the estimated diameter at 20 mmHg effective transmural pressure. Vessels were then exposed to 30 mmol/L KCl twice. Endothelial integrity of pulmonary arteries was verified by observing dilation to 10 nmol/L substance P after precontraction with 100 nmol/L of the stable thromboxane A_2 analogue pyridoxalphophate-6-azophenyl-2'4'-disulfonic acid (U46619). Vessels were then subjected to 100 mmol/L KCl to determine the

maximal vascular contraction. Thereafter, pulmonary arteries were allowed to equilibrate in fresh Krebs solution for 30 min before initiating different experimental protocols.[22]

Experimental protocols

After 30 minutes of equilibration in fresh Krebs, pulmonary small arteries were precontracted with 100 nmol/L U46619 before starting with one of four different experimental protocols.[22] Only one protocol was executed per vessel and within one protocol, each vessel were obtained from a different animal.

Pulmonary small arteries of male and female swine were subjected to the endothelium-dependent vasodilator bradykinin (BK) in incremental dosage ranging from 10^{-10} to 10^{-7} mol/L in the absence (15 male, 16 female) or presence of NO synthase inhibition with N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME 10^{-4} mol/L, 11 male, 12 female), PDE5 inhibition with 10^{-8} mol/L sildenafil (8 male, 10 female) or combined NO synthase- and PDE5 inhibition (8 male, 10 female).

Data analysis and statistical analysis

Vascular relaxation response to BK was expressed as percentage of contraction to U46619.

Statistical analysis was performed using SPSS version 21.0 (IBM) and Prism version 5.0 (Graphpad Software, Inc., La Jolla, CA). Statistical significance was accepted at $P \leq 0.05$. Data are presented as mean ± SEM.

The maximal relaxation (E_{max}) and half maximal effective concentration (EC50) in each experiment was calculated using the GraphPad Prism version 5 for Windows (Graphpad Software, San Diego, CA). Statistical analysis of maximal relaxation and EC50 to bradykinin was performed using two-way (sex and drug intervention) analysis of variance (ANOVA) for repeated measures using SPSS version 21.0 (IBM). Statistical analysis of the effect of drug intervention (vs. control) at baseline and at bradykinin-induced maximal relaxation was performed using unpaired t-test to compare data from male and female swine (SPSS version 21.0, IBM).

Quantitative real-time PCR analysis

For detection of eNOS, soluble guanylyl cyclase (sGC) and PDE5 mRNA, pulmonary small arteries (diameter ≈ 300 µm) were isolated from the same lungs as used in the myograph experiments, and snap frozen in liquid nitrogen. Small pieces of the frozen arteries (<30 mg) were homogenized by adding RLT lysisbuffer (Qiagen) using a homogenizer. After a prot K treatment at 55°C for 10 minutes, total RNA was isolated using RNeasy Fibrous Tissue Mini Kit (Qiagen). RNA was eluted in water and stored at –80°C. The concentration was determined by using a nanodrop and RNA integrity was confirmed by Bioanalyzer. cDNA was synthesized from 100ng of total RNA with SensiFAST cDNA Synthesis Kit (Bioline). Quantitative real-time PCR (CFX-96, Bio-Rad) was performed with SensiFAST SYBR

Table 2. Primer information

	Sequence	
Genes	**Forward**	**Reverse**
GAPDH	5′-GCTCATTTCCTCGTACGACAAT-3′	5′-GAGGGCCTCTCTCCTCCTCGC-3′
Actin	5′-TCCCTGGAGAAGAGCTACGA-3′	5′-AGCACCGTGTTGGCGTAGAG-3′
Cyclophilin	5′-AGACAGCAGAAAACTTCCGTG-3′	5′-AAGATGCCAGGACCCGTATG-3′
cGC	5′-AATGGTACCCAGGAGTCACGC-3′	5′-ACGAACCAGGGAGAAGACAGA-3′
eNOS	5′- GGACACACGGCTAGAAGAGC-3′	5′-TCCGTTTGGGGCTGAAGATG-3′
PDE5A	5′-GCCACTCAATCATGGAGCATC-3′	5′-GGAGAGGCCACTGAGAATCTG-3′

& Fluorescein Kit (Bioline). Target gene mRNA levels were normalized against β-actin, glyceraldehyde-3-phosphate dehydrogenase (GADPH) and Cyclophilin using the CFX manager software (Bio-Rad). Primer sequences are shown in Table 2.

RESULTS

Effect of sex on hemodynamics during treadmill exercise.

Exercise up to 5 km h^{-1} produced a significant increase in cardiac output, while the mean aortic pressure was minimally affected (Table 3). Exercise resulted in a twofold increase in pulmonary artery pressure (Figure 1A), as a consequence of the increase in cardiac output, which caused an increase in the pressure drop across the pulmonary vasculature, in combination with the increase in left atrial pressure (Table 3). Due to the small vasodilator capacity of the lung vasculature PVC increased only slightly during exercise in both male (16 ± 7%) and female (9 ± 6%) swine (Figure 1B). The hemodynamic responses during exercise were similar in both sexes (Table 3, Figure 1).

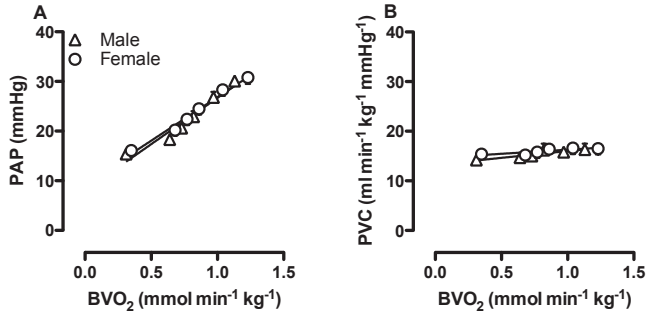


Figure 1. Changes in pulmonary hemodynamics during progressive levels of exercise in male and female swine. Relation between body oxygen consumption (BVO2) and (A) mean pulmonary arterial pressure (PAP) and (B) pulmonary vascular conductance (PVC). Values are mean ± SEM. There were no significant differences between male versus female swine.

Table 3. Hemodynamics in male and female swine

		Male		Female	
		Rest	Maximum Exercise	Rest	Maximum exercise
HR	Control	136 ± 3	249 ± 4	138 ± 5	254 ± 5
(Beats min^{-1})	Control	136 ± 4	251 ± 4	138 ± 5	252 ± 6
	LNNA	112 ± 3 *	221 ± 5 *	119 ± 3 *	223 ± 6 *
	Control	139 ± 5	262 ± 12	137 ± 8	255 ± 6
	EMD360527	147 ± 4	274 ± 12 *	164 ± 6 *‡	260 ± 6
	Control	131 ± 5	256 ± 10	135 ± 7	250 ± 10
	LNNA	108 ± 3 *	231 ± 12 *	116 ± 6 *	228 ± 9 *
	LNNA + EMD360527	115 ± 7 *	237 ± 13 *	129 ± 10 *	236 ± 8 *
MAP	Control	86 ± 2	88 ± 2	86 ± 2	90 ± 2
(mmHg)	Control	86 ± 2	89 ± 2	88 ± 2	90 ± 2
	LNNA	117 ± 2 *	116 ± 2 *	117 ± 2 *	121 ± 2 *
	Control	84 ± 3	85 ± 3	84 ± 3	92 ± 4
	EMD360527	74 ± 4 *	77 ± 3 *	78 ± 3	83 ± 4 *
	Control	76 ± 3	85 ± 2	88 ± 5	90 ± 4
	LNNA	111 ± 5 *	111 ± 3 *	112 ± 2 *	119 ± 4 *
	LNNA + EMD360527	106 ± 7 *	104 ± 3 *†	104 ± 7	107 ± 6 *†
PAP	Control	16 ± 1	30 ± 1	16 ± 1	31 ± 1
(mmHg)	Control	15 ± 1	30 ± 1	17 ± 1	31 ± 1
	LNNA	23 ± 1 *	40 ± 1 *	26 ± 2 *	44 ± 2 *
	Control	18 ± 1	31 ± 1	15 ± 1	34 ± 2
	EMD360527	14 ± 1 *	26 ± 3 *	13 ± 1 *	28 ± 2 *
	Control	26 ± 1	43 ± 2	32 ± 1 ‡	50 ± 2 ‡
	LNNA	34 ± 2 *	51 ± 3 *	41 ± 2 *‡	59 ± 3 *‡
	LNNA + EMD360527	26 ± 4	41 ± 3 †	33 ± 3 † ‡	47 ± 3 † ‡
LAP	Control	3 ± 1	10 ± 1	3 ± 1	9 ± 1
(mmHg)	Control	4 ± 1	10 ± 1	4 ± 1	11 ± 1
	LNNA	5 ± 1	10 ± 1	6 ± 1	11 ± 1
	Control	6 ± 1	12 ± 1	1 ± 1 ‡	9 ± 2
	EMD360527	5 ± 1	11 ± 2	1 ± 1	10 ± 2
	Control	6 ± 1	12 ± 1	3 ± 1	9 ± 1
	LNNA	8 ± 1	12 ± 1	4 ± 2	9 ± 1
	LNNA + EMD360527	8 ± 3	14 ± 2	3 ± 2	10 ± 1
CI	Control	0.17 ± 0.01	0.30 ± 0.01	0.19 ± 0.01	0.32 ± 0.01
(L min^{-1} kg^{-1})	Control	0.18 ± 0.01	0.32 ± 0.01	0.19 ± 0.01	0.31 ± 0.01
	LNNA	0.14 ± 0.01 *	0.27 ± 0.01 *	0.15 ± 0.01 *	0.27 ± 0.01 *
	Control	0.21 ± 0.01	0.36 ± 0.01	0.19 ± 0.01	0.33 ± 0.02
	EMD360527	0.23 ± 0.01	0.38 ± 0.01 *	0.21 ± 0.01	0.35 ± 0.02 *

Table 3. Hemodynamics in male and female swine (*continued*)

		Male		Female	
		Rest	Maximum Exercise	Rest	Maximum exercise
	Control	0.19 ± 0.01	0.34 ± 0.01	0.21 ± 0.01	0.37 ± 0.01 ‡
	LNNA	0.15 ± 0.01 *	0.28 ± 0.02 *	0.18 ± 0.01 *	0.32 ± 0.02 *
	LNNA + EMD360527	0.17 ± 0.01	0.31 ± 0.01 *†	0.21 ± 0.01 †‡	0.37 ± 0.01 †‡
PVCi	Control	14 ± 1	16 ± 1	15 ± 1	17 ± 1
(ml min^{-1} kg^{-1}	Control	15 ± 1	18 ± 1	15 ± 1	16 ± 1
mmHg^{-1})	LNNA	9 ± 1 *	10 ± 1 *	8 ± 1 *	9 ± 1 *
	Control	18 ± 1	20 ± 3	13 ± 1 ‡	15 ± 2
	EMD360527	25 ± 2 *	30 ± 4 *	19 ± 2 *	20 ± 3 *‡
	Control	15 ± 1	16 ± 2	15 ± 1	17 ± 2
	LNNA	8 ± 1 *	10 ± 2 *	9 ± 1 *	10 ± 2 *
	LNNA + EMD360527	14 ± 2 †	16 ± 2 †	22 ± 4 †	17 ± 2 †

Values are means ± SEM; n = 31 male and 28 female swine in the control group, n = 24 male and 19 female swine in the control/LNNA group, n = 7 male and 12 female swine in the control/EMD360527 group, and n = 5 male and 5 female swine in the control/LNNA/EMD360527 group. Maximum exercise is 5 km h^{-1}.

HR, heart rate; MAP, mean arterial pressure; PAP, pulmonary artery pressure; LAP, left atrium pressure; CI, cardiac index; PVCi, pulmonary vascular conductance indexed for bodyweight.

* P ≤ 0.05 versus the corresponding control; † P ≤ 0.05 LNNA + EMD360527 versus. LNNA; ‡ P ≤ 0.05 female versus male swine at corresponding treadmill speed.

Effect of sex on the response to bradykinin

Cumulative concentrations of bradykinin produced a concentration-dependent vasodilation up to 100% in precontracted isolated porcine pulmonary small arteries from either male or female sex. This vasodilator response to bradykinin was similar in pulmonary small arteries from male as compared to female swine, both in terms of E_{max} and EC50 (Figure 2).

Effect of sex on the response to NO synthase inhibition

Administration of the NO synthase inhibitor LNNA in vivo increased mean aortic pressure (Table 3) and decreased systemic vascular conductance (Figure 3) to a similar extent in male and female swine, both at rest and during incremental levels of exercise. This increase in aortic pressure was accompanied by a, probably baroreceptor reflex mediated, decrease in heart rate, and CI (Table 3). Similar to the systemic hemodynamic response, PAP markedly increased after LNNA administration. This increase in PAP was the result of extensive pulmonary vasoconstriction, as evidenced by a marked decrease in PVC. However, neither the LNNA-induced decrease in PVC at rest, nor the effect of LNNA on PVC during exercise was significantly different between male and female swine (Table 3, Figure 4A-D).

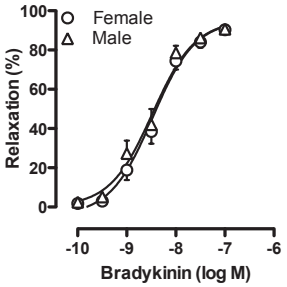


Figure 2. Concentration-response to bradykinin in pulmonary small arteries from male and female swine precontracted with U46619 (100nmol/L). Values are mean ± SEM. There were no significant differences between male versus female swine.

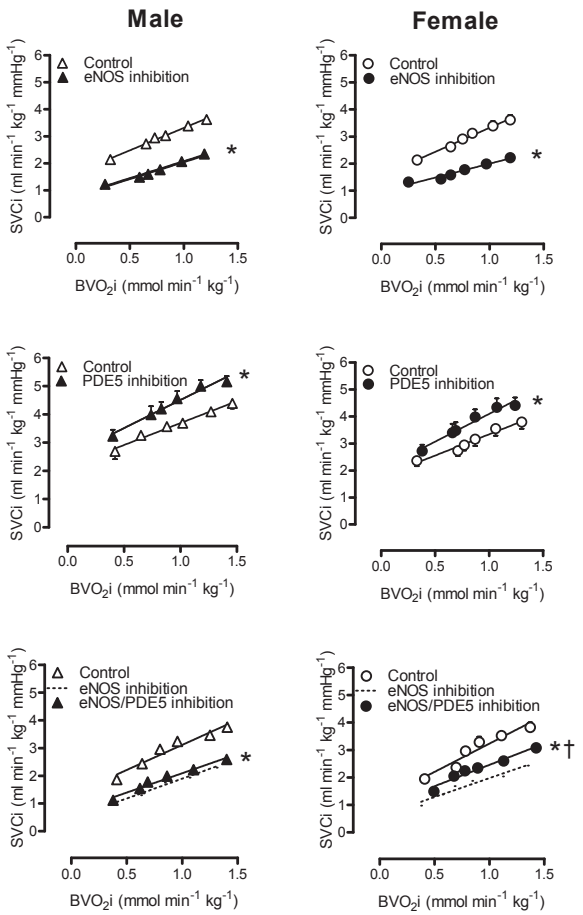


Figure 3. Effect of NO synthase and/or PDE5 on systemic vascular conductance in vivo at rest and during exercise. Values are mean ± SEM. * P ≤ 0.05 versus corresponding control; † P ≤ 0.05 female versus male swine. SVC, systemic vascular conductance; BVO_2, body oxygen consumption

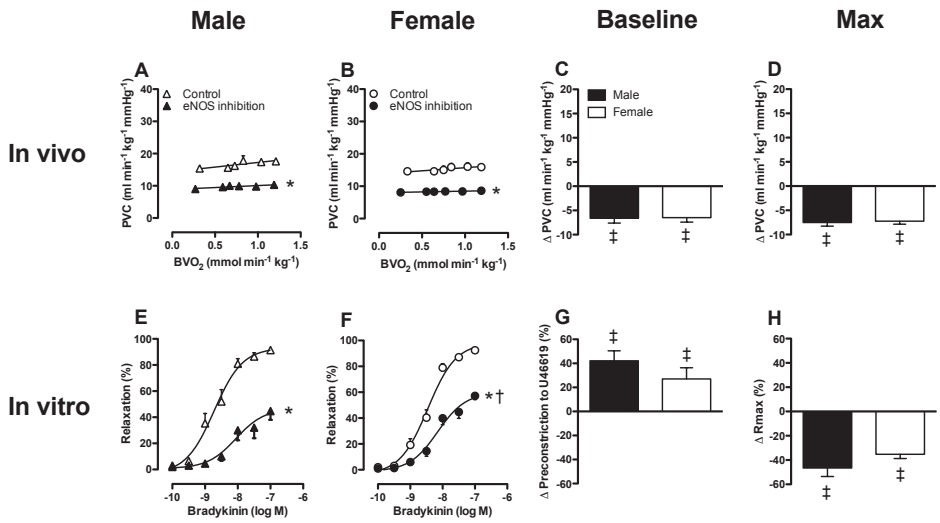


Figure 4. Effect of NO synthase on pulmonary vascular conductance in vivo at rest and during exercise (Panels A-D) and on bradykinin-induced vasodilation in vitro (Panels E-H) of male and female swine. Panels C and G show the change at baseline and panels D and H show the change at maximum compared to control. Values are mean ± SEM. * P ≤ 0.05 versus corresponding control; † P ≤ 0.05 female versus male swine; ‡ P ≤ 0.05 versus no change in pulmonary vascular conductance (eg., vs. zero). PVC, pulmonary vascular conductance; Rmax, maximum relaxation.

In vitro, addition of the NO synthase inhibitor L-NAME (10^{-4} mol/L) significantly enhanced the precontraction to U46619 (Figure 4G), and reduced the maximum bradykinin-induced relaxation (Figure 4E, F, and H). The concentration-response of pulmonary arteries from both male and female swine to bradykinin was shifted significantly to the right in the presence of L-NAME (EC50: Male 1.9 nmol/L vs. 8.8 nmol/L, P = 0.01; Female 3.5 nmol/L vs. 7.0 nmol/L, P = 0.02). In contrast to the comparable effect of NO synthase inhibition in both sexes in vivo, the BK-induced relaxartion after L-NAME administration was significantly smaller in male swine as compared to female swine (Figure 4E and F). However, the mRNA expression levels of eNOS in small pulmonary arteries (P = 0.84) were not statistically different between sexes.

Effect of sex on the response to PDE5 inhibition

PDE5 inhibition resulted in a significant increase in systemic vascular conductance at rest (Figure 3) that was accompanied by a decrease in mean aortic pressure (Table 3). The effect of PDE5 inhibition on both SVC and MAP were sustained with graded treadmill exercise (Table 3). In the pulmonary circulation, PDE5 inhibition decreased pulmonary artery pressure significantly both at rest and during exercise. This decrease in PAP was the result of pulmonary vasodilation, as PDE5 inhibition increased PVC at rest and during exercise (Table 3,

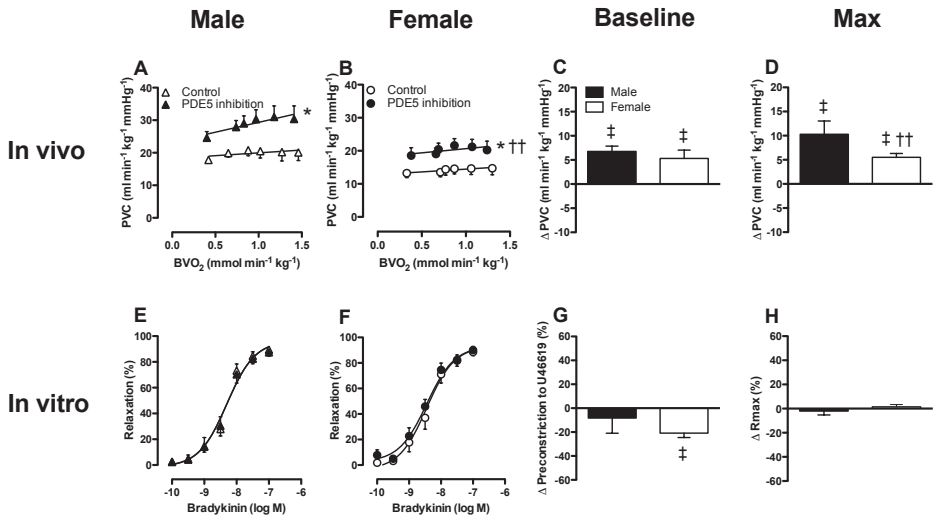


Figure 5. Effect of PDE5 on pulmonary vascular conductance in vivo at rest and during exercise (Panels A-D) and on bradykinin-induced vasodilation in vitro (Panels E-H) of male and female swine. Panels C and G show the change at baseline and panels D and H show the change at maximum compared to control. Values are mean ± SEM. * P ≤ 0.05 versus corresponding control; † P ≤ 0.05 female versus male swine; †† P ≤ 0.1 female versus male; ‡ P ≤ 0.05 versus no change in pulmonary vascular conductance (eg., vs. zero). PVC, pulmonary vascular conductance; Rmax, maximum relaxation.

Figure 5A-D), while left atrial pressure was unaffected and CO increased, particularly during exercise. However, neither the increase in PVC at rest produced by PDE5 inhibition, nor the effect of PDE5 inhibition during exercise were different in male as compared to female swine (Table 3, Figure 5A-D), although a trend toward a significantly larger effect of PDE5 inhibition during exercise in male swine was found (P=0.077 for PDE5 inhibition x sex, Figure 5A, B, and D).

Although PDE5 inhibition by sildenafil (10^{-8} mol/L) tended to attenuated the precontraction to U46619 (Figure 5G), it failed to augment the bradykinin-induced vasodilation in small pulmonary arteries isolated from either male or female swine. Hence, no sex differences in response to PDE5 inhibition were found in vitro (Figure 5E, F, and H). Similarly, no differences were found in PDE5 mRNA expression levels (P = 0.12).

Effect of sex on the response to PDE5 inhibition in the presence of NO synthase inhibition

In the systemic circulation, PDE5 inhibition following NO synthase inhibition decreased mean aortic pressure only in female swine, at rest as well as during exercise. (Table 3). This decrease in mean aortic pressure was accompanied by a significant increase in CI and SVC (Table 3, Figure 3). In male swine, the effect of PDE5 inhibtion in the systemic vasculature

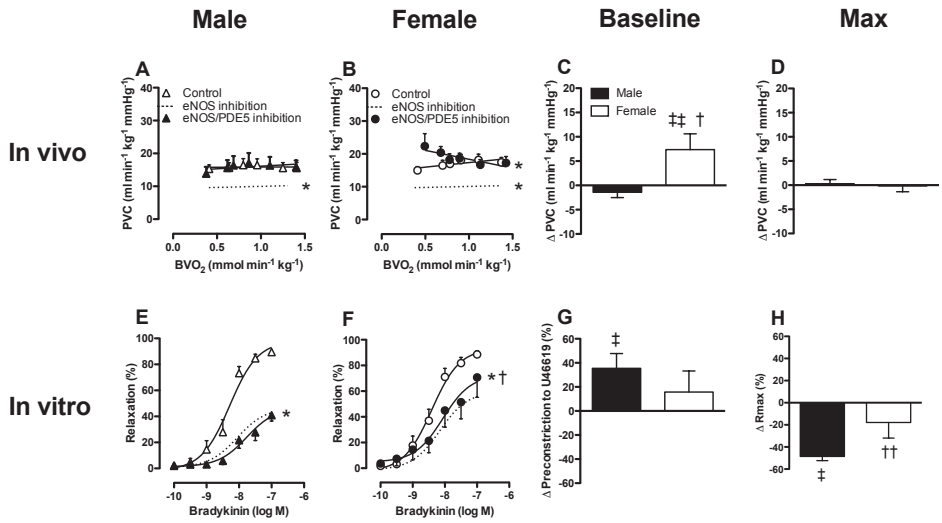


Figure 6. Effect of NO synthase and PDE5 on pulmonary vascular conductance in vivo at rest and during exercise (Panels A-D) and on bradykinin-induced vasodilation in vitro (Panels E-H) of male and female swine. Panels C and G show the change at baseline and panels D and H show the change at maximum compared to control. Values are mean ± SEM. * P ≤ 0.05 versus corresponding control; † P ≤ 0.05 female versus male swine; †† P ≤ 0.1 female versus male; ‡ P ≤ 0.05 versus no change in pulmonary vascular conductance (eg., vs. zero); ‡‡ P ≤ 0.1 versus no change in pulmonary vascular conductance (eg., vs. zero). PVC, pulmonary vascular conductance; Rmax, maximum relaxation.

following NO synthase inhibition only reached statistical significance during exercise. However, mean aortic pressure remained significantly higher as compared to control conditions in both male and female swine. In contrast, CI in the presence of combined NO synthase- and PDE5 inhibition remained significantly lower as compared to control conditions in male swine only (Table 3).

PDE5 inhibition, with EMD360527, following NO synthase inhibition, restored pulmonary artery pressure and PVC to control levels in both male and female swine (Table 3, Figure 6A-D). In female swine, PVC at rest tended to be even higher as compared to control (P = 0.08). The change in PVC in response to PDE5 -and NO synthase inhibition was significantly smaller in male as compared to female swine at rest (Figure 6C). However, no significant sex differences in the PDE5 inhibition induced increase in PVC during exercise were observed (Table 3, Figure 6A, B, and D).

BK-induced vasodilation was significantly blunted by combined NO synthase -and PDE5 inhibition in pulmonary small arteries from male swine and to a lesser extent in female pulmonary small arteries (Figure 6E, F, and H). Furthermore, there was a significant rightward shift of the concentration-response curve to bradykinin in the presence of L-NAME and sildenafil compared to control, but this shift was identical in male and female swine.

Altogether, these data suggest that there is a pathway different from the NO pathway that results in activation of sGC or pGC to produce cGMP. Furthermore, both in vivo and in vitro data indicate that in the presence of NO synthase inhibition, PDE5 inhibition has a larger pulmonary vasodilator effect in female, as compared to male swine. Since the mRNA expression levels of sGC did not differ between male and female swine (P = 0.20), this larger pulmonary vasodilator effect in female swine is unlikely to be explained by a higher presence of sGC.

DISCUSSION

This study compared the pulmonary vascular responses of healthy, young adolescent male and female swine both in vivo and in vitro to investigate whether sex differences are noted at an early age. The main findings of this study were that (1) pulmonary hemodynamics at rest and during incremental exercise did not differ between male and female swine. (2) NO synthase inhibition with LNNA resulted in pulmonary vasoconstriction to a similar extent in male and female swine at rest and during exercise. (3) NO synthase inhibition with L-NAME also reduces BK-induced vasodilation in isolated pulmonary small arteries, however this reduction was significantly larger in male than in female swine. (4) PDE5 inhibition with EMD360527 resulted in pulmonary vasodilation to a similar extent in male and female swine at rest and during exercise, but PDE5 inhibition with sildenafil failed to augment the bradykinin-induced vasodilation in small pulmonary arteries isolated from either male or female swine. (5) In the presence of NO synthase inhibition, the pulmonary vasodilator effect of PDE5 inhibition was significantly larger in female swine both in vivo (at rest) and in vitro. The implications of the present findings are discussed below.

Methodological consideration

Determination of PVC

PVC is defined as cardiac output divided by mean PAP minus mean pulmonary backpressure. Pulmonary backpressure can be measured either by pulmonary wedge pressure or by left atrial pressure.[23] In the clinical setting, pulmonary wedge pressure is routinely used to determine the pulmonary backpressure, because it can be easily obtained by the same catheter that is used to measure pulmonary artery pressure, thereby circumventing the need to insert a catheter in the left atrium. It is measured by inflating a balloon in a branch of the pulmonary artery to empty the vasculature distal to the balloon. The pressure distal to the balloon rapidly falls, and after several seconds, reaches a stable lower value that is very similar to the pulmonary backpressure. To prevent overestimation of wedge pressure, it is important to allow sufficient time for emptying the vasculature distal to the balloon.[23] Direct measurement of left atrial pressure, meanwhile, circumvents these concerns associated with

wedge pressure measurement and allows continuous assessment of PVC. Importantly, left atrial pressure measurements[24-26] correspond very well with wedge pressure measurements[27] at comparable levels of heart rate in swine at rest and during exercise, suggesting that either measurement provides a good measurement of pulmonary backpressure.

Selectivity of NO synthase inhibitors

Nitric oxide synthases (NOSs) are enzymes catalyzing the production of nitric oxide from L-arginine. Two classes of NOSs exist; the constitutive isoforms endothelial NOS (eNOS) and neuronal NOS (nNOS), and the inducible isoform (iNOS). Both LNNA and L-NAME demonstrate a 10- to 30-fold selectivity for eNOS and nNOS over iNOS.[28]

It has been previously shown that LNNA 20 mg kg^{-1} i.v. blunts the vasodilator response to ATP, but not to SNP, indicating that the effect of LNNA is specific to the endothelium-dependent vasodilation.[29] The dose we used in the present study was based on a study in swine from our laboratory that has shown that higher doses (40 mg kg^{-1} i.v.) produced similar hemodynamic responses compared to 20 mg kg^{-1}.[29] LNNA in the concentration used in this study, however, blocks all three isoforms of NOS.[28] Since iNOS blockade with aminoguanidine had no effect on basal pulmonary vascular tone at rest or during exercise in either healthy swine or in swine with myocardial infarction[30], it is unlikely that iNOS is involved in regulation of pulmonary vascular tone. However, since NO released from perivascular nerves has been shown to exert a vasodilator effect on the pulmonary vasculature[31], an effect of nNOS inhibition by LNNA on pulmonary vascular tone cannot be excluded in this study.

In our in vitro experiments, L-NAME was used in a dose of 10^{-4} mol/L. This dose of LNAME results in a complete blockade of eNOS (IC_{50} = 0.09 µmol/L) and nNOS (IC_{50} = 0.05 µmol/L).[28] Since pulmonary small arteries were dissected from the lung, thereby interrupting perivascular nerve signaling, it is unlikely that NO produced by nNOS played a role in the vasodilator response to bradykinin.

Selectivity of PDE5 inhibitors

Phosphodiesterases are enzymes responsible for the degradation of cAMP and cGMP in a wide variety of cell types. To date, at least 11 different families of PDEs have been identified, all with different kinetic properties, localization, and function. In vascular smooth muscle, PDE1 and particularly PDE5 are responsible for the degradation of the bulk of cGMP, while PDE3 is responsible for degradation of cAMP.[32] PDE5 inactivates cGMP by hydrolyzing it to 5' GMP. PDE inhibitor sildenafil demonstrates at least fivefold selectivity for PDE5 (IC_{50} = 0.0085 µmoil/L) compared with PDE6 (IC_{50} = 0.049 µmol/L), 41-fold selectivity for PDE1 (IC_{50} = 0.35 µmol/L), 376-fold selectivity for PDE4 (IC_{50} = 3.2 µmol/L), 447-fold selectivity for PDE10 (IC_{50} = 3.8 µmol/L), and >1,000-fold selectivity for PDE2, PDE3, and PDE7 (IC_{50} > 10 µmol/L).[33] PDE inhibitor EMD-360527 demonstrates at least 45-fold

selectivity for PDE5 (IC_{50} = 0.007 µmol/L) compared with PDE6 (IC_{50} = 0.32 µmol/L), 94-fold selectivity for PDE1 (IC_{50} = 0.66 µmol/L), 137-fold selectivity for PDE10 (IC_{50} = 0.96 µmol/L), and >1,400-fold selectivity for PDE2, PDE3, PDE4, and PDE7 (IC_{50} > 10 µmol/L).[21] In this study, we used EMD360527 in a dose of 300 µg kg^{-1} min^{-1} i.v., resulting in plasma drug concentration of 15 µmol/L.[34] Although inhibition of PDEs other than PDE5 may have occurred at this concentration, this is unlikely given our observation that EMD360527 has negligible effects on LV dP/d*t*max compared with the PDE3 inhibitor pimobendan that increases cAMP.[34]

Experimental animals

In male swine, the production of sex hormones starts at approximately 3 months of age and sexual maturity is attained at the age of 6-7 months. Puberty in female swine usually takes place at an age of 5-7 months.[35] Animals entering the study for the in vivo exercise experiments were juvenile swine at an age of 2-3 months old; male swine had been neutered. The use of juvenile, neutered animals eliminates long-term exposure to sex hormones, which potentially leads to lasting changes in pulmonary vascular function and structure in adult animals. Hence, the influence of sex hormones on the sex differences in pulmonary vascular control in our in vivo experiments was likely negligible. Pulmonary small arteries for the in vitro myograph experiments were isolated from lungs from 4- to 6-month old swine, obtained at a local slaughterhouse; male swine were not neutered (prohibited by Dutch law since 2015). Since the production of sex hormones has already started at this age, it cannot be excluded that sex hormone exposure, especially male swine that attained sexual maturity, influenced structure and function of the pulmonary vasculature. However, although an influence of sex hormones on pulmonary vascular structure cannot be excluded in the in vitro experiments, when isolated and mounted in Mulvany organ baths, the influence of circulating estrogens on eNOS activity in the pulmonary arteries is no longer present, and hence it is unlikely that sex hormones influence the outcome of the in vitro experiments.

Sex differences in pulmonary vascular control

Sex differences in the NO pathway have been observed in the systemic vasculature. Thus, eNOS protein expression is higher in both skeletal muscles of female swine[36], and coronary and cerebral arteries of female rats.[37,38] Consistent with the higher eNOS protein expression in females, NO urine excretion was also higher in healthy women as compared to men.[39] To the best of our knowledge, our study is the first to comprehensively investigate sex differences in the NO pathway in the healthy pulmonary vasculature.

Pulmonary hemodynamics at rest and during exercise were similar in male and female swine. The vasodilator response to bradykinin was also similar in pulmonary small arteries from male as compared to female swine, both in terms of E_{max} and EC50. In contrast to the above-mentioned studies in the systemic vascular endothelial cells, we observed no differ-

ences in eNOS mRNA expression in pulmonary small arteries from sexually mature male as compared to female swine. Although it is possible that the translation from mRNA to protein differs between male and female subjects, the unchanged mRNA expression suggests that the influence of sex on eNOS expression depends on the vascular bed studied (systemic vs. pulmonary). These findings are consistent with the comparable pulmonary vasoconstriction produced by NO synthase inhibition both at rest and during exercise in vivo between male and female swine. In vitro, NO synthase inhibition reduced BK-induced vasorelaxation in pulmonary small arteries from male swine to a greater extent as compared to female swine. Although this apparent discrepancy between in vivo and in vitro findings is not readily explained, several possible explanations could be forwarded. First, eNOS activation by circulating estrogen in the pulmonary circulation[11,14] is present in vivo but not in vitro. In the present study, the influence of estrogens is rather unlikely because the in vivo experiments were performed prior to puberty. Second, both eNOS and nNOS produce NO in the pulmonary vasculature in vivo, and thereby contribute to regulation of pulmonary vascular tone.[31] Miller et al. showed that deletion of the eNOS gene has a greater impact on the pulmonary circulation of male than female mice.[40] Together with the findings in the present study that LNNA (inhibition of both eNOS and nNOS) had a similar effect on pulmonary vascular tone in male and female swine, a higher eNOS/nNOS ratio in male as compared to female subjects is likely. This is consistent with our observation that in vitro, in the absence of a contribution of nNOS, NO synthase inhibition has a greater effect in male as compared to female pulmonary arteries. Third, receptor-mediated eNOS activation is known to act through a different signaling pathway than shear stress-mediated eNOS activation. Thus, receptor-mediated activation of eNOS occurs through a calcium calmodulin-dependent pathway[41], whereas shear stress activates eNOS through Akt-mediated phosphorylation[42], resulting in calcium-independent activation of eNOS. Several studies have shown that estrogen rapidly activates eNOS via a phosphoinositide-3 (PI-3) kinase-dependent pathway.[43-45] Hence, it is possible that sex affects calcium handling of the endothelial cells. Finally, sex differences have been found in NO signaling, which occurred solely through cGMP-PKG-PDE5 in males, whereas an unidentified alternative vasodilator pathway was present in females.[46,47] Consistent with these findings, we have previously shown that in the pulmonary vasculature, a significant part of the NO-mediated vasodilator effect resulted from inhibition of endothelin-signaling, although we did not investigate the effect of sex in that study.[48] Alternatively, endothelium-derived hyperpolarizing factor (EDHF) pathways may play a pivotal role in governing vascular tone in the pulmonary vasculature of female, but not in male subjects, like it has been shown previously in the systemic circulation.[46,49,50] Future studies are required to investigate in more detail the mechanisms underlying the different responses in vitro and in vivo, in male versus female swine.

The vasodilator effect of PDE5 inhibition on the intact pulmonary vasculature in awake resting swine, was similar in male and female swine. During exercise, a trend towards an

increased vasodilator effect of PDE5 inhibition during exercise in male as compared to female swine (P=0.077), was observed suggesting a more active endogenous PDE5 in male swine. This is consistent with recent post hoc analyses of the PHIRST and SUPER trials[51,52], showing that PDE5 inhibition lead to a greater improvement of 6 min walking distance in male as compared to female patients with PAH. Interestingly, PDE5 inhibition had no significant effect on BK-induced vasodilation of isolated pulmonary arteries from swine of either sex under baseline conditions, but blunted the LNAME-mediated inhibition of BK vasodilation in pulmonary small arteries from female but not male swine. Similarly, in the presence of NO synthase inhibition, PDE5 inhibition produced a larger vasodilator effect in the intact pulmonary vasculature of female swine as compared to male swine at rest, while this difference was abrogated during exercise. These divergent responses to PDE5 inhibition during control conditions (larger in males during exercise) and during concomitant NO synthase inhibition (larger in females at rest and during BK), in male versus female swine are not readily explained. However, we have previously shown that the sensitivity of the pulmonary vasculature to cGMP is enhanced by NO synthase inhibition.[21] This study suggests that an increased sensitivity to cGMP produced by NO synthase inhibition is particularly pronounced in female swine, which might be related to different signaling pathways of NO in female versus male swine.[46,47] The exact mechanisms underlying these divergent responses in male and female swine should be the subject of future studies.

Conclusions and clinical implications

In conclusion, the present study demonstrated significant sex differences in the regulation of pulmonary vascular tone. Thus, NO synthase inhibition reduced BK-induced vasorelaxation to a greater extent in male as compared to female pulmonary small arteries, which is consistent with observations that pulmonary vascular diseases is often more severe in men as compared to women. The increased vasodilator effect of PDE5 inhibition during exercise in male as compared to female swine reflects the sex-specific heterogeneity in treatment response. Finally, concomitant NO synthase inhibition enhanced the vasodilator responses to PDE5 inhibition at rest and during BK-induced vasodilation, but only in females, suggesting that loss of endothelial function may not interfere with (males) or even enhance (females) the pulmonary vasodilator responses to PDE5 in patients with pulmonary hypertension. Future studies are required to investigate the mechanisms underlying these sex-related differences in pulmonary vascular control mechanisms.

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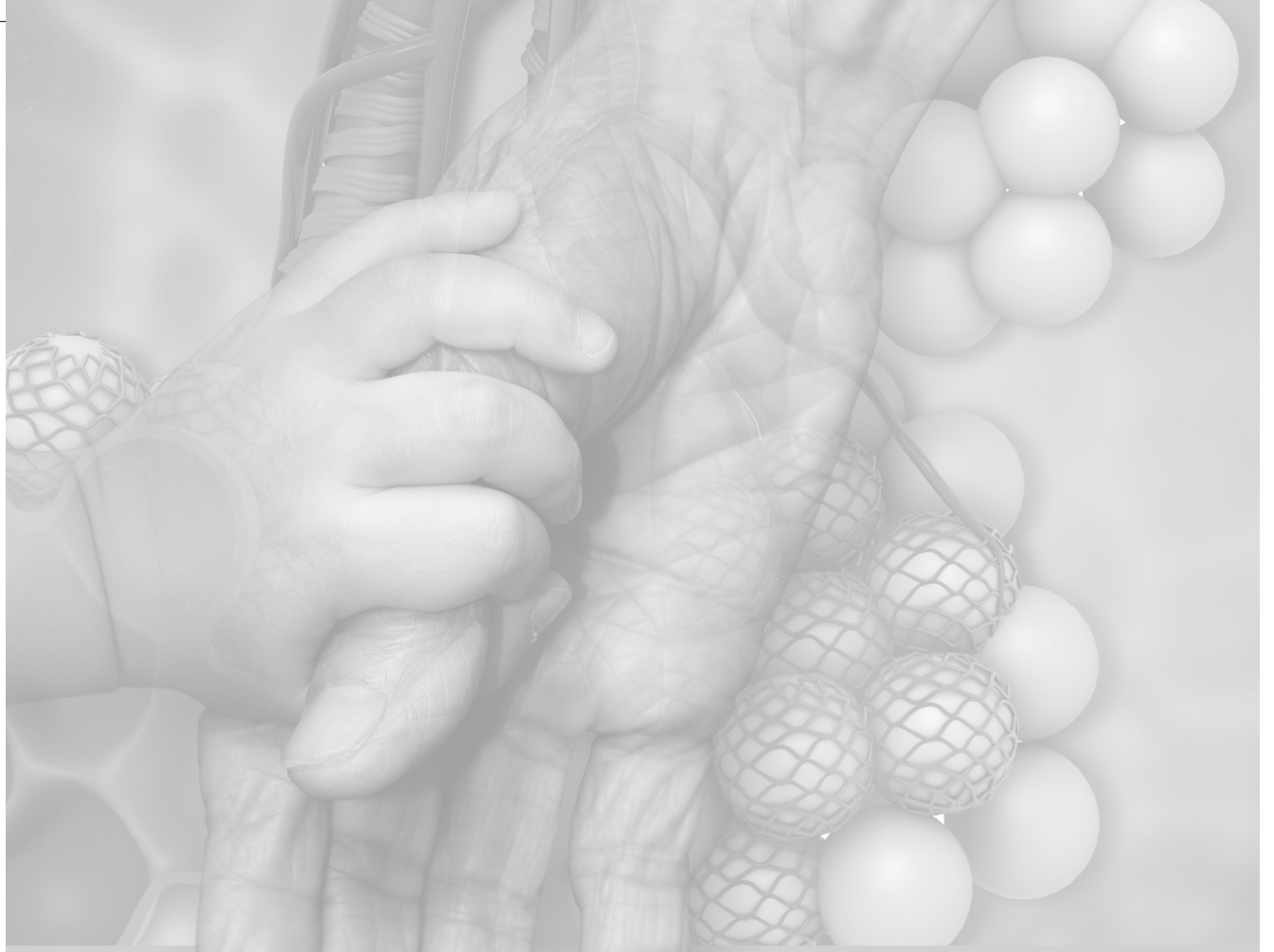

Chapter 7

Normalization of hemoglobin-based oxygen carrier-201 induced vasoconstriction: targeting nitric oxide and endothelin

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ABSTRACT

Hemoglobin-based oxygen carrier (HBOC)-201 is a cell-free modified hemoglobin solution potentially facilitating oxygen uptake and delivery in cardiovascular disorders and hemorrhagic shock. Clinical use has been hampered by vasoconstriction in the systemic and pulmonary beds. Therefore, we aimed to 1) determine the possibility of counteracting HBOC-201-induced pressor effects with either adenosine (ADO) or nitroglycerin (NTG); 2) assess the potential roles of nitric oxide (NO) scavenging, reactive oxygen species (ROS), and endothelin (ET) in mediating the observed vasoconstriction, and 3) compare these effects in resting and exercising swine. Chronically instrumented swine were studied during rest and during exercise after administration of HBOC-201 alone or in combination with ADO. The role of NO was assessed by supplementation with NTG or administration of the eNOS inhibitor N^{ω}-nitro-L-arginine. Alternative vasoactive pathways were determined via intravenous administration of the ET_A/ET_B receptor blocker tezosentan or a mixture of ROS scavengers. The systemic and to a lesser extent the pulmonary pressor effects of HBOC-201 could be counteracted by ADO; however, dosage titration was very important to avoid systemic hypotension. Similarly, supplementation of NO with NTG negated the pressor effects but also required titration of the dose. The pressor response to HBOC-201 was reduced after eNOS inhibition and abolished by simultaneous ET_A/ET_B receptor blockade, while ROS scavenging had no effect. In conclusion, the pressor response to HBOC-201 is mediated by vasoconstriction due to NO scavenging and production of ET. Further research should explore the effect of longer-acting ET receptor blockers to counteract the side effect of hemoglobin-based oxygen carriers.

New & noteworthy

Hemoglobin-based oxygen carrier (HBOC)-201 can disrupt hemodynamic homeostasis, mimicking some aspects of endothelial dysfunction, resulting in elevated systemic and pulmonary blood pressures. HBOC-201-induced vasoconstriction is mediated by scavenging nitric oxide (NO) and by upregulating endothelin (ET) production. Pressor effects can be prevented by adjuvant treatment with NO donors or direct vasodilators, such as nitroglycerin or adenosine, but dosages must be carefully monitored to avoid hypotension. However, hemodynamic normalization is more easily achieved via administration of an ET receptor blocker.

INTRODUCTION

Hemoglobin-based oxygen carrier (HBOC)-201 is a cell- and endotoxin-free, glutaraldehyde-polymerized hemoglobin solution produced by chemical modification of hemoglobin extracted from isolated bovine red blood cells.[1] HBOCs may be used in the treatment of cardiovascular disorders, and hemorrhagic shock, in particular; however, side effects include systemic and pulmonary blood pressure elevations, plasma volume expansion, lower cardiac output and reduction in heart rate.[2,3] Despite these potentially unfavorable effects, studies in human subjects with documented coronary disease showed that HBOC-201 had no effect on left ventricular (LV) stroke work index or any of the measured coronary function parameters.[2]

The most important HBOC side-effect is systemic and pulmonary vasoconstriction. Consequently, this study first aimed to determine the possibility of reversing HBOC-201 pressor effects via simultaneous administration of adenosine (ADO), a nitric oxide (NO)-independent vasodilator, or the NO-donor nitroglycerin (NTG). The pressor effect of HBOC has been ascribed to scavenging of NO, an important endogenous vascular relaxing factor.[4-9] Free hemoglobin (Hb) undergoes rapid ($\sim 10^7$ $M^{-1}s^{-1}$)[10,11] and irreversible reaction with NO to form metHb, where Hb kinetically behaves as a dioxygenase enzyme.[12,13] In the following slower processes, iron-NO complexes are formed that may further deplete NO concentrations.[4] However, although disruption of the NO-mediated cascade may be an important contributor to transient systemic and pulmonary hypertension, it is not the only possible pathway.

Oversupplying oxygen (O_2) can stimulate vasoconstriction, to protect against the oxygen burst, but can also stimulate reactive oxygen species (ROS) formation that may result in further scavenging of NO.[14,15] By scavenging NO, conversion from pro-endothelin to endothelin (ET) is no longer inhibited, thereby increasing the release of this vasoconstrictor.[2,16,17] Also, free radicals generated by the auto-oxidation of hemoglobin may contribute to the enhanced release of ET.[18] Therefore, the second aim of this study was to address the potential roles of nitric oxide scavenging, ROS, and/or endothelin in the HBOC-201 systemic and pulmonary pressor effects.

NO has been shown to contribute to exercise-induced vasodilation in skeletal muscle, the heart, as well as the pulmonary vasculature in many[19-21], but not all studies.[22] A state of decreased NO and increased ROS and ET production resembles some aspects of endothelial dysfunction, a phenomenon that may have exaggerated effects during exercise. Hence, the third aim of this study compared the effects of HBOC-201 in resting and exercising swine.

METHODS

Animals

Studies were performed in accordance with the Council of Europe Convention (ETS123)/ Directive (86/609/EEC) for the protection of vertebrate animals used for experimental and other scientific purposes, and with approval of the Animal Care Committee at Erasmus MC, University Medical Centre Rotterdam. A total of 16 Yorkshire × Landrace swine (2–3 months old, 22 ± 1 kg at the time of surgery) of either sex (11 female and 5 male) entered the study. After completing all experimental protocols, animals were euthanized by an intravenous overdose of pentobarbitone sodium.

Surgical procedures and experimental protocol

Detailed surgical procedures are previously described.[23,24] In brief, under deep anesthesia, a thoracotomy was performed in the fourth left intercostal space. Fluid-filled catheters were placed in the aorta, pulmonary artery, left atrium and LV for measurement of pressure, infusion of drugs and blood sampling. In addition, a flow probe (Transonic Systems) was placed around the ascending aorta for measurement of cardiac output. All catheters were exteriorized at the back of the animal and filled with heparinized saline. The thorax was closed in layers, and the animal was allowed to recover for at least one week. Antibiotic prophylaxis (amoxicillin 25 mg/kg iv) was provided for 5-7 days starting immediately before surgery. Immediate postoperative analgesia was provided by buprenorphine (0.015mg/kg im), while a slow-release fentanyl patch (12 µg/h) maintained postoperative analgesia for 72 h. Studies were performed 1–3 wk after surgery, with animals resting and exercising on a motor-driven treadmill up to 85–90% of maximal heart rate. Three main protocols (as described below) were performed on different days and in random order. All chemicals were obtained from Sigma, and HBOC-201 (13 g/dl) was obtained from OPK Biotech.

Hemodynamic effects and reproducibility of HBOC-201 infusion

With swine lying on the treadmill, resting hemodynamic measurements consisting of heart rate (HR), LV pressure, first derivative of LV pressure (dP/dt), mean aortic pressure (MAP), pulmonary artery pressure (PAP), left atrial pressure, and cardiac output were obtained. Subsequently, swine were subjected to a five-stage exercise protocol (1–5 km/h) while hemodynamic variables were continuously recorded, and blood samples collected during the last 60 s of each 3-min exercise stage at a time when hemodynamics had reached a steady state. Blood samples were used for determination of Hb, oxygen content, and lactate using an automated blood gas analyzer (ABL210, Radiometer). After the exercise protocol was completed, animals were allowed to rest on the treadmill for 90 min, after which HBOC-201 (10 ml/kg iv) was infused over a period of 30 min. At the end of infusion, the exercise protocol was repeated. We have previously

shown excellent reproducibility of the hemodynamic response in consecutive bouts of exercise.[19,25]

Also, in three pigs, we assessed the reproducibility of the hemodynamic responses to HBOC-201 infusion by administration of three separate doses of HBOC-201 (10 ml/kg iv), separated by 5 ±1 days.

Reversal of pressor effect of HBOC-201 by nitroglycerin and adenosine

After performing a control run, six animals received HBOC-201 combined with the vasodilator ADO. Administration of ADO was started 10 min after the start of HBOC-201 infusion and continued till the end of the second run. The infusion rate of ADO (25 mg/ml) was titrated to obtain a stable MAP similar to that before HBOC administration.

Role of NO

To determine the involvement of NO in HBOC-201-induced hypertension, in six swine, the NO donor nitroglycerin (NTG) was infused starting 10 min after the start of HBOC-201 infusion. To prevent a direct interaction between the NO donor and HBOC-201, HBOC-201 and NTG were infused through separate catheters. The infusion rate of NTG (1 mg/ml) was titrated to obtain a stable MAP similar to that before HBOC administration.

To further investigate the role of endogenous NO, NO production was inhibited using the NO synthase inhibitor N^{ω}-nitro-L-arginine (L-NNA, 20 mg/kg iv) in five swine.[19] After administration of L-NNA, swine underwent an L-NNA exercise trial. Ninety minutes later, HBOC-201 (10 ml/kg iv) was given to the animals, and they underwent a second exercise trial. As previously shown[26], L-NNA has a long-lasting effect so no additional L-NNA was administered before the second exercise protocol.

Other vasoactive pathways

Loss of NO reduces ROS scavenging and may increase the production of the potent vasoconstrictor ET. To determine the involvement of ET and ROS in HBOC-201-induced hypertension, HBOC-201 was infused after prior administration of an ET-receptor antagonist or a cocktail of ROS scavengers.

ET receptor blockade

After completing a control exercise protocol, animals were allowed to rest on the treadmill for 90 min. Then, the mixed ET_A and ET_B receptor (ET_A/ET_B) antagonist tezosentan was intravenously administered over 10 min in a dose of 3 mg/kg iv (slow bolus), followed by a continuous infusion of 6 $mg \cdot kg^{-1} \cdot h^{-1}$ iv in four swine.[24] HBOC-201 (10 ml/kg iv) was started upon completion of the tezosentan slow bolus. When HBOC-201 infusion was completed the exercise protocol was repeated.

ROS scavengers

We used a mixture of different substances to scavenge all ROS during HBOC administration. The mixture consisted of *N*-acetylcysteine (NAC, 150mg/kg iv), 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (Tempol, 30 mg/kg iv), and mercaptopropionyl glycine (MPG, 1mg/kg iv).[15]

NAC is an aminothiol and synthetic precursor of intracellular cysteine and glutathione and is thus considered an important antioxidant.[27] It is generally assumed that the antioxidant and free radical scavenging activities of NAC are attributable to increasing intracellular glutathione levels; however, NAC also possesses a reducing property through its thiol-disulfide exchange activity.[27,28] Tempol is a stable piperidine nitroxide and scavenges superoxide anions in vitro and may act as a SOD mimetic.[29,30] Tempol also reduces the formation of hydroxyl radicals either by scavenging superoxide anions or hydroxyl radicals (via the Fenton or Haber-Weiss reactions).[3,29] N-2-mercaptopropionylglycine (MPG) is a synthetic thiol compound which is not highly radical specific and scavenges different types of ROS, including $O_2^{\cdot-}$, $ONOO^-$ and OH.[31,32]

After completing a control exercise protocol, the animals were allowed to rest on the treadmill for 90 min. Then, the scavenger mixture was administered in five swine, starting 10 minutes before the HBOC-201 infusion. The administration of NAC and Tempol was completed before administration of HBOC-201, while MPG-infusion continued throughout HBOC-201 administration and the subsequent exercise protocol.

Data analysis and statistical analysis

Digital recording and offline analysis of hemodynamic variables have been described in detail elsewhere.[33-35] Systemic vascular resistance (SVR) was computed as mean aortic blood pressure divided by cardiac output. Pulmonary vascular resistance (PVR) was computed as mean pulmonary arterial pressure minus mean left atrial pressure divided by cardiac output. Body lactate production/consumption was calculated as the product of cardiac output and arterio-mixed venous lactate difference.

Statistical analysis of hemodynamic data was performed with SPSS 22 (IBM, Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY). Since no differences between male and female swine were found in the response to HBOC-201 administration alone, data from both sexes were pooled. The effects of drug treatment and exercise were compared using a two-way (ANOVA for repeated measures. When significant effects were detected, post hoc testing was performed using paired or unpaired t-test, with Bonferroni correction. Statistical significance was accepted when $P \leq 0.05$. Data are presented as mean ± SE.

RESULTS

Hemodynamic Effects and Reproducibility of HBOC-201 administration.

Administration of HBOC resulted in significant pressor effects in the systemic and pulmonary circulations with an increase in MAP (27±3 mmHg) and PAP (14±1 mmHg). These pressor responses were accompanied by a probably baroreflex-mediated decrease in HR, which together with a slight decrease in stroke volume, resulted in a decrease in cardiac output (Table 1). These pressor effects were the result of significant systemic and pulmonary vasoconstriction, as evidenced by significant increases in SVR and PVR. There was no sign of anaerobic metabolism, as arterial and mixed venous lactate levels and body lactate consumption (not shown) were maintained. The hemodynamic responses to HBOC-201 administration occurred during the first 10 min of HBOC-201 administration after which they stabilized. Moreover, a second and third HBOC administration with 5-7 days washout in between yielded hemodynamic responses similar to the first administration (Figure 1).

Table 1. Hemodynamics effecfs of HBOC-201 at rest and during exercise.

		Rest	Exercise level (km/h)				
	Treatment	Lying	1	2	3	4	5
Systemic hemodynamics							
HR (bpm)	Control	124 ± 5	170 ± 9*	177 ± 9*	188 ± 8*	218 ± 10*	244 ± 10*
	HBOC-201	100 ± 3†	138 ± 5†*	148 ± 6†*	161 ± 5†*	192 ± 7*	221 ± 8*
MAP (mmHg)	Control	89 ± 3	83 ± 3	83 ± 3	83 ± 2	84 ± 2	87 ± 3
	HBOC-201	113 ± 3†	105 ± 3†	105 ± 2†*	103 ± 2†*	104 ± 2†*	104 ± 2†*
SV (ml/beat)	Control	38 ± 2	43 ± 2*	43 ± 2	43 ± 2	40 ± 2	39 ± 2
	HBOC-201	42 ± 2	46 ± 2	44 ± 2	44 ± 2	40 ± 2	40 ± 2
CO (l/min)	Control	4.7 ± 0.2	7.4 ± 0.3*	7.6 ± 0.3*	8.1 ± 0.2*	8.8 ± 0.3*	9.8 ± 0.3*
	HBOC-201	4.3 ± 0.2	6.4 ± 0.2†*	6.7 ± 0.2†*	7.2 ± 0.2†*	8.2 ± 0.2*	9.0 ± 0.3*
SVR (l/min.mmHg^{-1})	Control	19 ± 1	11 ± 0*	11 ± 0*	10 ± 0*	10 ± 0*	9.0 ± 0*
	HBOC-201	27 ± 1†	17 ± 1†*	16 ± 1†*	15 ± 1†*	13 ± 1†*	12 ± 0†*
Pulmonary hemodynamics							
MPAP (mmHg)	Control	14 ± 1	21 ± 2*	21 ± 1*	23 ± 1*	28 ± 1*	31 ± 1*
	HBOC-201	26 ± 2†	32 ± 2†	34 ± 3†*	35 ± 2†*	39 ± 2†*	42 ± 2†*
LAP (mmHg)	Control	1.5 ± 1	2.4 ± 1	3.3 ± 0.6	4.9 ± 0.6*	7.7 ± 1*	8.3 ± 1*
	HBOC-201	9.8 ± 1.7†	7.7 ± 1.1†	7.0 ± 1†	8.1 ± 1.1†	9.0 ± 0.7	9.6 ± 1
PVR (l/min.mmHg^{-1})	Control	2.7 ± 0.2	2.5 ± 0.2	2.4 ± 0.2	2.3 ± 0.2	2.3 ± 0.2	2.4 ± 0.2
	HBOC-201	3.8 ± 0.4†	4.1 ± 0.4†	4.3 ± 0.5†	4.1 ± 0.4†	4.0 ± 0.4†	3.8 ± 0.4†

Data are means ± SE. HR, Heart rate; MAP, mean arterial pressure; SV, stroke volume; CO, cardiac output; SVR, systemic vascular resistance; MPAP, mean pulmonary artery pressure; LAP, left atrial pressure; PVR, pulmonary vascular resistance; HBOC-201, hemoglobin-based oxygen carrier 201. *P ≤ 0.05 vs. $Rest_{Lying}$, † P ≤ 0.05 vs. Control.

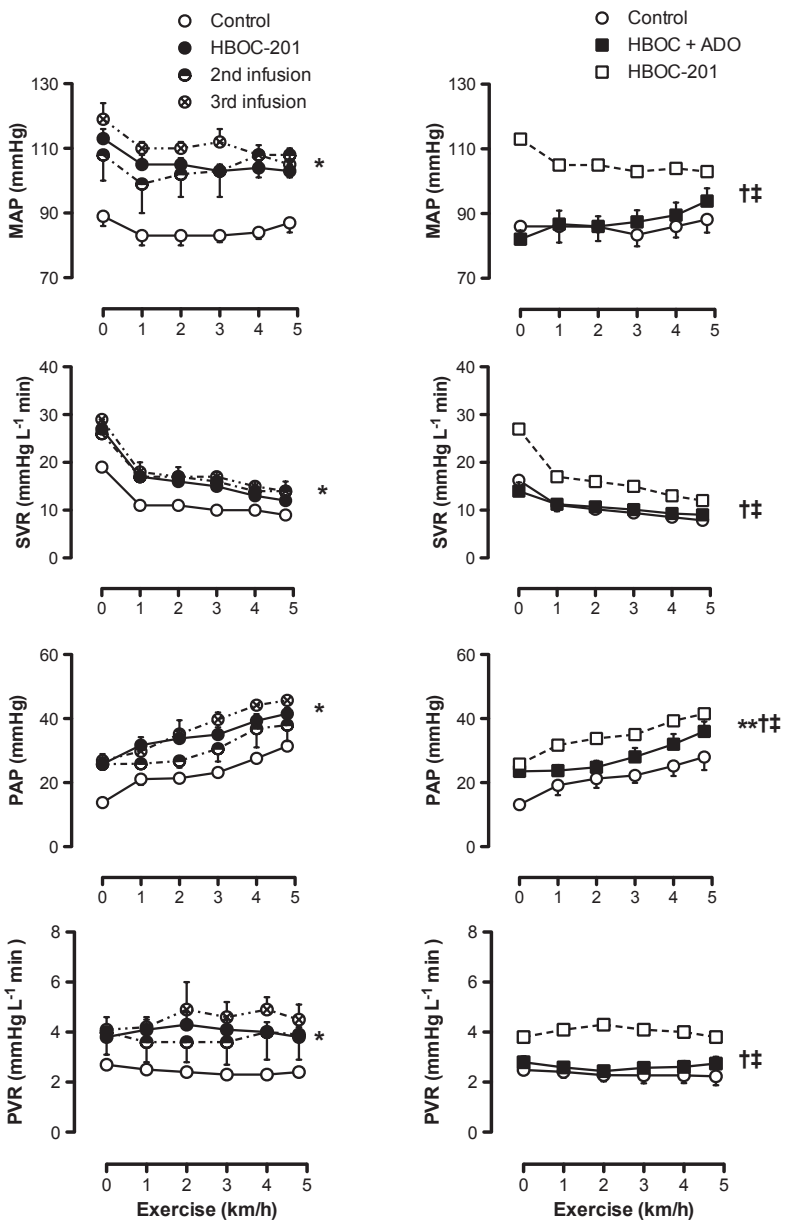

Figure 1. Systemic and pulmonary hemodynamics at rest and during exercise following administration of hemoglobin-based oxygen carrier (HBOC)-201 alone (n = 3 pigs, left), demonstrating reproducibility of 3 separate administrations of HBOC-201, and in combination with infusion of adenosine (ADO) (n = 6 pigs in full crossover study design, right). * $P \leq 0.05$, ** $P \leq 0.10$, compared with control; † $P \leq 0.05$, compared with HBOC-201 alone; ‡ $P \leq 0.05$, effect of HBOC+ Ado different from HBOC-201 alone.

The pressor response to HBOC-201 was maintained during exercise (Figure 1). In the systemic circulation, both MAP and SVR remained elevated throughout the exercise protocol as compared to control, although the elevation of SVR tended to wane with incremental levels of exercise. Similarly, in the pulmonary circulation both PAP and PVR remained elevated at all exercise intensities.

Reversal of pressor effect of HBOC-201 by adenosine in the systemic and pulmonary vasculature.

Coinfusion of ADO was carefully titrated to maintain MAP at a level similar to MAP before HBOC-201 infusion (Figure 1). Dosages required to stabilize MAP fluctuated throughout the experiment, but they were on average 0.17±0.01 mg/kg/min (range between 0.08 and 0.38 mg/kg/min). Although the HBOC-201-induced changes in SVR and PVR were abolished by ADO (Figure 1), PAP tended to remain slightly higher (P=0.08) due to a slight increase in left atrial pressure (not shown). MAP increased by ~15 mmHg upon cessation of the ADO (not shown).

Role of NO

The exogenous administration of NO, by coinfusion of the NO-donor NTG, was also titrated to counteract systemic pressor responses to HBOC-201. The dose of NTG required to stabilize MAP increased from 0.11±0.01 mg/kg/min at 20 min of HBOC-201 infusion to 0.22±0.06 mg/kg/min upon completion of HBOC-201 infusion (P=0.05) and remained essentially unchanged during the exercise protocol, being 0.16 mg/kg/min at maximal exercise (range from 0.06 to 0.49 mg/kg/min). This dose of NTG negated the HBOC-201-induced increase in SVR as well as PVR and, thereby, the elevated pressures in these vascular beds (Figure 2). Similar to ADO, MAP increased upon cessation of the NTG-infusion (not shown).

Endothelial NOS (eNOS) blockade with L-NNA resulted in peripheral vasoconstriction, as evidenced by a significant increase in SVR and an increase in MAP. The increase in MAP was accompanied by increases in LV systolic pressure, as well as left atrial pressure, and probably, by a baroreflex-mediated decrease in HR and CO, as stroke volume was not altered (Figure 2). However, subsequent infusion of HBOC-201 did not result in a further increase in MAP or SVR. In contrast to the findings in the systemic vasculature, HBOC-201 induced an increase in mean PAP and PVR even in the presence of L-NNA (Figure 2). Thus, HBOC-201 induced further pulmonary vasoconstriction following the vasoconstriction induced by L-NNA, both at rest and during exercise, suggesting that, in addition to scavenging of NO, HBCO-201 exerts its vasoconstrictor effect through another pathway in pulmonary vasculature. Of note, when HBOC-201 and L-NNA were coinfused, systemic pressor responses appeared to be increased as compared to the effect of HBOC-201 alone (Figure 2), indicating that not all NO is scavenged by HBOC-201.

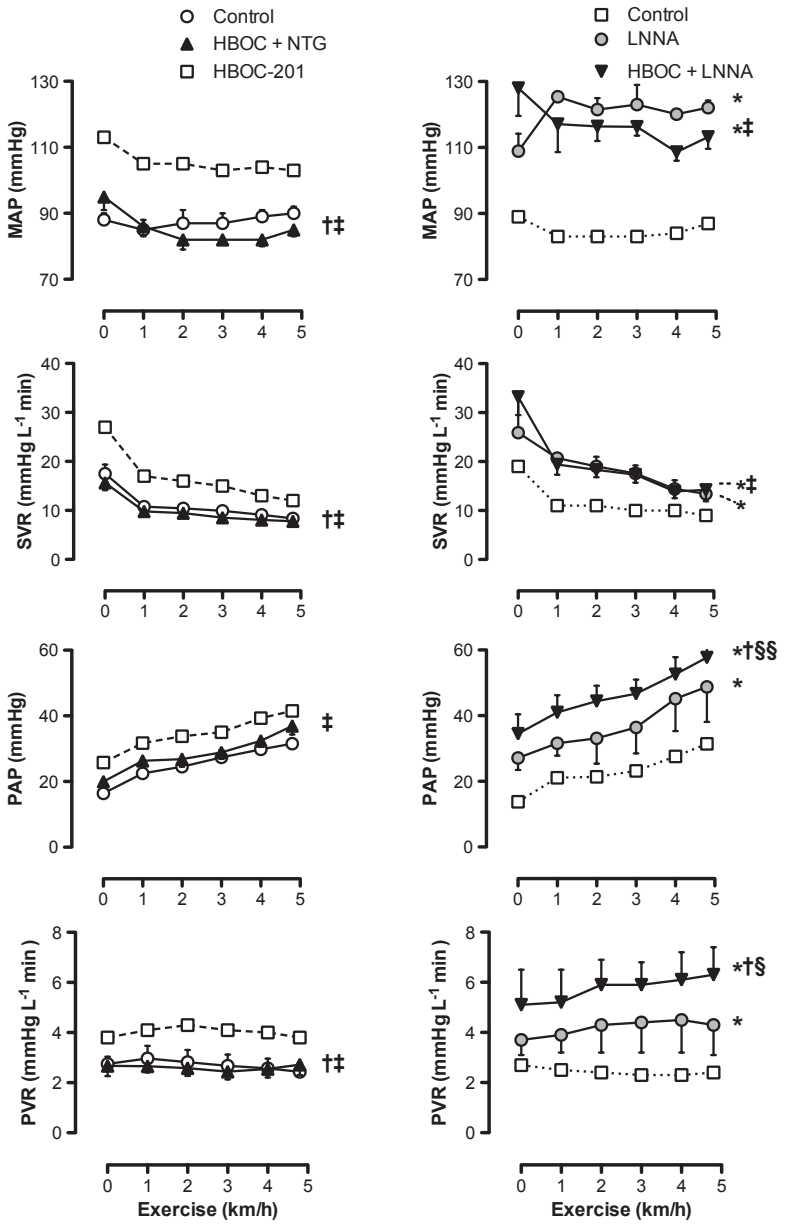

Figure 2. Systemic and pulmonary hemodynamics at rest and during exercise following administration of the nitric oxide (NO)-donor nitroglycerin (NTG; left) or the eNOS inhibitor N^{ω}-nitro-L-arginine (L-NNA; right) in combination with HBOC-201. * P ≤ 0.05, compared with control; † P ≤ 0.05, compared with HBOC-201 alone; ‡ P ≤ 0.05, effect of HBOC+ NTG different from HBOC-201 alone; § P ≤ 0.05, §§ P ≤ 0.1 compared with L-NNA alone; n = 6 pigs (NTG) or 5 pigs (L-NNA) in a full cross-over study design. For the sake of clarity, statistics comparing HBOC-201 with control are not shown, but they are identical to Figure 1.

Other vasoactive pathways

Administration of the mixed ET_A and ET_B receptor antagonist tezosentan reduced MAP by 10±4 mmHg ($P<0.05$), and negated the systemic hypertension caused by subsequent HBOC-201 by preventing the increase in SVR (Figure 3). Also, in the pulmonary vasculature, HBOC-201 had no effect on either pulmonary pressure or PVR, in the presence of tezosentan (Figure 3). These data suggest that activation of the endothelin system is an important contributor in the vasoconstrictor response to HBOC-201.

ROS scavenging in itself had no significant effect on MAP (ΔMAP 12±8 mmHg, $P=0.21$). Coinfusion of ROS scavengers with HBOC-201 slightly reduced the effect of HBOC-201 on mean arterial pressure but did not significantly affect SVR (Figure 3). Similarly, in the pulmonary vascular bed, no reduction of pressor effects could be detected at rest, and the effects of HBOC-201 tended to be exacerbated during exercise following administration of the ROS scavenger cocktail (Figure 3).

DISCUSSION

In the present study we report, in accordance with previous publications[9,36-40], that intravenous administration of HBOC-201 resulted in systemic and pulmonary hypertension as a result of vasoconstriction, which was maintained during exercise. Pressor responses could be prevented by coinfusion of NTG or ADO both at rest and during exercise; however, this required careful titration of the dosage of these vasodilators. eNOS inhibition prevented HBOC-201-induced increase in systemic vasoconstriction, and it reduced but did not abolish HBOC-201-induced pulmonary vasoconstriction. ET_A/ET_B blockade with tezosentan prevented the HBOC-201–induced pressor responses in the systemic and pulmonary vasculature, while ROS-scavenging tended to blunt the pressor response in the systemic but not pulmonary vasculature.

Abolishing pressor effects

The main hemodynamic effects of HBOC-201 occurred during the first 10 min of its administration and were maintained throughout the entire infusion and after infusion. Repeated administration of HBOC-201, following complete washout, induced virtually identical effects, corroborating results from ECMO priming with HBOC-201 in piglets[41,42] and indicating that no immune reaction occurred in response to the protein and that repeated administration is safe. Also, plasma clearance of HBOC-201, which has been shown to follow first-order pharmacokinetics with an elimination half time of 20 h, for either single or multiple dosage regimens[1], was comparable with previous studies.[1,43] As anticipated, HBOC-201 produced an increase in arterial Hb, metHb and plasma metHb (Table 2); however, the level of metHb in this study was well below toxic levels[44], and coinfusion of NTG with HBOC did not elevate metHb levels further (not shown).

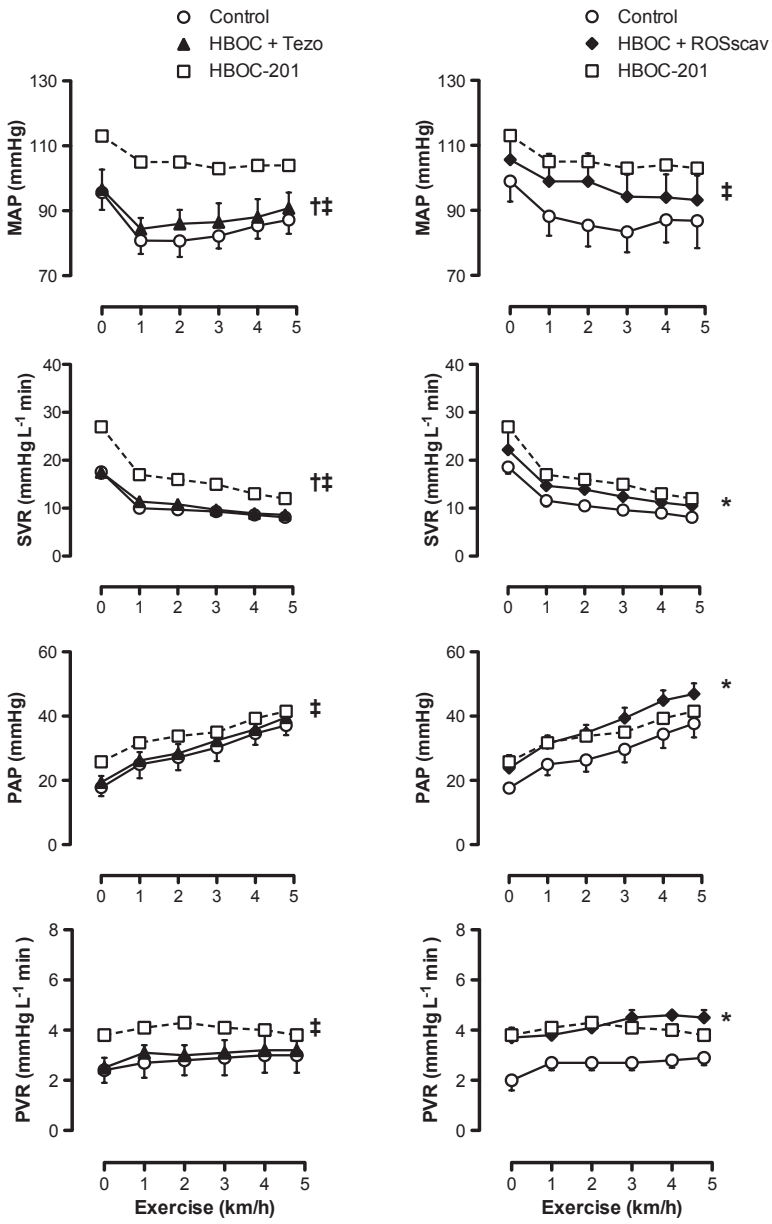

Figure 3. Systemic and pulmonary hemodynamics at rest and during exercise following administration of the endothelin ET_A/ET_B- receptor blocker Tezosentan (n = 4 pigs in full cross-over study design; left) or a reactive oxygen species (ROS) scavenger cocktail comprised of N-acetylcysteine (NAC), Tempol, and mercaptopropionyl glycine (MPG; n = 5 pigs in full cross-over study design; right) in combination with HBOC-201. * $P \leq 0.05$, compared with control; † $P \leq 0.05$, compared with HBOC-201 alone; ‡ $P \leq 0.05$, effect of HBOC+ Tezo or ROS different from HBOC-201 alone. For the sake of clarity, statistics comparing HBOC-201 with control are not shown, but they are identical to Figure 1.

Table 2. Effects of HBOC-201 on blood gas values at rest and during exercise

		Rest	Exercise level (km/h)				
	Treatment	Lying	1	2	3	4	5
Arterial							
Hemoglobin (g%)	Control	8.4 ± 0.2	8.7 ± 0.2	8.8 ± 0.2	8.9 ± 0.2	9.0 ± 0.2	9.3 ± 0.2*
	HBOC-201	9 ± 0.2	9.3 ± 0.1†	9.5 ± 0.2	9.6 ± 0.2†*	10.1 ± 0.2†*	10.4 ± 0.2†*
Met-hemoglobin (%)	Control	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0	0.3 ± 0	0.3 ± 0.1	0.3 ± 0.1
	HBOC-201	1.1 ± 0.1†	1.2 ± 0.1†	1.2 ± 0.1†	1.2 ± 0.1†	1.2 ± 0.1†*	1.0 ± 0.1†
Plasma hemoglobin (g%)	Control	0.02 ± 0	0.01 ± 0	0.02 ± 0	0.02 ± 0	0.02 ± 0	0.02 ± 0
	HBOC-201	2.06 ± 0.05†	2.08 ± 0.04†	2.04 ± 0.05†	2.03 ± 0.05†	2.03 ± 0.05	2.05 ± 0.04†
SaO_2 (%)	Control	97 ± 0.3	95 ± 1	98 ± 1	98 ± 1	96 ± 1	94 ± 1*
	HBOC-201	91 ± 0.4	90 ± 1†	90 ± 1†	90 ± 1†	90 ± 1†	90 ± 1†
O_2 Hb (%)	Control	96 ± 0.3	94 ± 1	95 ± 0.5	95 ± 1	95 ± 1	94 ± 1*
	HBOC-201	89 ± 0.4†	88 ± 1†	89 ± 1†	89 ± 1†	89 ± 1†	88 ± 1†
pO_2 (mmHg)	Control	102 ± 2	94 ± 3*	97 ± 2	96 ± 3	94 ± 2	90 ± 3*
	HBOC-201	102 ± 2	94 ± 3*	98 ± 3	94 ± 3	92 ± 3*	90 ± 3*
pCO_2 (mmHg)	Control	42 ± 1	41 ± 1	40 ± 1	40 ± 1	39 ± 1	38 ± 1*
	HBOC-201	43 ± 1	41 ± 1	41 ± 1	43 ± 3	39 ± 1*	38 ± 1*
pH	Control	7.44 ± 0.04	7.46 ± 0.01	7.47 ± 0*	7.47 ± 0.01*	7.48 ± 0.01*	7.48 ± 0.01*
	HBOC-201	7.45 ± 0.01	7.46 ± 0.01	7.46 ± 0	7.46 ± 0.01	7.47 ± 0.01*	7.47 ± 0.01
Lactate (mmol/L)	Control	1.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.3 ± 0.1	1.9 ± 0.2*
	HBOC-201	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	1.3 ± 0.1	2.2 ± 0.4*
Mixed venous							
SaO_2 (%)	Control	50 ± 1	39 ± 1*	37 ± 1*	37 ± 1*	33 ± 1*	26 ± 2*
	HBOC-201	39 ± 2†	30 ± 2†*	29 ± 2†*	29 ± 2†*	25 ± 2†*	33 ± 4
pO_2 (mmHg)	Control	42 ± 1	37 ± 1	36 ± 1*	36 ± 1*	33 ± 0.5	31 ± 1*
	HBOC-201	38 ± 1†	33 ± 1†	33 ± 1†*	33 ± 1†*	30 ± 1†	35 ± 2
pCO_2 (mmHg)	Control	51 ± 1	50 ± 2	52 ± 1	51 ± 1	51 ± 1	51 ± 1
	HBOC-201	53 ± 1	54 ± 1	54 ± 1	52 ± 1	53 ± 2	52 ± 2
pH	Control	7.36 ± 0.01	7.36 ± 0.01	7.38 ± 0.01	7.37 ± 0	7.38 ± 0.01	7.35 ± 0
	HBOC-201	7.35 ± 0.01	7.35 ± 0.01	7.35 ± 0.01	7.35 ± 0.01	7.35 ± 0.01	7.34 ± 0.01
Lactate (mmol/L)	Control	1.0 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	1.6 ± 0.2*
	HBOC-201	0.9 ± 0	0.9 ± 0	0.9 ± 0.1	0.8 ± 0	1.1 ± 0.1	1.9 ± 0.4*

Data are means ± SE; n = 11. SaO_2, oxygen saturation; O_2 Hb, fraction of oxyhemoglobin in total hemoglobin; Po_2, O_2 tension; Pco_2, CO_2 tension. *P ≤ 0.05 vs. $Rest_{lying}$; † P ≤ 0.05 vs. control.

The pressor effect of HBOC has been ascribed to scavenging NO, primarily by plasma ferrous heme, thereby lowering NO concentration.[38] Our results support previous findings that NTG is capable of negating HBOC-201-induced vasoconstriction and the accompanying increases in systemic and pulmonary blood pressures.[45] NTG reduces vascular resistance in small and large vessels through endothelium-independent, but NO-mediated, vasodilation[46] However, earlier studies were skeptical using NTG coadministration as a therapeutic option to negate the pressor effect of HBOC due to its short half-life, requiring continuous infusion. Moreover, profound systemic vasodilation and hypotension might occur in response to NTG, potentially jeopardizing resuscitation from hemorrhagic shock.[45,47] In the present study, we avoided NTG-induced hypotension through careful NTG titration to maintain MAP within physiological limits. Importantly, the NTG dosage required to normalize systemic pressures was also capable of normalizing pulmonary pressures.

NTG was compared to ADO, a purine nucleoside and NO-independent vasodilator.[24,48] At ADO infusion rates that eliminated HBOC-induced systemic hypertension, the pulmonary pressor effect was not fully eliminated, despite restoration of normal PVR. The persistence of pressor effect was likely due to elevated left atrial pressure, secondary to adenosine-induced negative cardiac inotropy.[49]

Indirect oxidation of hemoglobin involves a process of cooxidation in which the methemoglobin-forming agent is cooxidized with heme iron by hemoglobin-bound oxygen (HbO_2).[50] $O_2^{\cdot-}$ and H_2O_2 are produced when HbO_2 accepts electrons from ferrous heme and the methemoglobin-forming agent. However, ROS scavenging only marginally influence the pressor response to HBOC-201 in the systemic vasculature, while the pulmonary pressor response was unaffected, suggesting that ROS do not play a major role in the pressor effect of HBOC-201. Alternatively, it is possible that oxidative stress indirectly modulates vascular tone. Indeed, it has been shown that oxidative stress enhances ET production through stabilization of prepro-endothelin mRNA.[51-53] In the present study, the ET-receptor blocker tezosentan was capable of negating the pressor responses of HBOC-201 in both the systemic and pulmonary vasculature. Therefore, it is plausible that pressor effects of HBOC-201 may result from disinhibition of endothelin synthesis and release.[54,55] ET is a potent and long-lasting vasoconstrictor and ET-receptor blockade could, therefore, potentially provide another strategy to oppose HBOC-201-induced vasoconstriction. Although tezosentan is a relatively short-acting receptor blocker with a half-life of 3 h[56], longer-acting ET-receptor blockers are available. ET-receptor blockade would require less patient monitoring and have a lower risk of inducing hypotension.

Effects of HBOC-201 during exercise

To our knowledge, this is the first study to analyze the effect of a cell-free oxygen carrier on exercise hyperemia. In the normal healthy vasculature, exercise-induced vasodilation is regulated via an intricate interplay of vasoactive molecules, including NO, ROS, and endo-

thelin.[19,20,57] As outlined above, HBOC-201 could potentially scavenge NO and enhance production of ROS and ET and could, therefore, interfere with exercise-induced vasodilation. However, although the pressor responses of HBOC-201 were essentially unaffected by exercise, SVR did decrease during exercise following administration of HBOC-201, indicating that exercise-induced vasodilation is essentially intact.

To assess the role of NO in HBOC-201 pressor effects at rest and during exercise, HBOC administration was repeated following eNOS-inhibition. If indeed, scavenging of NO is the main contributor to the pressor effect of HBOC-201, eNOS inhibition would be expected to block the pressor effect by HBOC-201. Indeed, in accordance with previous studies[39,58], following inhibition of eNOS and the consequent vasoconstriction, no additional vasoconstriction was induced by HBOC-201 infusion in the systemic vasculature. In contrast, HBOC-201 did result in a further increase in PVR. It is not clear why the pulmonary and systemic vasculature responded differently to the combination of L-NNA and HBOC-201. However, it is possible that L-NNA did not completely inhibit eNOS in the pulmonary circulation, although this is unlikely given the high dose (20 mg/kg iv) of L-NNA administered. An alternative explanation for the divergent effects in the systemic and pulmonary vasculature is that it has been shown that the nature of the chemical interaction between NO and Hb is dependent on the amount of oxygen present. Formation of $Fe^{II}NOHb$ occurs principally when Hb is deoxygenated (T-state) in peripheral tissue. NO bound to the heme-group can be transferred to a specific cystein residue (β93Cys) upon reoxygenation of Hb in the lung, resulting in formation of SNO-Hb.[59,60] This SNO-Hb formation can also occur directly but only when Hb is oxygenated (R-state). From SNO-Hb, NO can be either released or transferred to another thiol group, thereby preserving part of NO signalling.[61,62] Thus, S-nitrosylation of Hb is governed, in part, by the state of the Hb molecule undergoing an allosteric shift from R to T shift during passage in the circulatory system.[63] These varying degrees of Hb S-nitrosylation at different molecular states (R and T) may explain, at least in part, the different hemodynamic responses to HBOC-201 in the systemic and pulmonary vasculature following NOS inhibition by L-NNA. In peripheral micro vessels, because of low oxygen tension, SNO-Hb levels are low and NO released from SNO-Hb may be consumed by biological targets, such as those mediated by GSNO reductase.[62] Consequently, the availability of bioactive NO may be closely coupled to de novo synthesis by NOS that is inhabitable by L-NNA, leaving little residual NO for scavenging by HBOC-201. By contrast, bioactive NO in the form of SNO-Hb is abundant in lungs and well protected inside erythrocytes but, upon release, is susceptible to scavenging by free Hb, manifesting as a further increase in PVR following eNOS inhibition.

A third explanation may be that the vasoconstrictor effect of HBOC-201 is not solely mediated through scavenging of NO. A ROS scavenging cocktail failed to appreciably alter hemodynamic responses to HBOC-201 and, unlike either NTG or ADO, failed to restore SVR or PVR to control levels. As glutaraldehyde-polymerized HBOC in itself was shown

to exhibit catalase-like properties, it is possible that the increase in free radicals induced by administration of this HBOC was negated by HBOC itself.[64] Indeed, lipid peroxidation as measured by thiobarbituric acid reactive substances was not significantly elevated by glutaraldehyde-polymerized HBOC. Similar to our study, these observations suggest that HBOC-201 fails to significantly stimulate ROS formation or that any HBOC-induced increase in ROS is adequately scavenged by endogenous antioxidants. However, it is possible that in certain disease states generally characterized by elevated oxidative stress[26], such as reperfusion following myocardial infarction or in the presence of severe endothelial dysfunction, HBOC may exacerbate oxidative stress, either directly or through scavenging NO. In vitro studies have suggested that HBOCs may amplify ROS formation that could, in turn, react with NO to generate nitroxide radicals[15] and/or uncouple NO synthase secondary to insufficient cofactors tetrahydrobiopterin (BH4) and NADPH required to convert L-arginine to L-citrulline and NO.[65]

The pattern of SVR and PVR during exercise with HBOC-201 very much resembles the pattern found with ET antagonism, as we previously showed that the vasodilator effect of ET-receptor blockade waned with increasing exercise intensity in the systemic circulation while it increased in the pulmonary vasculature.[35] Moreover, reduced bioavailability of NO and/or oxidative stress could contribute to overexpression of ET.[16] Although we did not measure plasma ET-levels in the present study, an increase in ET-mediated vasoconstriction as a cause of the pressor effects of HBOC-201 is consistent with the ability of tezosentan to negate these pressor effects both at rest and during exercise. Importantly, dosing of the ET-receptor blocker tezosentan did not need to be altered during exercise, and endothelin-antagonists by themselves have only very modest effects on hemodynamics, making endothelin-antagonists clinically attractive antagonists of the pressor effect of HBOC-201.

Finally, several clinical trials have shown that other HBOC products with other compositions than HBOC-201 may increase the risk of myocardial infarction and death.[66] However, clinical studies conducted with HBOC-201 in patients with documented cardiovascular disease showed both intravenous and intracoronary infusion of HBOC-201 to be safe and well tolerated.[2]

Methodological Considerations

Although plasma ET-1 measurements could possibly strengthen the conclusion that HBOC-201 induced vasoconstriction is ET-mediated, a number of both physiological and methodological issues complicate interpretation of such measurements. First, ET is released for more than 80% into the abluminal side, while less than 20% is secreted into the lumen side. Hence circulating ET-1 does not reflect the local concentration of ET-1 in the vessel wall. Second, an ET-mediated pressor effect may be caused by changes in ET-receptor sensitivity through altered nitrosylation of the ET-receptors. Finally, we have previously found that L-NNA and indomethacin yield a false-positive result in plasma ET-1 measure-

ments. Similarly, an interaction with HBOC-201 cannot be excluded, making it difficult to interpret the results. Therefore, blocking ET receptors with tezosentan is the best way to assess the interaction of HBOC-201 with the ET-system.

Conclusion and future directions

HBOC-201 can disrupt hemodynamic homeostasis, mimicking some aspects of endothelial dysfunction, resulting in elevated systemic and pulmonary blood pressures. HBOC-201 induced vasoconstriction is mediated by scavenging NO and likely by upregulating ET production. Pressor effects can be restored by NO donors or direct vasodilators, such as nitroglycerin or ADO, but dosages must be carefully monitored to avoid hypotension. However, hemodynamic normalization was more easily achieved via administration of an ET receptor blocker. Future studies should focus on coadministration of long-acting ET_A receptor antagonists (e.g., ambrisentan or sitaxentan) and, although oxygen-derived free radicals do not appear to play a significant role in HBOC-201-induced pressor responses of healthy subjects, the possible role of ROS in HBOC-induced vasoconstriction in subjects with documented preexisting endothelial dysfunction would be of interest.

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GRANTS

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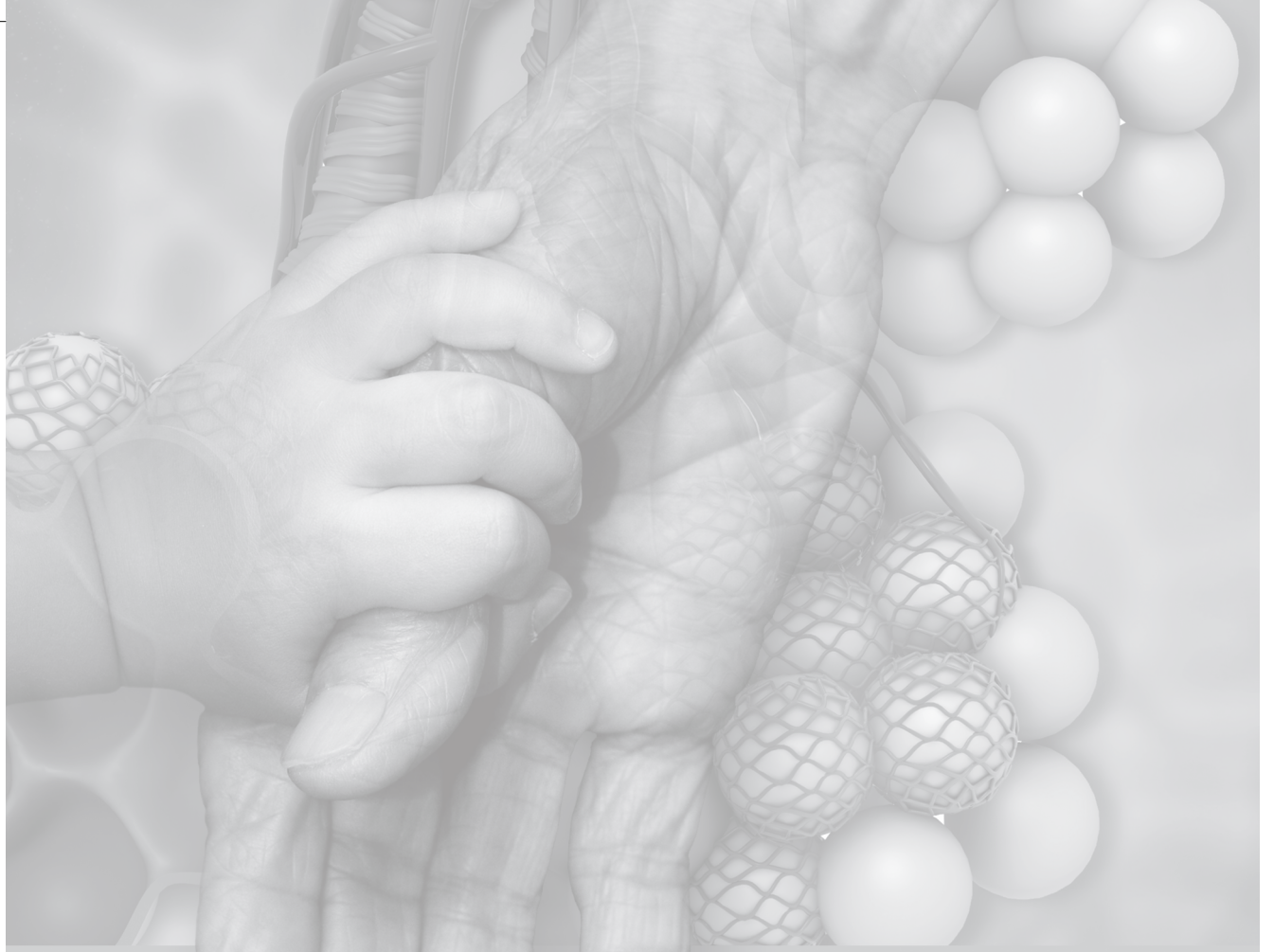

Chapter 8

Pulmonary vasoconstrictor influence of endothelin in exercising swine depends critically on phosphodiesterase 5 activity

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ABSTRACT

Both phosphodiesterase 5 (PDE5) inhibition and endothelin (ET) receptor blockade have been shown to induce pulmonary vasodilation. However, little is known about the effect of combined blockade of these two vasoconstrictor pathways. Since nitric oxide (NO) exerts its pulmonary vasodilator influence via production of cyclic guanosine monophosphate (cGMP) as well as through inhibition of ET, we hypothesised that interaction between the respective signaling pathways precludes an additive vasodilator effect. We tested this hypothesis in chronically instrumented swine exercising on a treadmill by comparing the vasodilator effect of the PDE5 inhibitor EMD360527, the ET_A/ET_B antagonist tezosentan, and combined EMD360527 and tezosentan. In the systemic circulation, vasodilation by tezosentan and EMD360527 was additive, both at rest and during exercise, resulting in 17±2% drop in blood pressure. In the pulmonary circulation, both EMD360527 and tezosentan produced vasodilation. However, tezosentan produced no additional pulmonary vasodilation in the presence of EMD360527, either at rest or during exercise. Moreover, in isolated preconstricted porcine pulmonary small arteries (~300 μm) EMD360527 (1nM-10 μM) induced dose-dependent vasodilation, whereas tezosentan (1nM-10 μM) failed to elicit vasodilation irrespective of the presence of EMD360527. However, both PDE5 inhibition and 8Br-cGMP, but not 8Br-cAMP, blunted pulmonary small artery contraction to ET and its precursor Big ET in vitro. In conclusion, in healthy swine, either at rest or during exercise, PDE5 inhibition and the associated increase in cGMP produce pulmonary vasodilation that is mediated in part through inhibition of the ET pathway, thereby precluding an additional vasodilator effect of ET_A/ET_B receptor blockade in the presence of PDE5 inhibition.

INTRODUCTION

Under basal resting conditions, the pulmonary circulation is a low-pressure, low-resistance system.[1,2] During exercise, however, flow through the pulmonary vasculature increases, which is accompanied by an increase in pulmonary arterial pressure. This requires the right ventricle not only to pump more blood but to do so against a higher afterload.[3] Although vascular tone in the pulmonary vasculature is low as compared to the systemic vasculature, pulmonary vasodilation during exercise does occur and limits the increase in pulmonary artery pressure (PAP) and thereby the increase in right ventricular afterload.[1,2]

Pulmonary vascular tone is determined by an interplay between vasodilators and vasoconstrictors, such as nitric oxide (NO) and endothelin (ET). Exercise-induced pulmonary vasodilation is largely NO mediated[1,4], and can be enhanced by prolonging the half-life of its second messenger cGMP through inhibition of phosphodiesterase 5 (PDE5).[5] Since PDE5 is abundantly expressed in pulmonary vascular smooth muscle in particular[6,7], PDE5 inhibition has clinically been used to selectively evoke pulmonary vasodilation without inducing systemic hypotension in patients with pulmonary hypertension.[8-10] NO induces pulmonary vasodilation not only through a direct cGMP-mediated effect on vascular smooth muscle but also indirectly by blunting ET mediated pulmonary vasoconstriction in swine.[11,12] Nevertheless, ET exerts a vasoconstrictor influence on the pulmonary vasculature[13,14], that is relatively small under basal resting conditions, but, surprisingly, becomes more pronounced during exercise, thereby limiting the exercise-induced pulmonary vasodilation.[11,15] Thus both PDE5 inhibition and ET receptor blockade cause vasodilation in the pulmonary vasculature, particularly during exercise, and combined inhibition of both pathways may have an additive vasodilator effect and may therefore synergistically decrease right ventricular afterload. However, since NO blunts ET-mediated pulmonary vasoconstriction[11] and since ET can enhance NO production via ET_B receptor stimulation[1], it is also possible that interaction between the respective signaling pathways precludes such additive vasodilator effect. Therefore, the aim of the present study was to evaluate the vasodilator effect of ET receptor blockade on the pulmonary vasculature in vivo in the presence of PDE5 inhibition not only at rest but also during exercise.

Since we found no additive pulmonary vasodilator effect of ET receptor blockade following PDE5 inhibition, we further investigated whether this lack of effect was the result of a direct interaction between the NO-cGMP and the ET pathway or due to a lack of residual tone in the pulmonary vasculature following PDE5 inhibition, using isolated pulmonary small arteries. For this purpose, we investigated whether PDE5 inhibition and ET_A/ET_B receptor blockade could act synergistically when pulmonary tone was increased with the stable thromboxane A_2 analogue U46619, and we evaluated the responsiveness of the isolated pulmonary small arteries to ET and its precursor Big ET, in the absence and presence of PDE5 inhibition as well as increased cGMP levels. Finally, to test whether the

interactions were specific for cGMP signalling, we studied pulmonary artery ET and Big ET responsiveness in the absence and presence of cAMP.

METHODS

In vivo studies

Animals

Studies were performed in accordance with the Council of Europe Convention (ETS123)/ Directive) (86/609/EEC) for the protection of vertebrate animals used for experimental and other scientific purposes, and with approval of the Animal Care Committee of the Erasmus University Medical Center Rotterdam. Thirteen crossbred Yorkshire X Landrace swine (2-3 mo old, 22±1 kg at the time of surgery, 9 females and 4 neutered males) entered the study. Daily adaptation of animals to laboratory conditions started 1 wk before surgery and continued during the first week after surgery.

Surgical procedures

Swine were sedated with ketamine (20 mg/kg im), and midazolam (1 mg/kg im), anesthetised with thiopental (10 mg/kg iv), intubated, and ventilated with a mixture of O_2 and N_2 (1:2) to which 0.2-1% (vol/vol) isoflurane was added.[4,11,16] Anesthesia was maintained with midazolam (1 mg kg^{-1} per hour iv) and fentanyl (10 µg kg^{-1} per hour iv). Under sterile conditions, the chest was opened via the fourth left intercostal space and fluid-filled polyvinylchloride catheters were directly inserted into the aortic arch, left atrium, and pulmonary artery by puncture of these structures for blood sampling and blood pressure measurement (Combitrans pressure transducers, Braun, Melsungen, Germany). A Transonic flow probe (16 mm; Transonic Systems) was positioned around the ascending aorta for measurement of cardiac output. Catheters were tunnelled to the back and animals were allowed to recover, receiving analgesia (0.3 mg buprenorphine im) for 2 days and antibiotic prophylaxis (25 mg/kg amoxicillin and 5 mg/kg gentamycin iv) for 5 days.

Exercise protocols

Experimental design

Studies were performed 1-2 weeks (11±1 days) after surgery with animals exercising on a motor-driven treadmill. Swine (n=13) were subjected to two different experimental protocols. In the first group, animals (n=7) performed *1)* control exercise and *2)* exercise in the presence of ET_A/ET_B antagonist tezosentan (a gift from Actelion Pharmaceuticals, Allschwil, Switzerland). In the second group, animals (n=7, one animal overlapping with the first group) performed *1)* control exercise, *2)* exercise in the presence of PDE5 inhibitor

EMD360527 (a gift from Merck, Darmstadt, Germany) and *3)* exercise in the presence of combined EMD360527 and tezosentan.

Effects of ET_A/ET_B receptor blockade during exercise

With swine resting (lying and standing) quietly on the treadmill, resting hemodynamic measurements consisting of left atrial, aortic, and pulmonary artery blood pressures; heart rate; and cardiac output were obtained; arterial and mixed venous blood samples were collected (lying); and rectal temperature measured. Subsequently, a five-stage exercise protocol (1, 2, 3, 4 and 5 km/h) was started, with each stage lasting 2-3 min. Hemodynamic variables were continuously recorded and blood samples collected during the last 30 s of each exercise stage, at a time when hemodynamics had reached a steady state. Following the exercise trial, animals were allowed to rest for 90 min, resulting in a complete return of hemodynamic variables to baseline values.[4] In the second exercise protocol, tezosentan was infused intravenously over 10 min in a dose of 3 mg/kg, followed by a continuous intravenous infusion of tezosentan in a dose of 6 mg kg^{-1} h^{-1}, and the five-stage exercise protocol was repeated. We have previously shown that this dose of tezosentan abolishes the increase in blood pressure in response to endothelin.[15] Tezosentan has a pA_2 of 9.5 for ET_A and a pA_2 of 7.7 for ET_B receptors, indicating only a 63-fold selectivity for ET_A receptors compared with ET_B receptors.[17,18]

Effects of ET_A/ET_B receptor blockade in the presence of PDE5inhibition during exercise

Animals from the second group underwent the same exercise protocol as those in the first group, but consisting of three consecutive exercise trials. First, a control exercise trial was performed as described above. Following the exercise trial, animals were allowed to rest for 90 min, resulting in a complete return of hemodynamic variables to baseline values. Subsequently, the EMD360527 was infused continuously in a dose of 300 µg min kg^{-1} min^{-1} intravenously, and 10 min after starting the infusion the five-stage exercise protocol was repeated while the infusion was continued.[19] EMD360527 demonstrates at least 45-fold selectivity for PDE5 (IC_{50}=0.007 µM) compared to PDE6 (IC_{50}=0.32 µM), 94-fold selectivity for PDE1 (IC_{50}=0.66 µM), 137-fold selectivity for PDE10 (IC_{50}=0.96 µM), and > 1400-fold selectivity for PDE2, PDE3, PDE4 and PDE7 (all IC_{50}>10 µM). In these assays PDE1-4 was from guinea pig heart muscle, PDE5 from human thrombocytes, PDE6 from bovine retina, whereas PDE7 and PDE10 were obtained by expression of cDNA in COS-7 cells and Escherichia coli, respectively.

Subsequently, the EMD360527 infusion was stopped and the animals were allowed to rest for another 90 min. Then, animals received an intravenous infusion of EMD360527 together with the ET_A/ET_B antagonist tezosentan, in dosages identical to those described above for the individual infusions, and the five-stage exercise protocol was repeated. We have previously observed excellent reproducibility of consecutive exercise protocols.[16]

In vitro studies

Tissues

Pig lungs (n=23) were collected at a local slaughterhouse. Pulmonary small arteries (diameter ~300 μm) were removed and stored overnight in cold, oxygenated Krebs bicarbonate solution of the following composition (mM): 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, and glucose 8.3; pH 7.4.

The next day, pulmonary small arteries were cut into segments of ~2 mm length and mounted in microvascular myographs (Danish MyoTechnology) with separated 6-ml organ baths containing Krebs bicarbonate solution aerated with 95% O_2-5% CO_2 and maintained at 37°C. Changes in contractile force were recorded with a Harvard isometric transducer. Following a 30-min stabilization period, the internal diameter was set to a tension equivalent to 0.9 times the estimated diameter at 20 mmHg effective transmural pressure. Vessels were then exposed to 30 mM KCl twice. Endothelial integrity was verified by observing dilation to 10 nM substance P after preconstriction with 100 nM of the thromboxane A_2 analog U46619. Then vessels were subjected to 100 mM KCl to determine the maximal vascular contraction. Thereafter, we allowed vessels to equilibrate in fresh organ bath fluid for 30 min before initiating different experimental protocols.[20]

Effects of PDE5-inhibition and ET_A/ET_B blockade

Pulmonary small arteries were preconstricted with 100 nM U46619, and concentration-response curves were constructed to EMD360527 (1nM-10 μM; n=7), tezosentan (1nM-10 μM[21]; n=7), and combined EMD360527 and tezosentan (n=7).

Effects of PDE inhibition on ET receptor sensitivity and ET production

The responses to cumulative concentrations of ET (1-100 nM[22]) and Big ET (10 nM-1μM) were measured in control vessels and vessels pretreated with EMD360527 (3 μM, n= 7 for ET and Big ET), 8Br-cGMP (100 μM n= 4 for ET and Big ET), and 8Br-cAMP (300 μM n= 4 for ET and Big ET). Big ET has no direct vasomotor effect; therefore Big ET-induced vasoconstriction is used as an index of Big ET conversion to vasoactive ET.

Data analysis and statistics

Digital recording and offline analysis of hemodynamic data have been described in detail elsewhere.[16,23] Pulmonary vascular resistance (PVR) and systemic vascular resistance (SVR) were calculated as PAP minus left atrial pressure divided by cardiac output and mean aortic pressure divided by cardiac output, respectively. Pulmonary vascular conductance (PVC) and systemic vascular conductance (SVC) were calculated as 1/PVR and 1/SVR.[24] Total pulmonary resistance (TPR) was calculated as PAP divided by cardiac output.[25] Body oxygen consumption (BVO_2) was calculated as the product of cardiac output and the difference between arterial and mixed venous oxygen content of the blood. To accommodate for the

varying weights between animals and groups, cardiac output, PVC, SVC, TPR, and BVO_2 were indexed to body weight. Pulmonary distensibility (α) was estimated using the formula described by Linehan and Reeves[26,27], minimizing the difference between predicted and PAP measured with Solver in Excel using six data points (rest and 5 levels of exercise) per animal.

$$PAP = \frac{[(1 + \alpha * LAP)^5 + 5 * \alpha * R_0 * CO]^{1/5} - 1}{\alpha}$$

In this formula R_0 is assumed to be PVR measured at rest. Values for α could be obtained in all animals except one, which showed a paradoxical increase in PVR during exercise. Correlations between measured PAP and predicted PAP were calculated in Excel.

Vasodilator responses in vitro to EMD360527, tezosentan and combined EMD360527 and tezosentan were expressed as percentage of contraction to U46619. Vasoconstrictor responses to ET and Big ET were measured when contraction had reached a steady state, and normalized to maximal constriction to 100 mM KCl.

The effects of EMD360527 and tezosentan during exercise were analyzed by using linear regression analysis with BVO_2 as independent variable and assigning a dummy variable to each animal. The effect of EMD360527 and tezosentan on relation between the transpulmonary pressure gradient and cardiac index was analyzed by linear regression. Since we found no statistically significant differences between the hemodynamic response to exercise between male and female swine, in either the pulmonary or the systemic vasculature, data from both sexes were pooled.

The effects of EMD360527, tezosentan, and combined EMD360527 and tezosentan on the preconstricted isolated pulmonary arteries were assessed by two-way ANOVA for repeated measures. The effects of EMD360527, cGMP, and cAMP on the vasoconstrictor response to ET and Big ET were also assessed by two-way ANOVA for repeated measures. In all ANOVAs post hoc testing was performed by Bonferroni's method. Statistical significance was accepted when $P<0.05$ (two-tailed). Data are presented as means± SE.

RESULTS

Integrated effects of EMD360527 and tezosentan in the systemic circulation in vivo

Graded treadmill exercise up to 5 km/h resulted in an increase in heart rate up to 90% of estimated maximal heart rate[28], and a doubling of cardiac output (Table 1), which in combination with an increase in body oxygen extraction from 49±2% at rest to 72±2% during maximal exercise, resulted in a threefold increase in BVO_2 (Figure 1) up to 65% of estimated

Table 1. Hemodynamic effects of ET antagonism, PDE5 inhibition, and the combination

		Rest	Exercise (km/h)				
		Standing	1	2	3	4	5
HR	Control	144 ± 4	168 ± 4*	177 ± 6*	194 ± 8*	223 ± 10*	256 ± 8*
(beats/min)	Tezo	158 ± 6†	177 ± 6*†	188 ± 10*	207 ± 9*	233 ± 10*†	257 ± 8*
	Control	135 ± 14	152 ± 14*	167 ± 13*	181 ± 12*	216 ± 12*	249 ± 9*
	EMD	159 ± 7	195 ± 12*†	199 ± 11*†	223 ± 9*†	243 ± 8*	259 ± 8*
	EMD+Tezo	177 ± 9†‡	207 ± 11*†‡	214 ± 9*†	236 ± 8*†	257 ± 7*†	270 ± 6*†
MAP	Control	86 ± 6	81 ± 4	83 ± 5	85 ± 5	87 ± 5	91 ± 7*
(mmHg)	Tezo	71 ± 6†	73 ± 4†	74 ± 4†	76 ± 6†	78 ± 4†	82 ± 5†
	Control	83 ± 3	83 ± 4	83 ± 3	84 ± 3	86 ± 4	89 ± 4
	EMD	75 ± 4	73 ± 5†	73 ± 4†	74 ± 3†	73 ± 4†	76 ± 5†
	EMD+Tezo	68 ± 4†	68 ± 5†	65 ± 6†	67 ± 3†‡	67 ± 4†	72 ± 4†
PAP	Control	12 ± 2	15 ± 1	18 ± 1	20 ± 1*	24 ± 2*	28 ± 2*
(mmHg)	Tezo	8 ± 1†	12 ± 1	15 ± 1†	17 ± 1†	21 ± 1*	26 ± 1*
	Control	12 ± 1	16 ± 2	17 ± 2	19 ± 2*	24 ± 3*	27 ± 3*
	EMD	11 ± 1	13 ± 2†	15 ± 2†	17 ± 2*†	19 ± 2*†	22 ± 3*†
	EMD+Tezo	12 ± 1	14 ± 1	15 ± 1	17 ± 2†	20 ± 2*	24 ± 3*
LAP	Control	−0.4 ± 1.2	1.0 ± 0.8	3.9 ± 0.4*	4.8 ± 0.8*	6.4 ± 1.3*	8.7 ± 1.4*
(mmHg)	Tezo	−2.2 ± 0.4†	1.3 ± 0.7	3.3 ± 0.9*	4.1 ± 1.3*	7.0 ± 1.5*	10.0 ± 1.6*
	Control	−2.3 ± 1.8	1.2 ± 0.9	1.8 ± 1.1	3.5 ± 1.1*	5.6 ± 2.0*	5.9 ± 2.2*
	EMD	1.1 ± 2.1	0.8 ± 1.4	2.8 ± 1.2	3.9 ± 1.2	5.4 ± 2.1*	6.6 ± 2.5*
	EMD+Tezo	0.2 ± 1.4	1.6 ± 1.0	3.2 ± 1.3	4.1 ± 1.5*	6.6 ± 2.1*	9.1 ± 2.6*

Table 1. Hemodynamic effects of ET antagonism, PDE5 inhibition, and the combination (*continued*)

		Rest	Exercise (km/h)				
		Standing	1	2	3	4	5
CO	Control	0.20 ± 0.01	0.24 ± 0.01*	0.25 ± 0.01*	0.28 ± 0.01*	0.31 ± 0.01*	0.33 ± 0.02*
(L min^{-1} kg^{-1})	Tezo	0.20 ± 0.01	0.24 ± 0.01*	0.26 ± 0.01*	0.29 ± 0.01*	0.32 ± 0.01*†	0.35 ± 0.02*†
	Control	0.19 ± 0.02	0.22 ± 0.02*	0.24 ± 0.02*	0.26 ± 0.02*	0.30 ± 0.02*	0.34 ± 0.02*
	EMD	0.21 ± 0.02	0.26 ± 0.03*†	0.28 ± 0.03*†	0.31 ± 0.02*†	0.34 ± 0.02*†	0.36 ± 0.02*†
	EMD+Tezo	0.24 ± 0.02‡	0.28 ± 0.03*†	0.30 ± 0.03*†	0.32 ± 0.03*†	0.36 ± 0.03*†	0.37 ± 0.02*†
SV	Control	1.40 ± 0.11	1.44 ± 0.07	1.45 ± 0.06	1.46 ± 0.07	1.43 ± 0.09	1.41 ± 0.11
(ml kg^{-1})	Tezo	1.31 ± 0.07	1.41 ± 0.07	1.45 ± 0.06*	1.47 ± 0.07*	1.45 ± 0.10*	1.41 ± 0.10
	Control	1.42 ± 0.12	1.46 ± 0.10	1.47 ± 0.09	1.45 ± 0.08	1.39 ± 0.05	1.36 ± 0.07
	EMD	1.31 ± 0.08	1.34 ± 0.07	1.40 ± 0.07	1.38 ± 0.06	1.39 ± 0.08	1.39 ± 0.09
	EMD+Tezo	1.34 ± 0.10	1.33 ± 0.10	1.39 ± 0.10	1.35 ± 0.08	1.38 ± 0.09	1.38 ± 0.08

Values are mean±SE. ET, endothelin, PDE5, phosphodiesterase 5; HR, heart rate; MAP, mean aortic pressure; LAP, left atrial pressure; CO, cardiac output; SV, stroke volume; Tezo, tezosentan; EMD, EMD360527. * $P < 0.05$ vs. Standing; † $P < 0.05$ effect of Tezo; ‡ $P < 0.05$ effect of Tezo in the presence of EMD360527.

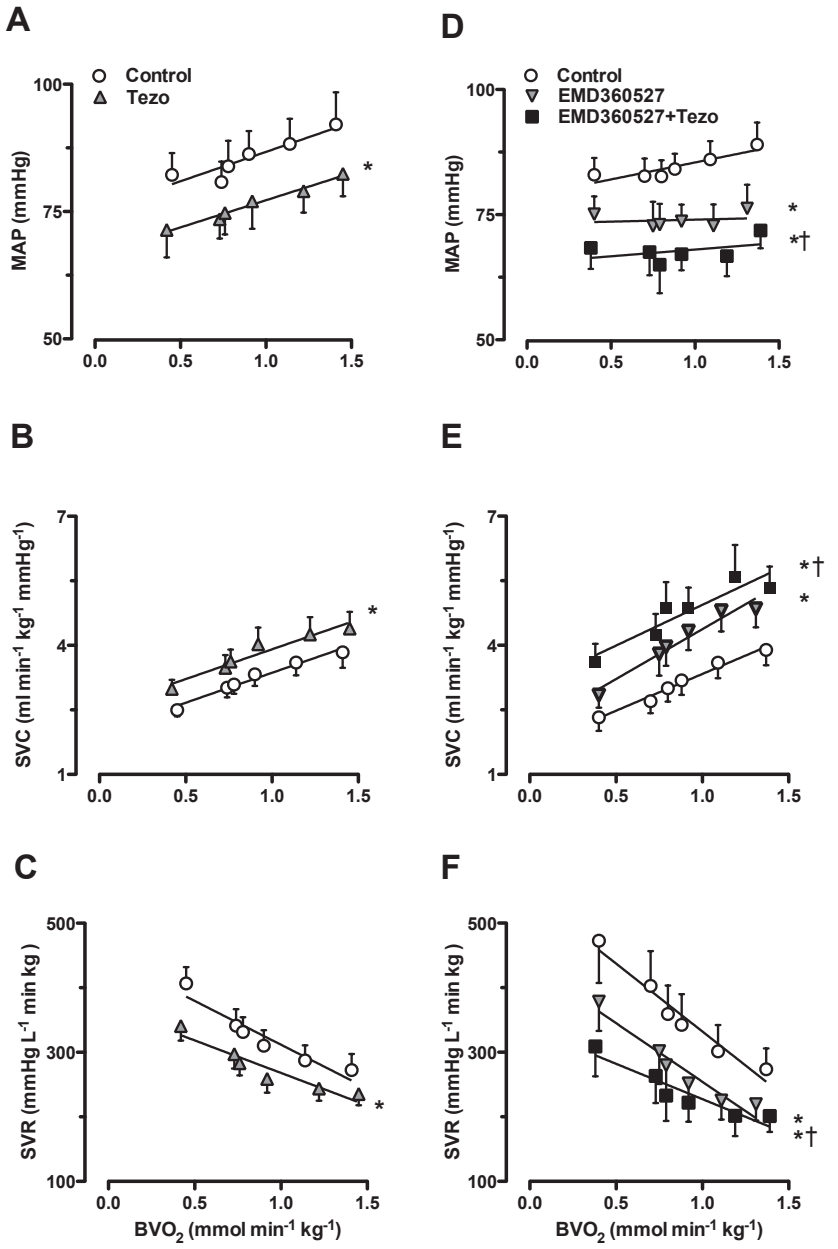

Figure 1. Effects of phosphodiesterase 5 (PDE5) inhibition and endothelin $(ET)_A/ET_B$ receptor blockade on the systemic vasculature in vivo. Shown are the effects of the ET_A/ET_B receptor blocker tezosentan (Tezo), the PDE5 inhibitor EMD360527, and Tezo in the presence of EMD360527 on mean aortic pressure (MAP), systemic vascular conductance (SVC), and systemic vascular resistance (SVR) at rest and during exercise. Values are means±SE. * $P < 0.05$, drug effects vs. corresponding control; † $P < 0.05$, Tezo in the presence of EMD360527 vs. EMD360527 alone.

maximal BVO_2.[29] Mean aortic pressure was maintained constant (Fig. 1, A and D), as the increase in cardiac output was balanced by a 66±9% increase in SVC (Fig. 1, B and E).

Infusion of either ET_A/ET_B receptor antagonist tezosentan or PDE5 inhibitor EMD360527 alone induced systemic vasodilation as evidenced by an increase in SVC (Fig 1, B and E), resulting in a decrease in mean aortic pressure (Fig 1, A and D), despite concomitant, probably baroreceptor reflex-mediated, increases in heart rate and cardiac output (Table 1).

Infusion of the ET_A/ET_B receptor antagonist tezosentan following EMD360527 resulted in a further decrease in mean aortic pressure and increase in SVC (Fig. 1, D and E), as well as a further increase in heart rate and cardiac output (Table 1). Hence, the effect of ET antagonism on the systemic vasculature was not affected by prior PDE5 inhibition, and the integrated vasodilator effect of PDE5 inhibition and ET antagonism on the systemic vasculature was larger than the effect of PDE5 inhibition or ET antagonism alone.

Integrated effects of EMD360527 and tezosentan in the pulmonary circulation in vivo

Exercise resulted in a significant increase in pulmonary arterial pressure (Fig. 2, A and D), which was principally the result of increase in cardiac output and to a lesser extent of increase in left atrial pressure (that is transmitted backward into the pulmonary vasculature) (Table 1). TPR increased (Fig. 2, C and F), suggesting an increase in right ventricular afterload. The transpulmonary pressure gradient (PAP minus left atrial pressure) increased slightly less than cardiac output (Table 1) indicating that exercise-induced pulmonary vasodilation occurred as evidenced by an increase in PVC (Fig. 2, B and E). Distensibility of the pulmonary vasculature under control conditions was on average 0.5±0.1%/mmHg and ranged from 0.1 to 1.1 %/mmHg (r^2= 0.91±0.03).

In accordance with previous studies from our laboratory[11,15], ET_A/ET_B blockade with tezosentan resulted in a decreased PAP (Fig. 2A), and a decrease in total pulmonary resistance (Fig. 2C) with minimal effect on left atrial pressure (Table 1). Tezosentan had little effect on cardiac output at rest and low levels of exercise, but it increased cardiac output significantly at 4 and 5 km/h compared to control exercise (Table 1). Tezosentan resulted in a downward rotation of the relation between cardiac output and transpulmonary pressure gradient (Fig. 3A), reflecting a significant increase in PVC (Fig. 2B). Distensibility of the pulmonary vasculature was not significantly altered by tezosentan.

Similar to previous observations from our laboratory[19], infusion of PDE5 inhibitor EMD360527 resulted in a decreased PAP (Fig.2D), with minimal effect on left atrial pressure, whereas cardiac output increased significantly at all levels of exercise (Table 1). The decrease in TPR in response to EMD360527 was larger at higher levels of exercise (Fig. 2F), suggesting a larger effect of PDE5 inhibition on right ventricular afterload with incremental levels of exercise. EMD360527 caused a downward rotation of the relation between cardiac

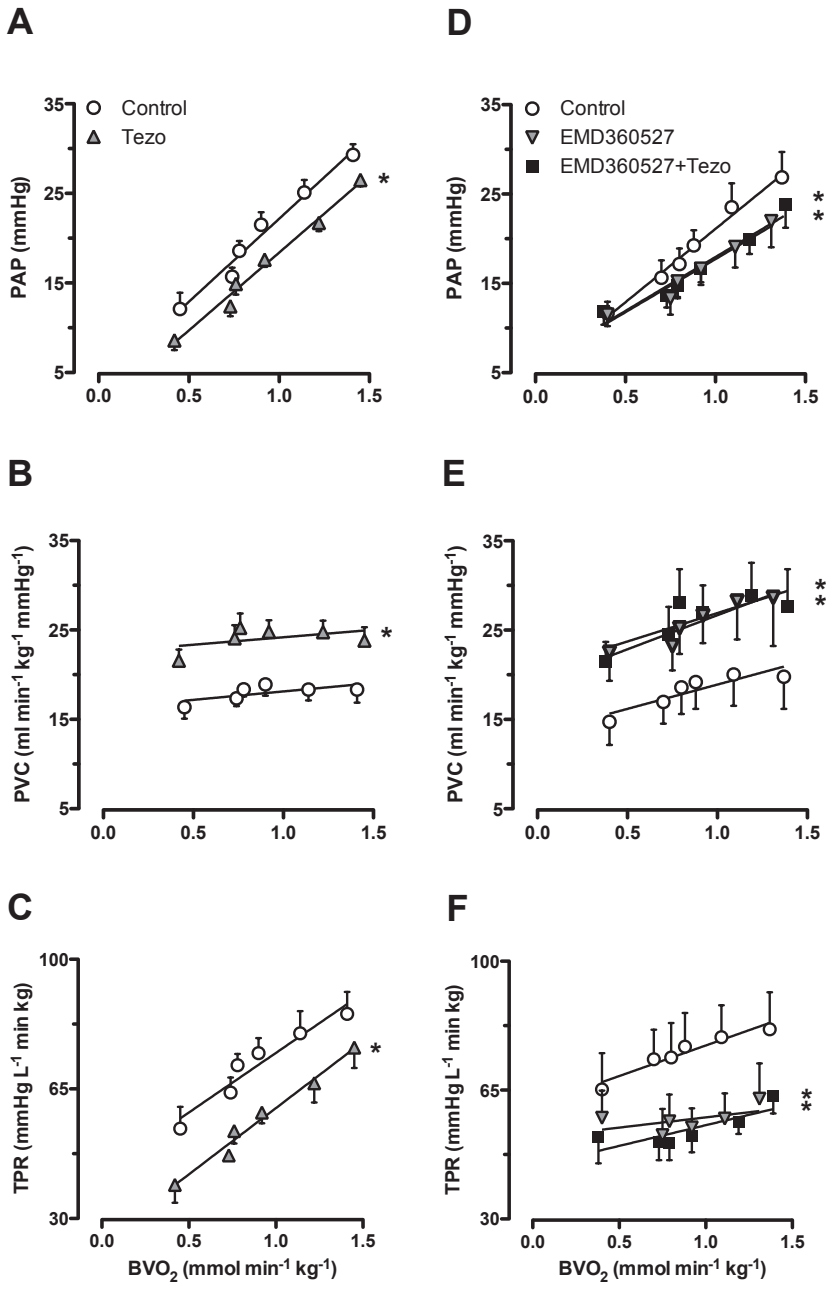

Figure 2. Effects of PDE5 inhibition and ET_A/ET_B receptor blockade on the pulmonary vasculature in vivo. Shown are the effects of the ET_A/ET_B receptor blocker Tezo, the PDE5 inhibitor EMD360527, and Tezo in the presence of EMD360527 on pulmonary artery pressure (PAP), pulmonary vascular conductance (PVC), and total pulmonary resistance (TPR) at rest and during exercise. Values are means±SE. * $P < 0.05$, drug effects vs. corresponding control.

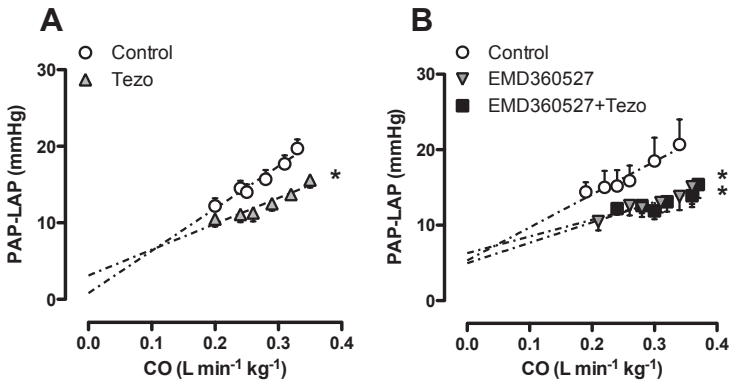

Figure 3. Effects of PDE5 inhibition and ET_A/ET_B receptor blockade on the pulmonary pressure-flow relationships. Shown are the effects of the ET_A/ET_B receptor blocker Tezo, the PDE5 inhibitor EMD360527, and Tezo in the presence of EMD360527 on the pressure-flow relationship at rest and during exercise. CO, cardiac output; LAP, left atrial pressure. Values are means±SE. * $P < 0.05$, drug effects vs. corresponding control.

output and transpulmonary pressure-gradient (Fig. 3C), reflecting a significant increase in PVC (Fig. 2E). Distensibility of the pulmonary vasculature was not significantly altered by EMD360527.

Infusion of tezosentan following PDE5 inhibition with EMD360527 did not result in further changes in PAP, PVC, or TPR (Fig 2, D-F) nor in the relation between the transpulmonary pressure gradient and cardiac output (Fig 3C), indicating that in the presence of PDE5 inhibition, ET_A/ET_B receptor blockade had no additional vasodilator effect on the pulmonary vasculature.

Interaction between the NO-cGMP and the ET pathways in isolated pulmonary small arteries

The lack of additive pulmonary vasodilation with tezosentan in the presence of EMD360527 could be due either to an interaction between the ET and cGMP pathways or to the fact that EMD360527 alone was sufficient to obtain maximal pulmonary vasodilation. Since these two scenarios are difficult to study in vivo, we performed dose responses of EMD360527, tezosentan, and combined EMD360527 and tezosentan in isolated pulmonary small arteries preconstricted with U46619. EMD360527 caused dose-dependent vasodilation of the preconstricted vessel segments (Fig. 4). Tezosentan failed to induce relaxation in vitro either in the absence or presence of EMD360527 (Fig. 4).

To further investigate whether the lack of vasodilator effect of tezosentan in the presence of EMD360527 was the result of a direct suppression of the ET pathway by the NO-cGMP pathway, we measured constriction to ET and Big ET in the absence and presence of EMD360527 and 8Br-cGMP in isolated pulmonary small arteries. Both ET and Big ET

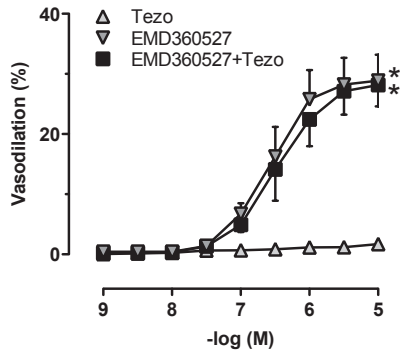

Figure 4. Effects of PDE5 inhibition and ET_A/ET_B receptor blockade on pulmonary small arteries in vitro. Shown are the effects of the PDE5 inhibitor EMD360527, the ET_A/ET_B receptor blocker Tezo, and Tezo in the presence of EMD360527 on isolated pulmonary arteries preconstricted with U46619 (100 nM). Each dose-response curve was obtained in 7 vessel rings (7 swine). Values are means±SE. * $P < 0.05$, EMD360527 or EMD360527+Tezo vs. Tezo.

produced dose-dependent vessel segment contraction. EMD360527 blunted the response to ET and Big ET (Fig. 5, A and D), whereas 8Br-cGMP blunted the response to ET slightly more than the response to Big ET (P<0.05, Fig. 5, B and E). These data indicate inhibition of PDE5 decreases the sensitivity of the pulmonary vasculature to ET by increasing cGMP but has no effect on the conversion of Big ET to ET in the pulmonary vasculature. 8Br-cAMP had no effect on the response to either ET or Big ET (Fig 5, C and F).

DISCUSSION

The main findings of the present study were as follows: 1) both ET_A/ET_B receptor blockade with tezosentan and PDE5 inhibition with EMD360527 resulted in systemic and pulmonary vasodilation. 2) ET_A/ET_B receptor blockade resulted in further vasodilation in the presence of PDE5 inhibition in the systemic circulation. 3) However, in the presence of PDE5 inhibition, ET_A/ET_B receptor blockade failed to produce additional vasodilation in the pulmonary circulation in vivo or in isolated preconstricted pulmonary small arteries in vitro. 4) Both PDE5 inhibition and 8Br-cGMP blunted ET and Big ET-induced pulmonary small artery contraction in vitro and to a similar extent. The implication of these findings will be discussed below.

Methodological Considerations

Conductance versus resistance

In general, changes in vasomotor tone of a given vascular bed are extrapolated from changes in either the conductance or the resistance of the vascular bed. Although these measures

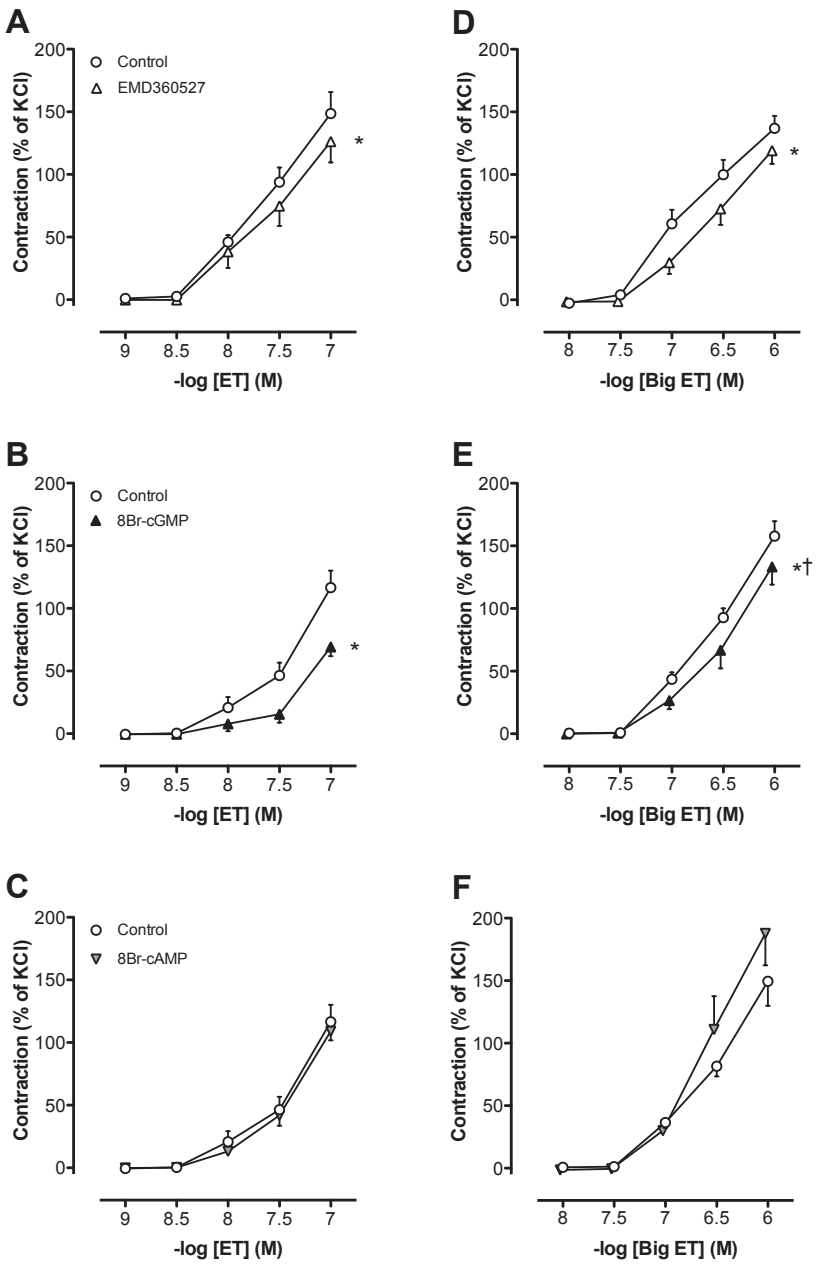

Figure 5. Effects of elevations in cGMP and cAMP on ET sensitivity and production from its precursor Big ET in pulmonary small arteries in vitro. Elevation of cGMP by either EMD360527 (3 μM, n=7, A and D) or the stable cGMP analog 8Br-cGMP (100 μM, n=4, B and E) attenuated constriction to ET and Big ET in isolated pulmonary small arteries, whereas elevation of cAMP with 8Br-cAMP (300 μM, n=4, C and F) had no effect. Values are means±SE. * $P < 0.05$, control vs. EMD360527, 8Br-cGMP, or 8Br-cAMP; † $P < 0.05$ effect of 8Br-cGMP different for ET and Big ET.

are mathematically related, interpretation can differ depending on whether one considers resistance or conductance.[24] Vascular conductance is calculated as flow corrected for pressure (flow/pressure) whereas vascular resistance is calculated as pressure divided by flow. These variables are interchangeable if one investigates the effect of only a single stimulus (e.g., exercise); however, interpretation of our results here are more complicated because we studied the effects of vasoconstrictor mechanisms at rest and during various levels of treadmill exercise in the systemic and pulmonary circulations.

The systemic circulation is a system with a low-flow state (high resistance, low conductance) at rest that transforms into a high-flow state (low resistance, high conductance) during exercise. Consequently, under low-flow conditions at rest, vasodilation causes a large decrease in resistance while the increase in conductance is small. In contrast, the same vasodilation under high-flow conditions during exercise causes a large increase in conductance, with only a small decrease in resistance. When quantifying the magnitude of the vasodilator responses, it appears in terms of conductance that a greater vasodilation occurs during exercise, whereas in terms of resistance it appears that vasodilation is larger at rest. This is illustrated by Fig. 1, which shows that the increase in SVC produced, for example, by the combination of PDE5 inhibition and ET_A/ET_B blockade is similar at rest and during exercise, whereas the decrease in vascular resistance wanes with incremental levels of exercise. Interpretation of vasomotor control thus critically depends on the variable examined. It has been forwarded that the variable (flow or pressure) that undergoes the primary change should be in the numerator of the index for vascular responses.[30] Since aortic blood pressure remains relatively constant while cardiac output markedly increases during exercise, the most appropriate measure for systemic vascular responses is SVC (cardiac output / aortic blood pressure). An additional argument in support of using conductance to determine systemic vascular responses is that the systemic circulation is comprised of vascular beds of various organs that are perfused principally in parallel. Parallel resistors add reciprocally, whereas parallel conductors add linearly, so that a change in conductance of one regional vascular bed results in an equal change of the total SVC.

The choice for either PVR or PVC as a measure of pulmonary vasomotor tone is less obvious as exercise increased both cardiac output and PAP (Table 1). However, the choice for either PVR or PVC also appears less critical, because exercise-induced changes in PVR and PVC are relatively minor compared to the vasodilation caused by PDE5 inhibition and ET_A/ET_B blockade. Consequently, the use of either resistance or conductance to assess the pulmonary vascular effects of a vasodilator will yield similar interpretations in the pulmonary bed.

Active vasodilation vs. passive distension

The increase in PVC during exercise in the present study could represent passive distension to the increase in pressure as well as vasodilation due to a decrease in pulmonary vascular tone. It is difficult to distinguish between passive distension and a decrease in pulmonary

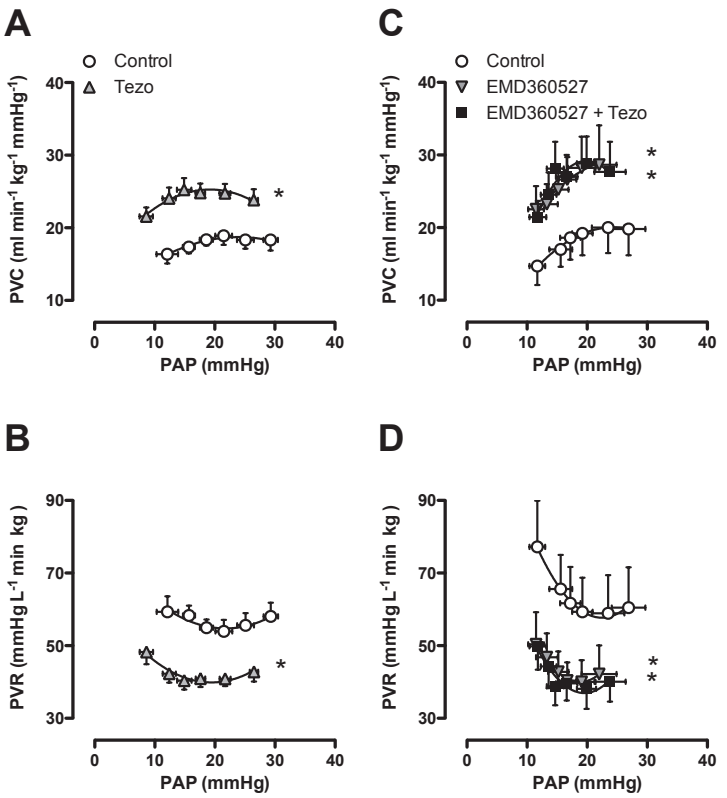

Figure 6. Effects of PDE5 inhibition and ET_A/ET_B receptor blockade on the relation between PAP and PVC and pulmonary vascular resistance (PVR). Shown are the effects of the ET_A/ET_B receptor blocker Tezo, the PDE5 inhibitor EMD360527, and Tezo in the presence of EMD360527. Values are means±SE. *P < 0.05, drug effects vs. corresponding control.

vascular tone. The pulmonary distensibility coefficient α of 0.5±0.1% is in accordance with the values found in literature.[27] To further address the question whether the increase in PVC is due to passive distension or active vasodilation, the relation between PAP and PVC in the different experiments was plotted in Fig 6. At low pressure, PVC increases with increasing PAP, whereas the relations show a plateau above ~20 mmHg, suggesting that the increase in PVC is not solely due to passive distension. Moreover, PDE5 inhibition or ET_A/ET_B receptor blockade resulted in an upward shift of the relation between PAP and PVC, which must be the result of a reduction in pulmonary vascular tone, since the increase in PVC occurred at any given level PAP.

Integrated control of pulmonary vascular tone by PDE5 and ET

The magnitude of these individual vasodilator effects of PDE5 inhibition and ET_A/ET_B receptor blockade in the systemic and pulmonary vascular beds are in good agreement with

their respective effects in previous studies from our laboratory.[11,15,19,24] Thus PDE5 inhibition resulted in pulmonary and systemic vasodilation that was similar in magnitude at rest and during exercise. PAP decreased in response to ET_A/ET_B receptor blockade at rest. The increase in PVC in response to ET_A/ET_B receptor blockade under resting conditions was nonsignificant (P=0.1), whereas the increase in PVC was significant at all levels of exercise. This smaller effect of ET_A/ET_B receptor blockade on PVC under resting conditions in vivo is consistent with previous studies from our laboratory[11,15] as well as with the observation that tezosentan failed to induce vasodilation in isolated pulmonary small arteries preconstricted with U46619. In the present study, we did not examine the effect of tezosentan on pulmonary veins, which are known to contribute to PVC as well and have been shown to be even more sensitive to ET than pulmonary arteries.[31]

Nevertheless, our in vivo and in vitro data, taken together, indicate that there is little ET released into the pulmonary vasculature under basal resting conditions. During exercise, however, pulmonary vasodilation occurred in response to ET_A/ET_B receptor blockade. It remains to be determined whether this vasodilator effect of ET_A/ET_B receptor blockade is due to its action on pulmonary arteries and/or pulmonary veins.

The vasodilator effect of ET_A/ET_B receptor blockade on the pulmonary vasculature in vivo was lost in the presence of PDE5 inhibition. These data seem in contrast with a recent study in isolated pulmonary arteries preconstricted with ET, in which PDE5 inhibition and ET receptor blockade produced additive vasodilation.[32] This additive effect is likely due to the preconstriction with ET, since we failed to observe an additive vasodilator effect of PDE5 inhibition and ET receptor blockade on isolated pulmonary arteries preconstricted with the stable thromboxane analog U46619 in the present study.

In contrast to the observations in the pulmonary vasculature, we found that the systemic vasodilation produced by ET_A/ET_B receptor blockade was not influenced by prior PDE5 inhibition. In fact, the systemic vasodilation induced by combined treatment resulted in an average reduction in blood pressure of 17±2 mmHg, with average systolic blood pressure being 90±6 mmHg and average diastolic blood pressure being 49±3 mmHg following treatment, indicating marked hypotension. One pig, which was excluded from the analyses, was even unable to perform treadmill exercise following the combination treatment. In contrast to the findings in the present study, a recent study showed that the combination of ET_A/ET_B blockade by bosentan and PDE5 inhibition by sildenafil, at dosages that were ineffective in single treatments, did result in pulmonary vasodilation in rats without the occurrence of systemic hypotension.[33] The lack of systemic hypotension observed in that study is likely to be due to the smaller increase in SVC (~47%) in combination with the larger increase in cardiac output (~60%) that was observed in the rats[33] compared with the larger increase in SVC (55±14%) and the smaller increase in cardiac output (23±9%) in the present study. This difference in increase of cardiac output was potentially due to the decreased cardiac output at baseline in the rats with pulmonary hypertension, which was normalized following

the reduction in afterload of the right ventricle. Alternatively, the prolonged duration of the treatment[33] may have resulted in recruitment of compensatory long-term blood pressure regulation mechanisms, i.e., activation of the renin-angiotensin-aldosterone system, that resulted in peripheral vasoconstriction (and hence limited the increase in SVC), thereby contributing to restoration of systemic pressure.

Multiple explanations could be forwarded for the different results of the combination treatment between the systemic and pulmonary vasculature in the present study. First, pulmonary vasomotor tone is lower compared with systemic vasomotor tone, whereas the vasodilator effect of PDE5 inhibition on the pulmonary is larger than that on the systemic vasculature. Thus maximal vasodilation may have been reached by PDE5 inhibition in the pulmonary circulation, whereas vasodilator reserve was still present in the systemic vasculature. However, even when tone was artificially increased in isolated pulmonary small arteries, a vasoconstrictor influence of ET either in the absence or presence of PDE5 inhibition was not uncovered, suggesting that the low pulmonary vascular tone is not a critical factor in explaining the different interaction between PDE5 inhibition and ET receptor blockade in the systemic vs. the pulmonary vascular bed. Second, since the systemic vasculature is comprised of different regional vascular beds in parallel, it is possible that vasodilation in response to ET_A/ET_B receptor blockade occurred in a different regional vascular bed as vasodilation in response to PDE5 inhibition. This also precludes analysis of isolated systemic small arteries, because it is unclear which vascular bed should be chosen. Third, different receptors are involved in ET-induced vasoconstriction in the systemic and pulmonary vascular beds. Thus the ET_B receptor is the main receptor involved in ET-induced vasoconstriction in the healthy porcine pulmonary vasculature, whereas the ET_A receptor is the predominant vasoconstrictor receptor in the systemic vasculature.[15]

We have previously shown that endogenous NO acts to suppress the pulmonary vasoconstrictor influence of endogenous ET[11], particularly during exercise, which together with our results in isolated pulmonary small arteries points toward a direct interaction between the NO-cGMP system and the ET system. NO has been shown to directly modulate binding of ET to the ET_A receptor.[34] In addition to such direct effect of NO that would not be enhanced by PDE5 inhibition, experiments in porcine aorta, rat hearts, and cultured pulmonary arterial endothelial cells show that an increase in cGMP induced by either NO or the nonhydrolyzable cGMP analog 8Br-cGMP suppresses ET production and release.[35-37] Moreover, plasma ET levels were lower in rats with pulmonary hypertension treated with the PDE5 inhibitor sildenafil.[38]

Although plasma ET levels do not always adequately reflect tissue ET levels, these data are consistent with a reduced ET release following PDE5 inhibition. In contrast, our experiments in isolated vessels, showing that elevation of cGMP levels either through administration of the cGMP analog 8Br-cGMP or through PDE5 inhibition attenuated the vasoconstrictor response to Big ET as well as to ET, indicating that the interaction between

the NO-cGMP system and the ET system in the porcine pulmonary vasculature occurs mainly at the level of the ET receptor, not ET production. The observation that 8Br-cAMP did not affect the response to either ET or Big ET suggests that this interaction in the pulmonary vasculature is specific for the NO-cGMP system.

Conclusions and implications

The present study shows that the interactions between the NO-cGMP system and the ET system in the pulmonary vasculature occurred at the level of ET receptor(s) and prevented an additive vasodilator effect of PDE5 inhibition and ET_A/ET_B receptor blockade in the healthy pulmonary vasculature. This inhibition of the ET_A/ET_B receptor-mediated vasoconstrictor influence by NO-cGMP signaling is already present at rest and is not further modulated during exercise. Future studies should investigate whether in pulmonary disease states the observed increased vasoconstrictor influences of PDE5[19] and ET[12] will unmask an additive vasodilator effect of combined PDE5 inhibition and ET receptor blockade.

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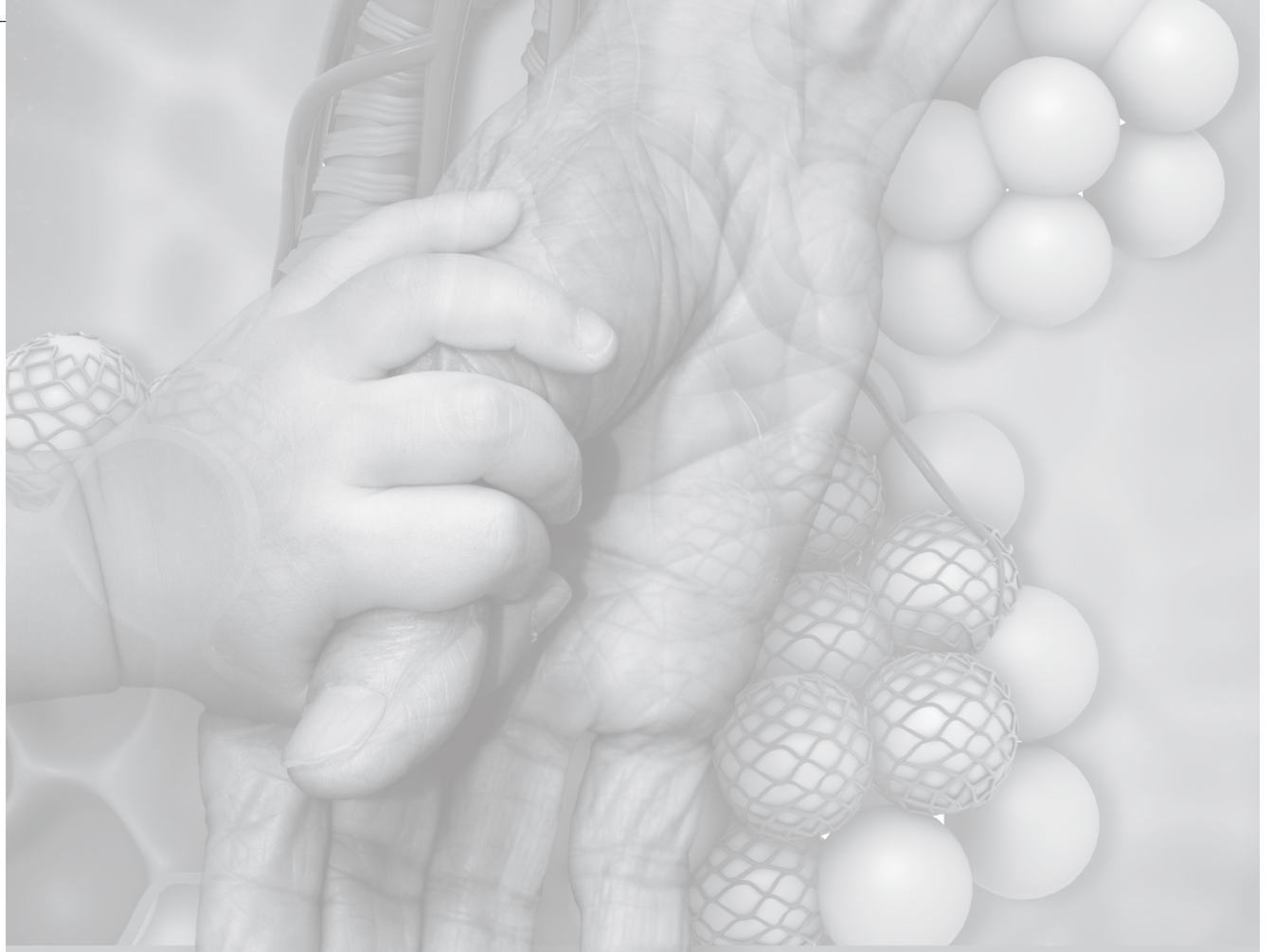

Chapter 9

Summary and general discussion

1. SUMMARY

Annually, over 15 million babies are born prematurely (<37 weeks gestational age), accounting for more than 1 in 10 births worldwide.[1,2] In the Netherlands, about 7% of all live births were preterm (in 2015).[3] Because these babies are born before they are physically ready to face the world, they often are at risk for developing complications that result from anatomic and functional immaturity.[4,5] Because the lungs develop late in the embryo and are even far from mature at birth,[6,7] they appear to be most susceptible to damage in premature babies. Disruption of normal pulmonary vascular development plays a pivotal role in the pathogenesis of several neonatal pulmonary vascular diseases (PVD), including bronchopulmonary dysplasia (BPD) and pulmonary hypertension (PH). These chronic lung diseases are one of the most common adverse outcomes in these preterm neonates.[8]

Neonatal PVD complicates cardiopulmonary adaptations after birth, is associated with exercise intolerance later in life and predisposes to long-term cardiopulmonary disease.[9] It develops as a result of lung injury caused by several injurious stimuli. Oxygen tension is a key player in the pathogenesis of neonatal PVD.[10] Both antenatal and postnatal exposure to either hypoxia and/or hyperoxia contributes to disruption of the normal development of the pulmonary vascular bed, resulting in endothelial dysfunction, pulmonary vascular remodeling, and ultimately right ventricular dysfunction (**Chapter 2**).

PVD represents an increasing clinical burden, both in the neonatal period and later in life. Despite decades of research, the exact pathophysiologic mechanisms underlying PVD as well as to what extent PVD contributes to a decreased lung function, decreased exercise tolerance, increased vulnerability to cardiopulmonary disease throughout life, and cardiovascular mortality later in life remain incompletely understood. There is growing evidence that PVD is associated with multiple disruptions in the nitric oxide (NO)-cGMP-phosphodiesterase 5 (PDE5) signalling pathway.[11-17] However, no studies have been performed to investigate the long-term consequences of disruptions in this signalling cascade, and (large) animal models of neonatal PVD performing long term follow-up are lacking. Therefore, we developed a swine model for neonatal PVD showing clinical features resembling those found in patients in terms of pulmonary hemodynamics and abnormalities in the structure of the right ventricle and pulmonary vascular remodeling (**Chapter 3-4**). In this model for neonatal PVD we observed structural and functional changes in the pulmonary microvasculature that are still present several weeks after re-exposure to normoxia. Evidence from the literature as well as our study suggests that there is an impaired endothelium-dependent vasodilatation in piglets with hypoxia-induced PH in the first three weeks after re-exposure to normoxia that is due to a reduced responsiveness to NO, probably caused by altered sensitivity and/or activity of soluble guanylyl cyclase (sGC), resulting is an impaired cGMP production (**Chapter 5**). In our neonatal swine model for PVD we also showed more severe and more persistent PVD, limited exercise capacity, and more pronounced right ventricular remodeling during

follow-up in normoxia in male compared to female swine, consistent with neonatal clinical practice (**Chapter 4**).

Just as in infancy, the severity and prognosis of PVD have been shown to be worse in male adults, while on the other hand the incidence of PH is higher in females.[18,19] Consistent with these sex-dependent clinical observations, we demonstrated that endothelial NO synthase (eNOS) inhibition reduced bradykinin-induced vasorelaxation to a greater extent in male as compared to female pulmonary small arteries. Furthermore, we found an increased vasodilator effect of PDE5 inhibition during exercise in male as compared to female swine, reflecting the sex-specific heterogeneity in treatment response (**Chapter 6**).

Prolonged endothelial dysfunction and persistent structural abnormalities in the pulmonary vasculature, as shown in this thesis, likely contribute to the exercise intolerance and increased vulnerability to cardiopulmonary disease throughout life. Thus, exposure of the developing lung to injurious stimuli in the perinatal period, may also contribute to the development of PVD later in life, trough so-called "fetal or perinatal programming". It is well known that endothelial dysfunction is not only a key factor in vascular development, but also in maintenance of vascular structure and function throughout life, and thus plays a crucial role in the pathogenesis of adult PVD.[20-22] While endothelial function of the systemic and coronary circulation has been extensively investigated, studies into the endothelial function of the pulmonary vasculature have received less attention. Therefore, in the second part of this thesis we present the results of studies concerning pulmonary endothelial function and vascular control, with a particular focus on the NO-cGMP-PDE5 pathway (**Chapter 6-8**).

Chapter 8 showed that PDE5 inhibition and the associated increase in cGMP produce pulmonary vasodilation that is mediated in part through inhibition of the endothelin (ET) pathway, thereby precluding an additional vasodilator effect of ET_A/ET_B receptor blockade in the presence of PDE5 inhibition. After administration of hemoglobin-based oxygen carrier (HBOC)-201, mimicking endothelial dysfunction, ET_A/ET_B blockade prevented, and eNOS inhibition reduced the pulmonary vasoconstriction (**Chapter 7**).

Together, the research described in **this thesis** shows that we successfully created and characterized a large animal model of neonatal PVD. In this model, the abnormalities of the pulmonary vasculature and right ventricle persists despite normalization of pulmonary artery pressure (PAP), suggesting that the effects of disruption of normal lung development early in life could have consequences in adulthood, as recognized in the developmental origin of health and disease (DOHaD) concept.[23] Furthermore, we found sex differences in response to early postnatal injury, but also found sex differences in pulmonary vasomotor control later in life. These significant sex differences in the regulation of pulmonary vascular tone by the NO-cGMP-PDE5 pathway may contribute to understanding sex differences in incidence, treatment response and prognosis of PVD. **This thesis** contributes to the increasing insight in the mechanisms underlying PVD as well as the impact on cardiovascular health later

in life, and may lead to novel therapeutic and medical management strategies, including early interventions in the neonatal period, to decrease both the short- and long-term health problems in these patients.

2. GENERAL DISCUSSION

2.1 Peri- and neonatal (mal)adaptation

There is growing evidence that disruption of normal pulmonary vascular development in the perinatal period contributes to the development of PVD in adulthood. This so-called "fetal or perinatal programming", also known as the DOHaD concept,[23] has gained a great deal of attention in recent years. In view of the growing cohort of adult survivors of prematurity and/or neonatal PVD, more research into the long-term consequence of perinatal pulmonary vascular events is imperative. Therefore, the main focus of the first section of this thesis was to study the short- and long-term effects of injurious stimuli in the peri- and neonatal period to the pulmonary vasculature.

2.1.1 Methodological considerations

To identify basic pathological mechanisms underlying neonatal PVD and to examine long-term sequelae of damage to the developing neonatal lung, we developed a neonatal swine model. Already, a number of animal models for neonatal PVD have been developed, relying on several injurious stimuli such as mechanical ventilation, oxygen toxicity and infection and sterile inflammation.[24-30] It is most commonly modeled in mice and rats, however these models are not without substantial drawbacks. Like patients with neonatal PVD, full-term mice and rats are born in the saccular stage of lung development. However, these newborn rodent pups are competent for proper gas exchange, which is in marked contrast to preterm human neonates. Furthermore, the small size leads to concerns in regard to parenteral administration of substances and difficulty of intubation and mechanical ventilation.[31] Other experimental animal models for neonatal PVD have proven to be useful. Both preterm rabbits and lambs appear to be translationally relevant in modeling neonatal PVD, and their larger size makes instrumentation and mechanical ventilation possible.[31] Piglets represent an alternative to other large animal models. Swine lungs share many anatomical, histological, biochemical, and physiological features with human lungs [32] and the relevance of the developing pulmonary circulation of neonatal piglets to human infants has been established already in the early '80s.[33,34] Although alveolar multiplication occurs faster in piglets (2-4 weeks compared to 3 years in human infants), the morphological development of pulmonary architecture in swine is comparable with that in humans.[32]

The use of oxygen as an injurious stimulus is likely to remain a key driver of pathology in experimental animal models of neonatal PVD. Both hyperoxia-based models,[35-38] as well

as models with hypoxia-induced PVD have been established.[39-42] In the clinical setting, however, premature infants experience episodes of intermittent hypoxia, normoxia (relative hyperoxia), and hyperoxia (due to high levels of supplemental oxygen). Therefore, we developed a neonatal swine model based on chronic exposure to a hypoxic environment, interspersed with daily re-exposure to normoxia for a short period and followed by hyperoxia during surgery and re-exposure to normoxia during follow-up. These alternations in oxygen tension in the neonatal period result in activation of various signal transduction pathways and transcription factors, especially hypoxia-inducible factors (HIF). Under hypoxic conditions, both HIF-1α and HIF-2α accumulate instantaneously, while HIF-1α, but not HIF-2α, protein disappears when hypoxia is sustained (>12h).[43] Re-oxygenation results in rapid degradation of both HIF-1α and HIF-2α.[44] 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.[45] Thus, in premature neonates as well as in this swine model, it is likely that both HIF-1α and HIF-2α contribute to the pulmonary vascular remodeling and PH.

To our knowledge, this new swine model for neonatal PVD is the first to comprehensively investigate long-term sequelae of damage to the developing lung. It confirms clinical observations that PH induced by chronic hypoxia is transient, although structural and functional changes in the right ventricle and the lung vasculature were still present after follow-up. Our model is an excellent model to examine the effect of exercise training and/ or pharmacotherapy on long-term outcome, as well as the molecular mechanisms underlying potential beneficial effects.

2.1.2 NO pathway

The NO-cGMP signalling pathway (figure 1) is important for the adjustments in the pulmonary vasculature that accompany the transition from pre- to postnatal life following birth. There is increasing evidence that alterations in the NO-cGMP signalling pathway play an important role in the pathogenesis of neonatal PVD.[11-13,41]. Perinatal hypoxia impacts the functionality of different parts of the NO-cGMP pathway.

First, hypoxia-induced PH is accompanied by impaired endothelium-dependent vasodilation in the pulmonary vasculature. Evidence from the literature as well as **Chapter 5** of this thesis suggests that the impaired endothelium-dependent pulmonary vasodilation is due to 1) a decreased eNOS activity and 2) a reduced responsiveness to NO.[11-15,17] Both Fike et al. and Berkenbosch et al. found an impaired production of NO through a reduced eNOS protein expression and/or activity [11,13] or dysfunction of eNOS [12,41,46]. In accordance with these findings, we showed a reduced vasodilator response to bradykinin in isolated pulmonary small arteries from hypoxia-exposed piglets that was abrogated after eNOS inhibition, also suggesting a reduced eNOS activity or eNOS dysfunction, resulting in an impaired NO production. In the pulmonary vascular endothelium, endogenous NO is produced by

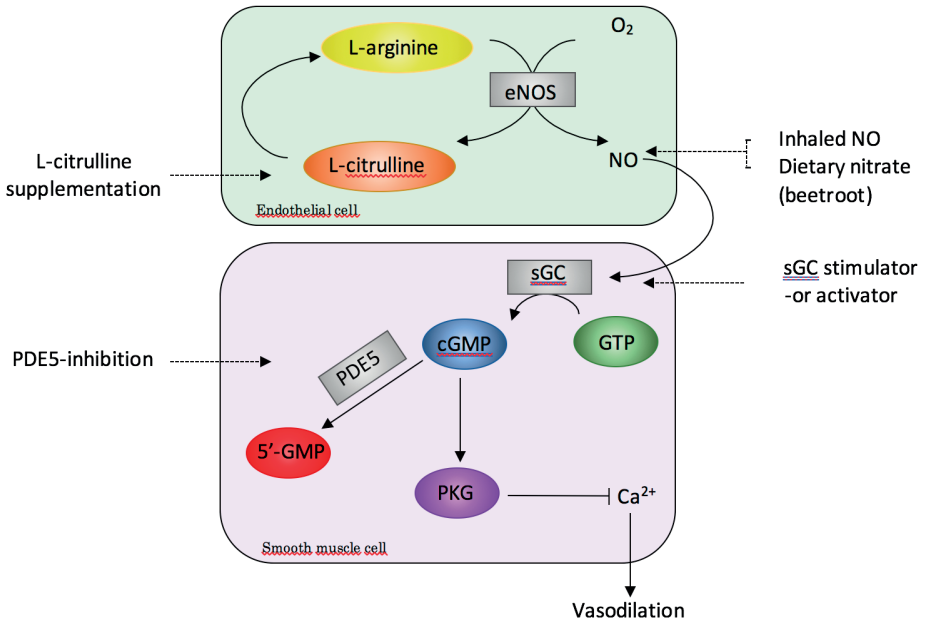

Figure 1. Schematic representation of NO-cGMP-PDE5 signalling pathway, highlighting several therapeutic strategies.

eNOS from the metabolism of L-arginine to L-citrulline. In turn, the citrulline produced is recycled to arginine, providing a recycling pathway for the conversion of L-citrulline to NO via L-arginine.[47] In addition to the evidence showing an impaired NO-production in hypoxia-induced PH, it has been shown that L-citrulline supplementation attenuates PH in oxygen-induced lung injury, both in rodents and swine.[48,49] Likewise, a recent case report showed that oral L-citrulline supplementation ameliorated chronic PH and reduced oxygen requirement in a premature infant born at 25 weeks of gestation with severe BPD.[50]

Besides the impaired production of NO, there is also evidence for a reduced responsiveness to NO. In agreement with findings of other studies,[11,46] chronic postnatal hypoxia was associated with a diminished vasodilator responsiveness to the exogenous NO-donor SNP in vivo in our study (**Chapter 5**). This reduced responsiveness of the pulmonary vasculature to NO could explain that no important clinical benefit was seen in preterm infant with respiratory failure treated with inhaled NO.[51-53]

Thus, secondly, the apparent reduction in responsiveness to NO suggests that there are disruptions in the NO-cGMP pathway more downstream to eNOS/NO production. sGC is the main enzyme activated by NO and catalyses the conversion of GTP into the second messenger cGMP in pulmonary vascular smooth muscle cells, causing vasorelaxation. A major prerequisite for the NO-induced activation is the presence of the reduced Fe^{2+} heme moiety. Oxidative stress, as for instance in hypoxia, causes removal or oxidation to Fe^{3+}, lead-

ing to the formation of an NO-insensitive form of the enzyme.[54,55] This implies that sGC also may be a fundamental mechanism that influences vascular structure and tone. Several in vivo and in vitro studies investigated the effect of sGC activators and stimulators in acute and chronic hypoxia. In acute hypoxia, it has been shown that sGC stimulation reversed the pulmonary vasoconstrictor response in pigs[56] and attenuate pulmonary hypoxic vasoconstriction in isolated perfused mouse lung.[57] In chronic hypoxia, sGC activation reduced PH, right ventricular hypertrophy and structural remodelling of the pulmonary vasculature in mice[57] and inhibit or reverse the development of chronic hypoxic PH in mice[58] and rats.[59,60] **Chapter 5** showed that the effect of PDE5 inhibition tended to be smaller at rest in the pulmonary vasculature of hypoxia-exposed as compared to normoxia-exposed piglets, suggesting an impaired cGMP production after chronic exposure to hypoxia. This implies that the reduced responsiveness to NO in vivo most likely is caused by altered sensitivity and/or activity of sGC. Interestingly, the effect of PDE5 inhibition during exercise was significantly larger in the pulmonary vasculature of hypoxia-exposed piglets. We hypothesize that the significant increase in PAP during incremental exercise causes secretion of natriuretic peptides by cardiomyocytes in response to cardiac stretch,[61] and thus particulate guanylyl cyclase activation, resulting in a normalisation of cGMP production. This hypothesis is supported by the highly correlated vasodilator effect of PDE5 and PAP. **Chapter 5** adds important information by showing that alterations in the NO-cGMP signalling pathway are still present several weeks after re-exposure to normoxia.

2.1.3 Sex differences

Throughout their lifespan, males generally have worse outcomes in PVD as compared to females. This gender disparity is particularly evident in preterm infants and is most marked in the respiratory morbidity of these preterm infants.[62,63] The prevalence of BPD is higher in male as compared to female premature infants,[64] and being male is also associated with more severe disease and thus a higher risk for the development neonatal PVD.[65] A meta-analysis by Liptzin et al. including data from over 500,000 preterm newborn infants highlighted a sex ratio ranging from 1.22 ($p < 0.05$) in favor of males for BPD compared to females.[66] The etiology of this disparity is mostly undetermined, but likely involves structural, genetic, physiologic and hormonal differences. Still, little is known about sex differences in the long-term outcome of neonatal PVD.

Consistent with clinical observations, significant sex differences were present in **Chapter 4** of this thesis. Male hypoxia-exposed piglets demonstrated more severe and more persistent disease that female hypoxia-exposed piglets, as evidenced by a higher PAP, that persisted for a longer period, and more pronounced right ventricular dilatation. Sartori et al. showed a greater altitude-induced increase in systolic PAP in young adults who had had transient PH.[44] Re-analyses of these data in **Chapter 4**, showed that males with perinatal transient PH displayed significantly higher systolic PAP at high altitude than both their controls and

females with perinatal transient PH, while baseline systolic PAP levels were not significantly different between all groups. These data suggest that a transient perinatal insult to the pulmonary circulation results in a higher pulmonary vasoreactivity in males, but not in females. Future studies are required to investigate the mechanisms underlying these sex-related differences in (neonatal) PVD.

2.1.4 DOHaD

The DOHaD hypothesis, formerly known as the "Barker" or "Fetal Origins of Adult Disease" hypothesis, postulates that exposure to certain environmental influences during crucial periods of development and growth may have significant consequences for an individual's short- and long-term health.[23,67] This concept has gained a great deal of attention in recent years, especially in pediatrics because of the dramatically increased survival of premature babies. Since approximately 10% of births are preterm, a growing cohort of prematurely born survivors reaches adolescence.[1,68] While the majority of research in this field has focused on the developmental origins of metabolic disease, it is increasingly recognized that disruption of normal pulmonary vascular development in the perinatal period contributes to the development of (pulmonary) vascular disease in adulthood. Already in the late 1990's, Sartori et al. showed a greater altitude-induced increase in systolic PAP in young adults who had had transient PH.[44] It has also been shown that PAP is elevated in offspring of mothers with preeclampsia, demonstrating that placental hypoxia causes pulmonary vascular dysfunction.[69] Lewandowski et al. showed with cardiac magnetic resonance (CMR) imaging that preterm birth is associated with global myocardial structural and functional differences even in adult life, with potentially clinically significant impairments in right ventricular systolic function.[70,71] In **Chapter 4** we demonstrated that exposure to chronic hypoxia in the neonatal period leads to a loss of pulmonary vasodilator capacity and a limited exercise capacity shortly after re-exposure to normoxia and, even more important, to structural and functional changes in the right ventricle and the lung vasculature that were still present after long-term follow-up. These data are consistent with the DOHaD hypothesis, 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, like right ventricular failure, thereby contributing disproportionately to the burden of adult cardiovascular disease in the future.

2.2 Endothelial function in the adolescent pulmonary vasculature

Prolonged endothelial dysfunction and persistent structural abnormalities in the pulmonary vasculature, as shown in **Chapter 4**, likely contribute to the exercise intolerance and increased vulnerability to cardiopulmonary disease throughout life (DOHaD hypothesis). It is well known that endothelial dysfunction not only is a key factor in vascular development, but also maintenance of vascular structure and function throughout life, and thus plays a

crucial role in the pathogenesis of adult PVD.[20-22] While the endothelial function of the systemic and coronary circulation has been extensively investigated, the endothelial function of the pulmonary vasculature has received less attention. Therefore, the main focus of the second section of this thesis was to study the pulmonary endothelial function and vascular control in adolescence, with a particular focus on the NO-cGMP-PDE5 pathway.

2.2.1 NO-pathway

PH is associated with alterations in pulmonary vascular function and structure, resulting in an increased pulmonary vascular resistance (PVR) and thereby right ventricular afterload. The regulation of PVR occurs by changing the diameter of blood vessels by both passive (structural) and active (smooth muscle tone) influences. Pulmonary vascular tone is the result of a complex interplay between vasodilator and vasoconstrictor influences.[72] The vascular endothelium releases a variety of these vasoactive substances, including NO, prostanoids and ET, which play an important role in vasomotor control. Endothelial dysfunction, therefore, plays a crucial role in the pathogenesis of adult PVD.

HBOC-201 can disrupt hemodynamic homeostasis, mimicking some aspects of endothelial dysfunction. HBOC-201-induced vasoconstriction has been ascribed to scavenging of NO.[73-76] Besides the disruption of the NO-mediated cascade, we found that the pressor effects of HBOC-201 results from an upregulation of ET production (**Chapter 7**). In accordance with these findings, several studies indicate that NO limits the influence of ET in the pulmonary vasculature. In swine pulmonary vasculature, combined ET_A and ET_B receptor blockade with tezosentan resulted in a larger decrease in pulmonary vascular resistance in the presence of NO synthase inhibition, as compared to the effect of combined ET_A and ET_B receptor blockade under control conditions.[77] Wiley et al. showed a direct modulatory effect of NO on the ET receptor binding,[78] whereas Kelly et al. showed that NO decreases ET secretion through the activation of sGC in pulmonary arterial endothelial cells.[79] Consistent with the findings of Kelly et al., we demonstrated that PDE5 inhibition and the associated increase in cGMP produce pulmonary vasodilation that is mediated in part through inhibition of ET, thereby precluding an additive vasodilator effect of combined blockade of these two vasoconstrictor pathways with PDE5 inhibition and ET receptor blockade.

Thus, NO induces pulmonary vasodilation not only through a direct effect on vascular smooth muscle cell via production of cGMP but also indirectly through inhibition of ET. Interactions between mechanisms involved in the regulation of pulmonary vascular tone must be considered in the development of new treatment strategies.

2.2.2 Sex differences

Just as there are sex difference in the prevalence and severity of neonatal PVD, there are also sex differences shown in PVD in adulthood. Although the incidence of PH is higher in females, the severity and prognosis of PVD, like in neonates, have been shown to be

worse in male subjects.[18,19] Until now, studies concerning sex differences in PH have mainly focused on the role of sex hormones, particularly female reproductive hormones. The effects of estrogens on the pulmonary vasculature are mediated trough both non-genomic (rapid) and genomic mechanisms. Via non-genomic mechanisms, it enhances the production of nitric oxide by upregulation of eNOS.[80-84] Estrogen receptor-dependent mechanisms, the genomic pathway, increases eNOS mRNA levels and eNOS activity in pulmonary endothelial cells.[82,84,85] Additionally, it is well known that estrogens downregulate gene expression of ET. However, the effects of estrogen on the pulmonary vasculature are complex and remain incompletely understood.[82,84,86]

Our study of sex differences in the regulation of pulmonary vascular tone also showed significant differences between male and female swine, both in vivo and in vitro (**Chapter 6**). NO synthase inhibition reduced bradykinin-induced vasorelaxation to a greater extent in male as compared to female in isolated pulmonary small arteries, which is consistent with observations that PVD is often more severe in men as compared to women. In vivo, however, we found comparable pulmonary vasoconstriction after administration of NO synthase inhibition. Possible explanations for this apparent discrepancy are 1) the absence of eNOS activation by circulating estrogens in vitro,[80-84] 2) the absence of contribution of nNOS in vitro,[87,88] 3) the different signaling pathways in receptor-mediated eNOS activation and shear stress-mediated eNOS activation[89,90] and 4) the presence of an unidentified alternative vasodilator pathway in NO signaling in females, in contrast to solely through cGMP-PKG-PDE5 in males.[91,92]

Furthermore, we demonstrated an increased vasodilator effect of PDE5 inhibition during exercise in male as compared to female swine, which is consistent with recent post-hoc analyses of the PHIRST and SUPER trials showing that PDE5 inhibition leads to a greater improvement of 6 minute walking distance in male as compared to female patients with PH.[93,94] Finally, we found that concomitant NO synthase-inhibition enhanced the vasodilator responses to PDE5 inhibition at rest and during bradykinin-induced vasodilation, but only in females, suggesting that loss of endothelial function may not interfere with (males) or even enhance (females) the pulmonary vasodilator responses to PDE5 in patients with PH. Together, these results reflect the sex-specific heterogeneity in treatment response. Future studies are required to investigate the mechanisms underlying these sex-related differences in pulmonary vascular control mechanisms. A better understanding of the sex differences in pulmonary vascular control may allow for future therapeutic interventions in adult patients with PVD.

3. CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

Several pathophysiologic insults in the prenatal and early postnatal period can disrupt normal pulmonary vascular development both in terms of the impaired alveolarization and dysmorphic vascular growth and, consequently, can lead to a variety of neonatal PVD. Preterm birth in itself already hampers the development of the lung, not only because preterm babies are born in a critical stage of lung development (saccular or alveolar stage), but also by exposing their immature lungs to several injurious stimuli, including hypoxia and/or hyperoxia, mechanical ventilation, infection, inflammation and oxidative stress.

According to the DOHaD concept,[23] disruption of normal pulmonary vascular development in the perinatal period not only contributes to morbidity and mortality in the neonatal period, but has also been shown to significantly increase the risk for a variety of health problems later in life. PVD can lead to endothelial dysfunction, vascular remodeling, and ultimately to cardiac dysfunction (right ventricular hypertrophy and failure). As improved neonatal care has dramatically improved the survival of premature babies, we may face a new epidemic of cardiovascular disease based on an as yet underestimated burden of PVD that originates from the time of birth.

Until now, most studies concerning long-term health outcomes of (extremely) premature infants have focused on respiratory outcomes. Indeed, it is well known that pulmonary function in childhood and adolescence is impaired in these patients, and this is even more pronounced in survivors of neonatal PVD like BPD.[95-100]

Less is known about cardiovascular function in survivors of neonatal PVD. The swine model of neonatal PVD we developed, shows clinical features resembling those found in patients with neonatal PVD (including PH), in terms of pulmonary hemodynamics, abnormalities in the structure of the right ventricle and disruptions in normal lung development (vascular remodeling). Consistent with clinical data, male swine develop more severe, and more persistent PVD, have a limited exercise capacity and exhibit more pronounced right ventricular remodeling during follow-up in normoxia. Therefore, our model is an excellent model to examine long-term sequelae of a perinatal hypoxic insult, the effect of therapy on long-term outcome and the course of the disease. It enables us to understand the mechanisms and potentially lead to new therapeutic strategies.

3.1 Long term sequelae of a perinatal hypoxic insult

It has been well-documented that exercise capacity is limited in long-term survivors of prematurity and/or neonatal PVD.[101-105] Exercise capacity is a resultant of pulmonary function and cardiovascular performance.[106,107] Until now, most studies concerning long-term health outcomes of (extremely) premature infants have focused on respiratory outcomes. Indeed, it is well known that pulmonary function in childhood and adolescence is impaired in these

patients. However, there is increasing evidence that the limited exercise capacity is not only due to the impaired pulmonary function, but is also due to cardiovascular dysfunction. Use of CMR imaging revealed distinct differences in mass, geometry and function of both the left and right ventricle.[70,71] Differences in right ventricular mass and function were proportionally greater than in the left ventricle. This observation corresponds well with our findings that, despite normalization of PAP, structural and functional changes in the right ventricle and the lung vasculature were still present after long-term follow-up. These alterations in cardiac function and structure may increase the risk for cardiovascular events later in life, thereby contributing disproportionately to the burden of adult cardiovascular disease in the future.

Nevertheless, the studies mentioned above were all performed under resting conditions. By subjecting the cardiopulmonary system to stress with exercise testing, subtle dynamic abnormalities that are not apparent during conventional static tests may be revealed. This may lead to early recognition of altered cardiac remodelling and heart failure, which is important for optimal management of these patients to improve long-term outcomes or even prevent future disease. Therefore, we are currently investigating cardiac function and structure during exercise in both controls and prematurely born adolescents, with and without neonatal PVD, by using CMR imaging.

3.2 Effect of therapy on long-term outcome and potential new therapeutic strategies

Several interventions in the neonatal period, including oral L-citrulline,[41,48] thromboxane inhibition,[108] angiotensin II type 1 receptor blockade[39] and ET-A receptor antagonists,[42] have been shown to ameliorate PH and/or pulmonary vascular remodeling in a similar model. However, the long-term outcome of these interventions remains to be established. Our porcine model is an excellent model to examine therapy on long-term outcome, as well as the molecular mechanisms underlying potential beneficial effects.

Evidence from several animal studies as well as evidence from our neonatal swine model suggests that the impaired endothelium-dependent vasodilatation found in PVD is due to a reduced responsiveness to NO, probably caused by altered sensitivity and/or activity of sGC, resulting is an impaired cGMP production. sGC stimulation and/or activation reduced or even completely reversed the pulmonary hypoxic pulmonary vasoconstriction, as well as reduce the structural remodelling of the pulmonary vasculature and right ventricular hypertrophy.[56,57,58,59,60] **Chapter 5** of this thesis adds important information to these previous studies by showing that alterations in the NO-pathway in a neonatal porcine model are still present several weeks after re-exposure to normoxia. Together, these data suggest that sGC stimulators/activators could be of benefit as a novel treatment strategy to stop or even reverse neonatal PVD, especially since the use of inhaled NO for preterm infants with respiratory failure is currently under debate.[51,109-111]

Besides pharmacological treatment, it would be very interesting to 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. Training is associated with improved pulmonary perfusion,[36] blood gas exchange[112] and right ventricular function.[113-115] Furthermore, exercise training has been shown to reduce smooth muscle cell proliferation.[116] Altogether, these beneficial effects improve exercise capacity and may reduce PAP, thereby improving quality of life.[116,117] Our swine model of neonatal PVD is the first that allows exercise-testing, but until now only the cardiovascular responses to acute exercise were assessed. Future studies should investigate the effect of exercise training on long-term outcome.

Beetroot juice provides another novel therapeutic target in PVD (figure 1). It has gained the attention of scientists because of its beneficial effects on cardiovascular health at both the macro-circulatory and the microcirculatory levels,[118-123] owing to the nitrate present in this food. Nitrate is reduced to nitrite in the oral cavity by commensal facultative anaerobic bacteria by the action of nitrate reductase enzymes, followed by the further reduction of nitrite to bioactive NO. This so-called nitrate-nitrite-NO pathway represents an important alternative source of NO to the classical L-arginine–NO-synthase pathway and is enhanced in hypoxia/ischaemia. Therefore, it might serve as a backup system to ensure NO bioactivity, particularly in situations when the endogenous NO-synthase dependent pathway is dysfunctional like in PVD.[124-126] We have recently performed some experiments to see if beetroot juice could mitigate PH and consequent right ventricular remodeling. However, the group was too small to detect significant changes, so it will be expanded in the future.

Finally, we aim to investigate if infusion of healthy neonatal endothelial colony forming cells (ECFCs) can be used as a therapy to prevent or reduce the development of neonatal PVD in our neonatal swine model of PVD. ECFCs are circulating bone-marrow-derived cells, which play two major roles in the cardiovascular system; endothelial healing and neoangiogenesis. Decreased numbers of these cells may directly hamper pulmonary vascular development and/or may contribute to the vulnerability of the developing pulmonary vasculature to injurious stimuli. Nowadays, there is increasing evidence that a decrease in number, but more importantly dysfunction of ECFCs in preterm infants may be a crucial step in the development of PVD in preterm infants.[127-129]

3.3 Course of disease

In view of the growing cohort of adult survivors of prematurity and/or neonatal PVD, more research into the long-term consequence of perinatal pulmonary vascular events represents an emerging field. Since this is a relatively new patient population, there is a lack of consensus for the follow-up of high risk (formerly premature) patients. The American Heart Association and American Thoracic Society have made a guideline for diagnosis, evaluation and monitoring of pediatric patients with PH.[130] They recommend monitoring of children with

PH (or other neonatal PVD) in a multidisciplinary setting. It is a future challenge to evolve a follow-up program for (neonatal) PVD that facilitates an optimal transition of the patient from the pediatric to the adult setting and ensure early detection of health problems in these patients, thereby diminishing cardiovascular morbidity and mortality and improving quality of life. Furthermore, development of an exercise training program tailored for prematurely born adolescents, who may be at higher risk for early-onset adult diseases, should be considered. Establishing early, adequate levels of fitness and activity will have beneficial effects on overall health, thereby playing an important role in the prevention of diseases at long term. Thus, it should be a cornerstone in the follow-up of formerly premature adults.

In conclusion, **this thesis** contributes to the rapidly increasing insight in the process that can lead to neonatal PVD and its long-term consequences. The described research can lead to specific therapies that reduces or even reverses structural and functional changes of the pulmonary vasculature and right ventricle, thereby preventing cardiovascular diseases and improving the long-term outcome of prematurely born adults.

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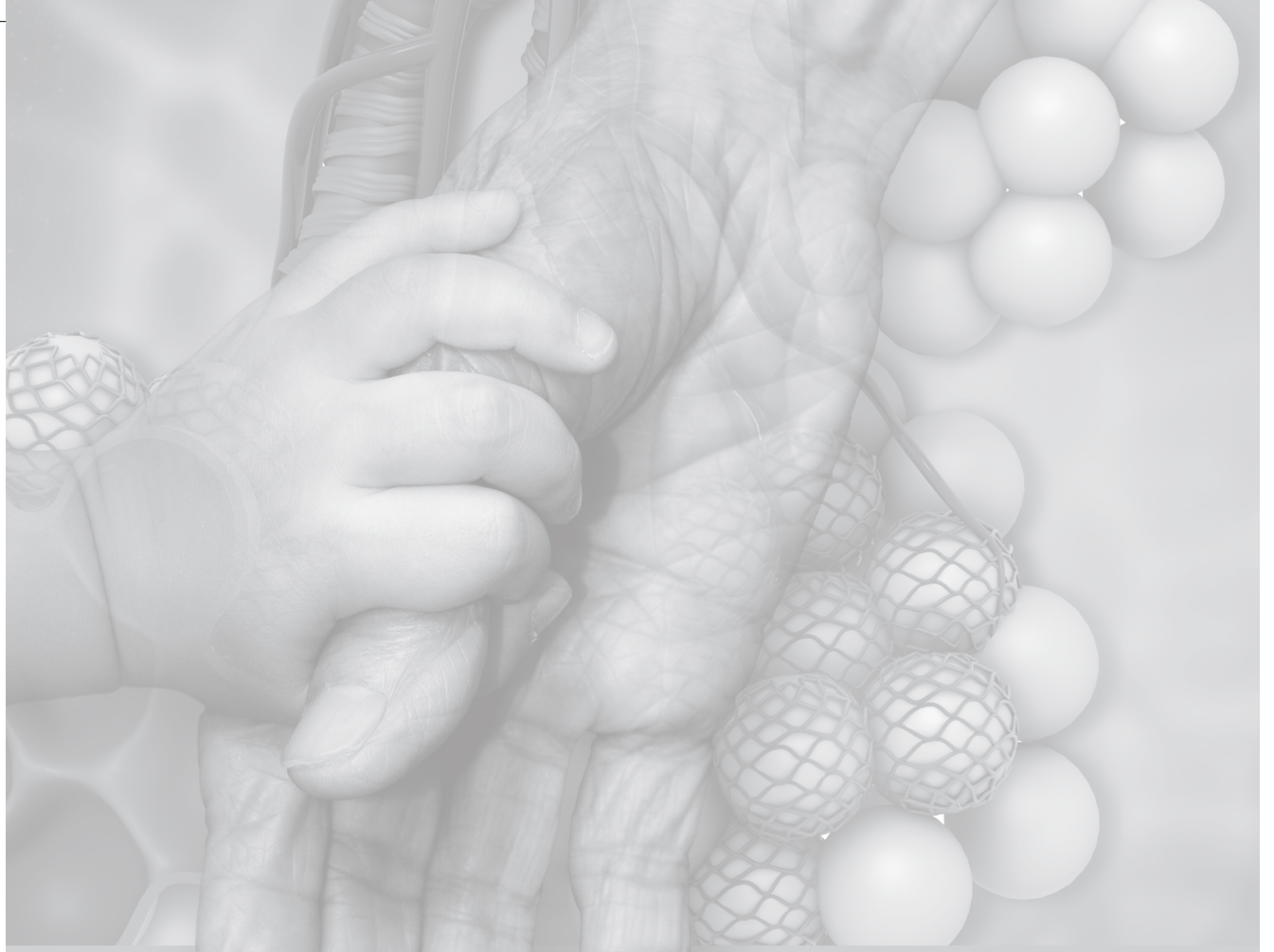

Chapter 10

Nederlandse samenvatting

Jaarlijks worden meer dan 25 miljoen baby's te vroeg (prematuur) geboren, wat neerkomt op meer dan 10% van alle geboortes wereldwijd. In Nederland wordt ongeveer 7% van alle levendgeborenen prematuur geboren (in 2015). Er wordt gesproken over een premature geboorte als de baby voor een zwangerschapsduur van 37 weken geboren wordt. Prematuur geboren kinderen hebben een verhoogd risico op diverse medische problemen, omdat hun organen nog niet volledig ontwikkeld zijn waardoor deze niet goed functioneren. Met name de longen zijn zeer gevoelig voor complicaties, omdat de uitrijping van de longen pas laat in de zwangerschap voltooid wordt. Bij een vroeggeboorte is er, naast een verstoring in de ontwikkeling van de luchtwegen, ook sprake van een verstoring in de ontwikkeling van de longvaten. Deze verstoring speelt een belangrijke rol in het ontstaan van verschillende ziekten van het longvaatstelsel van pasgeborenen, ook wel neonatale pulmonale vasculaire ziekten (PVZ) genoemd. Voorbeelden van neonatale PVZ zijn bronchopulmonale dysplasie, een ziekte waarbij de baby moeite heeft met ademhalen door onderontwikkelde longen, en pulmonale hypertensie (PH, te hoge bloeddruk in de longvaten). Deze chronische longaandoeningen behoren tot de meest voorkomende complicaties bij prematuur geboren kinderen.

Behalve gevolgen voor de gezondheid direct of kort na geboorte, kan vroeggeboorte ook gevolgen hebben voor de gezondheid op de lange termijn. Kinderen en volwassenen die prematuur geboren zijn, kunnen een verminderde inspanningscapaciteit hebben. Daarnaast hebben zij een verhoogd risico op hart- en vaatzieken. De toch al kwetsbare, onrijpe longen van prematuur geboren kinderen worden namelijk na de geboorte blootgesteld aan allerlei schadelijke factoren, die veroorzaakt kunnen worden door de beademing, die nodig is om de premature baby van voldoende zuurstof te voorzien, en infecties. Het zuurstofgehalte in het bloed speelt hierbij een sleutelrol. Zowel hypoxie (te laag zuurstofgehalte in het bloed) als hyperoxie (te hoog zuurstofgehalte in het bloed) leidt tot verstoringen in de ontwikkeling van de longvaten. Dit leidt tot het niet adequaat functioneren van de longvaten en het veranderen van vorm en samenstelling van de longvaten (remodelleren), waardoor de rechterharthelft, die het bloed door de longen moet pompen, uiteindelijk kan gaan falen (**Hoofdstuk 2**).

Door verbeteringen in de prenatale en neonatale zorg zijn de prognose en de kans op overleving van prematuur geboren kinderen de afgelopen decennia aanzienlijk verbeterd. Het aantal kinderen met PVZ daalde echter niet. Dit komt omdat de grens van levensvatbaarheid van premature baby's steeds verder verschuift naar een kortere zwangerschapsduur. Met andere woorden, steeds "jongere" baby's, met dus onrijpere longen, kunnen worden behandeld en overleven, wat leidt tot een groter risico op PVZ. Deze groep prematuur geboren kinderen bereikt hedendaags de volwassen leeftijd en vormt dus een geheel nieuwe, groeiende patiëntenpopulatie. Ondanks uitgebreid wetenschappelijk onderzoek blijft de exacte oorzaak, alsmede de lange termijn gevolgen van PVZ, onduidelijk. Wel zijn er steeds meer aanwijzingen dat PVZ geassocieerd zijn met verstoringen in de nitraat oxide (NO)-

cGMP-phosphodiesterase 5 (PDE5) pad. Echter, (grote) diermodellen met de mogelijkheid tot lange termijn follow-up en dus wetenschappelijk studies naar de lange termijn gevolgen van verstoringen in dit pad, ontbreken. Daarom hebben wij een varkensmodel ontwikkeld voor neonatale PVZ, waarbij gebruik is gemaakt van langdurige blootstelling aan hypoxie. In dit varkensmodel komen de afwijkingen in het longvaatbed en het rechterhart overeen met de afwijkingen die gevonden worden in pasgeborenen met PVZ (**Hoofdstuk 3-4**). We hebben aangetoond dat de structurele en functionele veranderingen in de kleine vaten van het longvaatbed (pulmonale microvasculatuur) ook nog aanwezig waren na een langdurige follow-up in een omgeving met een normaal zuurstofgehalte (normoxie). Zowel onze studie als andere wetenschappelijke onderzoeken hebben aangetoond dat er sprake is van een verminderde capaciteit tot vaatverwijding bij biggen met door hypoxie geïnduceerde PVZ in de eerste 3 weken follow-up, die inderdaad gepaard gaat met verstoringen in het NO-cGMP-PDE5 pad (**Hoofdstuk 5**). Verder hebben we in ons model aangetoond dat PVZ een ernstiger beloop heeft en langduriger aanwezig is in mannelijke biggen in vergelijking met vrouwelijke biggen. Dit blijkt onder andere uit een beperkte inspanningscapaciteit en meer uitgesproken veranderingen in de rechter ventrikel (remodellering) bij mannelijke biggen. Dit komt overeen met de klinische bevindingen van pasgeborenen met PVZ. (**Hoofdstuk 4**).

Tot dusver is er veel onderzoek gedaan naar het functioneren van het systemische en coronaire vaatbed in verschillende vormen van hart-en vaatziekten. Wetenschappelijk onderzoek naar het functioneren van het longvaatbed, daarentegen, is schaars. Daarom hebben we in het tweede gedeelte van dit proefschrift onderzoek gedaan naar de pulmonale vaatfunctie (**Hoofdstuk 6-8**). In **hoofdstuk 7** worden sommige aspecten van het disfunctioneren van de vaten nagebootst middels de toediening van HBOC-201. Hierdoor treedt vaatvernauwing op, die in het longvaatbed niet alleen wordt gemedieerd door het wegvangen van NO, maar ook door opregulatie van de endotheline productie. In de kliniek wordt naast PDE5 remming, ook endotheline receptor blokkade gebruikt voor behandeling van PH. In **hoofdstuk 8** hebben we gekeken naar de interactie tussen deze twee therapieën in het pulmonale vaatbed. Dit onderzoek heeft aangetoond dat pulmonale vaatverwijding door PDE5 remming, en de verhoogde cGMP die daarmee gepaard gaat, deels wordt gemedieerd door remming van het endotheline pad. Endotheline receptor blokkade heeft in gezonde varkens geen additioneel vaatverwijdend effect bij het gebruik van PDE5 remming.

We hebben tot slot onderzocht of vaatverwijdende en vaatvernauwende mechanismen anders zijn in het pulmonale vaatbed van vrouwen dan van mannen. Het is belangrijk om de verschillen tussen mannen en vrouwen te onderzoeken omdat in de kliniek gebleken is dat de ernst en de prognose van PVZ slechter is in mannen dan in vrouwen zowel bij kinderen als bij volwassenen patiënten. De incidentie van PVZ is daarentegen hoger in de vrouwelijke populatie. Inderdaad vonden we dat het longvaatbed van mannelijke varkens anders reageert dan dat van vrouwelijke varkens. Kleine pulmonale bloedvaatjes van mannelijke varkens

maken bijvoorbeeld meer NO als de vaatverwijder bradykinine wordt gegeven. Daarnaast was er een toegenomen vaatverwijdend effect van PDE5 remming in het longvaatbed van mannelijke varkens in vergelijking met vrouwelijke varkens tijdens inspanning op een loopband. Dit weerspiegelt de sterkere respons op behandeling van PH met een PDE5 remmer in mannen dan in vrouwen (**Hoofdstuk 6**).

In dit proefschrift laten we zien dat blootstelling aan schadelijke stimuli vroeg in het leven, in dit geval chronische hypoxie, leidt tot blijvende veranderingen in de structuur van het longvaatbed, die gepaard gaan met het langdurig disfunctioneren van deze vaten. Deze afwijkingen lijken bij te dragen aan de verminderde inspanningscapaciteit op latere leeftijd en vormen mogelijk een verklaring voor het verhoogde risico op hart- en vaatziekten gedurende de rest van het leven. Er kan dus gesteld worden dat blootstelling van de zich ontwikkelende long aan verschillende schadelijke factoren rondom de geboorte bijdraagt aan de ontwikkeling van PVZ op de lange termijn. Deze theorie past in de theorie van de "foetale en/of perinatale programmering". Het is wetenschappelijk bewezen dat het adequaat functioneren van de longvaten een belangrijker rol speelt in de ontwikkeling van de longvaten, maar ook in het behoud van de structuur en functie van longvaten. Disfunctioneren van de longvaten op jonge leeftijd speelt dus een cruciale rol in de pathogenese van PVZ op latere (volwassen) leeftijd.

Samenvattend hebben we met het onderzoek in dit proefschrift succesvol een groot diermodel voor neonatale PVZ ontwikkeld en gekarakteriseerd. In dit varkensmodel zijn er blijvende veranderingen in het longvaatbed en het rechterhart aantoonbaar, ondanks het normaliseren van de bloeddruk in het longvaatbed. Dit suggereert dat verstoringen in de ontwikkeling van de longvaten rondom de geboorte consequenties kunnen hebben op latere leeftijd, zoals gesteld in de "foetale en/of perinatale programmering" hypothese. Tevens hebben we sexe-verschillen gevonden in de ernst en prognose van PVZ, zowel op jonge als op volwassen leeftijd. Deze bevindingen kunnen bijdragen aan het beter begrijpen van de welbekende geslachtsverschillen in incidentie, respons op behandeling en prognose van PVZ. Dit proefschrift draagt in belangrijke mate bij aan het inzichtelijk maken van de onderliggende mechanismen van PVZ, maar ook van de impact op de cardiovasculaire gezondheid later in het leven. Het zou in de toekomst kunnen leiden tot nieuwe behandelopties, zoals vroege interventies in de neonatale periode, om zowel de korte als lange termijn gevolgen in deze groeiende patiënt populatie terug te dringen.

Appendix

PhD PORTFOLIO

Name PhD candidate	Daphne de Wijs-Meijler
ErasmusMC department	Neonatology, Experimental Cardiology
PhD period	2012-2019
Promotors	Prof. Dr. I.K.M. Reiss
	Prof. Dr. D.J. Duncker
	Prof. Dr. D. Merkus

1. PhD training	**Year**	**ECTS**
General academic skills		
Laboratory Animal Science (LUMC)	2012	3
Radiation Safety 5A	2012	1.5
MRI safety course	2014	0.1
Biostatistical Methods I (NIHES)	2014	5.7
In-depth courses		
NHS course "Cardiac function and adaptation"	2014	2
Coeur courses	2013-2014	4.5
Presentations		
Coeur research seminar- RV interaction	2013	0.8
Chiesi 5th Dutch Neonatal Fellow Meeting	2014-2015	2.2
Coeur PhD Day	2014-2015	1.6
NVF Papendal Symposium, Rotterdam	2014	1.1
Thalys Meeting, Amsterdam	2015	1.1
International Conferences		
Joint Symposium ECCPS/PVRI (Poster)	2014	1.9
FCVB Florence (Poster)	2016	1.3
Seminars and workshops		
Coeur PhD Day	2013	0.4
Coeur seminars	2013	0.8
Erasmus Lectures	2013-2016	0.4
Sophia Research Days (Poster)	2014-2015	1.3

2. Teaching activities	**Year**	**ECTS**
Lecturing		
Student education "Blood gas analysis"	2014-2016	0.2
Student education "Neonatal PH"	2014-2016	0.2
Supervising Bachelor theses		
Higher laboratory education (*Patty Kok, Ruben van Drie, Mitchell Nuijen, Geraldine de Bruijne, Metin Sahin)*	2012-2017	5
Supervising Master theses		
Medical students *(Herbert Kroon)*	2014	1

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5. **De Wijs-Meijler DP**, Stam K, van Duin RW, et al. Surgical Placement of Catheters for Long-term Cardiovascular Exercise Testing in Swine. J Vis Exp. 2016(108):e53772.

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13. **de Wijs-Meijler DPM**, van Duin RWB, Duncker DJ, et al. Structural and functional changes of the pulmonary vasculature after hypoxia exposure in the neonatal period: a new swine model of pulmonary vascular disease. Am J Physiol Heart Circ Physiol. 2018;314(3):H603-H615.

CURRICULUM VITAE

Daphne Petronella Maria Meijler was born on May 12, 1986 in Nieuw-Ginneken. She grew up in Bavel and completed secondary school in Breda at the Mencia de Mendoza Lyceum in 2004. Afterwards, she started her medical training at the Erasmus University in Rotterdam. During her scientific internship, she performed experiments on effects of nutritional supplementation on growth in the neonatal period in term lambs at Ngapouri Research Farm Laboratory, Liggins Institute, Auckland University, New Zealand. Furthermore, as student assistant she worked on a clinical study to feeding strategies and tolerance in preterm infants at the department of Neonatology at the Sophia Children's Hospital, Erasmus Medical Centre, Rotterdam, The Netherlands. She completed her study cum laude in 2011. Starting in 2011, she did a residence in pediatrics at Amphia Hospital in Breda. In 2012, she started her thesis in the department of Neonatology of the Sophia Children's Hospital, in collaboration with the Department of Experimental Cardiology of the Erasmus Medical Centre in Rotterdam (supervisors Prof. dr. I.K.M. Reiss, Prof. dr. D.J. Duncker and Prof. Dr. D. Merkus). Her research was focused on the short- and long-term effects of injury to the developing pulmonary vasculature. In 2018, she worked in juvenile healthcare for a year, after which she started working as a doctor in child rehabilitation.

DANKWOORD

Hoe vaak heb ik niet naar dit moment uitgekeken? Eindelijk is het dan zover. De laatste bladzijden van mijn proefschrift zijn een feit. Een verslag van een kleine vijf jaar onderzoek naar verstoringen in de ontwikkeling van de longvaten en de impact op de cardiovasculaire gezondheid later in het leven. De afgelopen twee jaar waren de bekende zware laatste loodjes, met de terugkeer in de kliniek, het stichten van een gezin en het tegelijkertijd afronden van de laatste zaken. Maar wanneer je dan eindelijk je proefschrift neerlegt, lijken de jaren werk bijna niet meer van belang. Wat echter wel van belang is en ik nooit zal vergeten, is de steun die ik al die tijd van verschillende personen heb gekregen. Daarom wil ik graag iedereen bedanken die op wat voor manier dan ook heeft bijgedragen aan de totstandkoming van dit proefschrift: BEDANKT! Een aantal mensen wil ik graag in het bijzonder noemen:

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