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## Antenatal Hypoxia and Pulmonary Vascular Function and Remodeling

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### Abstract

This review provides evidence that antenatal hypoxia, which represents a significant and worldwide problem, causes prenatal programming of the lung. A general overview of lung development is provided along with some background regarding transcriptional and signaling systems of the lung. The review illustrates that antenatal hypoxic stress can induce a continuum of responses depending on the species examined. Fetuses and newborns of certain species and specific human populations are well acclimated to antenatal hypoxia. However, antenatal hypoxia causes pulmonary vascular disease in fetuses and newborns of most mammalian species and humans. Disease can range from mild pulmonary hypertension, to severe vascular remodeling and dangerous elevations in pressure. The timing, length, and magnitude of the intrauterine hypoxic stress are important to disease development, however there is also a genetic-environmental relationship that is not yet completely understood. Determining the origins of pulmonary vascular remodeling and pulmonary hypertension and their associated effects is a challenging task, but is necessary in order to develop targeted therapies for pulmonary hypertension in the newborn due to antenatal hypoxia that can both treat the symptoms and curtail or reverse disease progression.

### Keywords

Hypoxia; pulmonary hypertension; sheep; fetal programming; newborns

### INTRODUCTION

A mother's uterus protects the unborn child and provides nutritional support. Unfortunately, but of great importance, prenatal stresses can alter uterine function and program the fetus causing disease after birth. The lung is particularly vulnerable since it undergoes a marked

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#### CONFLICT OF INTEREST

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transition with birth. The lung switches from low blood flow and not exchanging air to receiving large blood flow and respiring. Respiratory abnormalities due to bronchopulmonary dysplasia, diaphragmatic hernia, antenatal hypoxia and other complications associated with pregnancy are extremely important health problems afflicting ~2% of newborns in the United States [1-3]. Neonatal lung diseases are difficult to treat, often resulting in long-term complications or death.

The respiratory system exhibits significant developmental plasticity, making it especially sensitive to hypoxemia and other stresses during the prenatal and neonatal periods. Maternal prenatal hypoxemia due to living at high altitudes, placental insufficiency, and smoking are relatively common examples. There are as many as 140 million permanent residents at altitudes >2,500 m [4]. As of 2008 ~ 13% of pregnant women in the United States, carrying just over 500,000 unborn children, smoked during the last 3 months of gestation [2]. This is really a worldwide problem, however, as roughly 9% of all women smoke [5]. Together these place a significant number of newborns at risk of developing pulmonary hypertension [6-8], high altitude pulmonary edema [4], as well as pulmonary hypertension later in life [9].

Antenatal hypoxia, a relatively common prenatal stress, is not only associated with high altitude living and smoking but also with various other prenatal disorders including maternal anemia or placental insufficiency. The resultant fetal hypoxemia can cause respiratory distress and pulmonary hypertension (PH) in newborns [10, 11]. The high pulmonary vascular resistance (PVR) associated with antenatal hypoxia causes backpressure on the right heart leading to cardiac hypertrophy and insufficiency. The devastating results of PH on right ventricular (RV) function eventually lead to heart failure, which is a common characteristic of all sub-classes of pulmonary arterial hypertension. The lack of definitive treatment options coupled with treatments that only temporarily alleviate the symptoms result in a slow but progressive disease. This debilitating disease of infants is unacceptable and illustrates why we need to vigorously investigate all aspects of pulmonary hypertension of the newborn, especially in the setting of easily identifiable risk factors, including antenatal hypoxia. These investigations hold promise of discovering more definitive treatment options that can slow or even reverse the disease.

Focusing on specific etiological risk factors, high altitude living is especially relevant, since it affects large populations and the stress does not have as many confounding issues as smoking, maternal malnutrition, or placental insufficiency. Living in elevations greater than 2,500 m is common and pregnancy at such altitudes causes antenatal stress to the unborn fetus. Many of the resident populations live in the Himalayas and its surrounding ranges, the Andes of Central and South America, portions of the Rocky Mountains in the United States, as well as other high altitude areas around the world [12]. Of particular interest is why infants from one population adapt to the high altitude environment while those from another population do not. For example, native Tibetan and Peruvian populations appear to have adapted to the high altitude environment [12-14]. The lung structural and functional development of the infants is altered in order to enhance blood oxygenation, although these infants can still have cardio-pulmonary complications after birth. In comparison, infants born to Han Chinese who have recently moved to Tibet and Europeans that move to the high

Andes are more likely to have low birth weight and lung dysfunction, including elevated pulmonary pressures and reduced blood oxygenation [8, 14, 15].

In this review, we examine lung development to provide a frame of reference, and then focus on those elements that are likely to be vulnerable to low oxygen tension *in utero*. We examine adapted residents and review functional and genetic evidence from animal as well as human studies that works to explain divergence in the ability of infants born at high altitude to acclimatize. We then appraise our clinical understanding of the disease and the current treatment regimens as well as several relevant emerging therapeutic strategies.

## THE MAMMALIAN LUNG: DEVELOPMENTAL PROGRESSION AND FUNCTION

### Airway and vasculature structure

Developmental progression of the human lung is well described and outlined in a number of fine reviews over the past five decades [16-23] and in several books [24-26]. The progression of human lung development is summarized in Table 1 and then comparisons to other species are made in Table 2.

In brief, human lung development begins in the embryonic period during the 1<sup>st</sup> week of gestation up through week 7. This is followed by 3, somewhat overlapping, fetal stages of lung development. The pseudoglandular stage is between weeks 5 to 17, the canalicular stage between weeks 16 and 26, and the saccular stage from week 24 to term. Prior to birth, the alveolarization stage begins around week 36, and continues for as long as 2-3 years after birth [24, 25, 44, 45]. Finally, microvascular maturation begins shortly after birth and continues for the first few years of life, with less defined boundaries compared to the other stages [44, 46, 47]. These aforementioned stages are heavily based on airway formation and development, and although vascular development has been significantly less studied, it is just as integral to lung development.

The lung is distinct among all of the organs because it develops two vascular systems. The bronchial circulation arises from the thoracic aorta and provides oxygenation of various lung tissues while the pulmonary circulation transports deoxygenated blood from the right ventricle to the alveoli for blood oxygenation and then returns the oxygenated blood to the left heart. Vascular development of the lung is broken into three distinct processes. First, there is proximal angiogenesis, which is the formation of vessels from pre-existing ones. Second, there is distal vasculogenesis, which is the *de novo* formation of vessels from angioblasts or endothelial precursor cells within peripheral blood lakes of the mesenchyme. Lastly, there is proximal-distal vessel fusion during the pseudoglandular period [40, 53]. More recently, variations of this theory have emerged from different investigative groups. These differing views support either an all-vasculogenesis theory [54] or an all-angiogenesis theory [55], the latter of which heavily relies on vascular remodeling. Regardless of the actual mechanism, it is evident that vascular development starts early in fetal life, continues after birth and is heavily based on epithelial-mesenchymal cell interactions, often referred to as “cross-talk” [33-35, 53].

## Developmental stages of the lung

During the embryonic period, the lungs appear at approximately day 26 of gestation as a ventral out-pouching of the foregut. The newly formed lung bud elongates, dichotomizes and invades the surrounding mesenchyme. The airway tree is slowly formed with continuous dichotomous divisions, and during this period the entire bronchial tree forms down to the terminal bronchiole [36]. The vascular connections are also established at this early stage of development. The main pulmonary trunk through to the intrapulmonary arteries is formed, either through angiogenesis, vasculogenesis or a combination of these processes [29, 30]. The vascular development at this stage parallels the airway tree, with supernumerary arteries for alveoli situated on the airway walls [31]. The venous system develops in-between airway branches within connective tissue septa [32]. A capillary plexus also starts to form [39] and becomes a connecting bridge between pulmonary arteries and veins during this period [40]. The bronchial circulatory network of the lungs begins to form as well, which eventually connects to the pulmonary veins of the left atrium [56, 57]. Ultimately, the bronchial, arterial, and venous structural pattern is very similar to the adult lung by the end of the pseudoglandular stage.

The canalicular stage follows, and is characterized by respiratory epithelium growth, and formation of respiratory bronchioles and pulmonary acini, which are the gas-exchange units. The distal lung capillary bed increases dramatically, due to the “canalization” of the lung parenchyma by capillaries. The capillaries also come closer to the epithelial layer of the expanding air-spaces, and the cuboidal epithelium starts to flatten and differentiate into type I and type II airway epithelial cells. This marks a shift in the focus of lung development to the more functional elements needed for gas exchange [24, 45].

The saccular stage is the final prenatal stage of lung development, and is associated with the formation of saccules distally to the terminal bronchioles, significantly increasing the surface of the lung parenchyma through dichotomization. These saccules eventually morph into alveolar ducts and sacs during the alveolar stage [24, 25]. The interstitial tissue between air spaces is compressed and the capillary network is altered, increasing the number of capillaries within the inter-air-space walls. Elastic fibers are laid within this thinning interstitial tissue providing structural support, and differentiation further advances with maturation of type I and II alveolar cells, and increase in ciliated, Clara, basal and neuroendocrine cells. Finally, the larger vessels of the pulmonary vasculature muscularize [41].

A few weeks before birth and lasting close to 2-3 years after birth, the alveolar stage begins. More than 90% of the alveoli are formed post-natally with many in the first 6 months of life. This stage is characterized by changes of the terminal airway saccules such that they give rise to the alveolar ducts, alveolar sacs and single alveoli [42]. Microvascular maturation overlaps heavily with alveolar development, and transforms the capillary network of the lung parenchyma into its adult form. During this period the bi-layered capillary network will become the typical single-layered capillary network found in the more mature lung. The two main mechanisms through which this is accomplished include capillary fusions [43] and preferential growth [24]. The cellular growth and organization of the pulmonary vasculature

is integral to its formation and function (Fig. 1). Moreover vessel wall structure is based on the external diameter of the artery and the vascular generation where it is located. Pre-capillary microvessels and capillaries have only an endothelial barrier with the pneumocytes at the alveoli (~10  $\mu\text{m}$  external diameter). In slightly larger vessels, the walls become muscularized, with non-muscular and partially muscular vessels found at up to 150  $\mu\text{m}$  external diameter. The fully muscularized vessels, and thicker arteries have an elastic lamina that creates a more pronounced infrastructure and helps delineate the endothelial and smooth muscle layers [58]. Contacts between neighboring cells and matrix help determine cell phenotype and are important in responses to growth factors and regulators of vasomotor tone [25, 45].

The growth and organization of the pulmonary vasculature is coupled to its form and function. The endothelial cells are vital to vasculogenesis. They coalesce into tubes and form the vessels, creating junctional complexes with one another by fusing their membranes into a contiguous structure. This endothelial barrier is critical in all vessels, but particularly important in the microvessels and capillaries of the lung where the endothelial cell is buttressed against the pneumocyte of the alveoli. This provides the final barrier between the blood and the alveoli and the reduced membrane width facilitates gas diffusion. During development the interactions between endothelial and mural cells are very important because they promote vessel wall formation. The vessels that feed the terminal bronchioles and larger airways become muscularized. Mature larger diameter vessels, ~ 75  $\mu\text{m}$  and larger [58], have an elastic lamina that creates a more pronounced infrastructure and helps delineate the endothelial and smooth muscle layers. The endothelial cells comprise the intima and are on the luminal side of the elastic lamina. Smooth muscle myocytes make up the medial layer, which is on the outer surface of the elastic lamina. Fibroblasts are on the outer surface of the vessel, making up the adventitia. The endothelium, as mentioned, forms a tightly coupled syncytium and the myocytes of the medial layer are also connected via gap junctions. The endothelial and smooth muscle cells couple to one another through myoendothelial junctions that traverse the internal elastic lamina layer. There are also additional contacts between neighboring cells within the adventitia. Lastly, all of the cells are tethered into the matrix, which helps define cell phenotype and is important in determining the responses to growth factors and regulators of vasomotor tone [25, 26].

### **Vascular reactivity *in utero***

Vascular reactivity is fundamental to the ability of the lung to regulate its blood flow. Prior to birth the lung does not have any role for gas exchange. Because the lung does not serve its primary purpose it is not beneficial for the fetus to expend effort on providing high perfusion to this organ. The unique ability of the lungs' vascular bed to constrict in response to hypoxia and dilate in response to normoxia or hyperoxia helps the lung to limit its perfusion before birth. After birth, constriction in response to hypoxia facilitates blood oxygenation by shunting blood away from low oxygen-tension alveoli and towards ones rich in oxygen. As early as the 100<sup>th</sup> day of gestation sheep fetuses will exhibit increased PVR mediated by pulmonary vasoconstriction in response to low oxygen tension, and this response becomes progressively greater later in pregnancy and after birth [59, 60]. Conversely, an increase in

the oxygen tension of 5 mmHg can cause pulmonary arterial dilation, increased pulmonary blood flow and decreased PVR [61].

Several mechanisms keep fetal PVR high. These include low oxygen tension, low basal vasodilator production, and increased vasoconstrictor production in combination with enhanced myogenic tone [25, 62]. Moreover the fetal circulation is also characterized by progressive changes in vasore-activity. For example, we find that ovine fetuses have reduced constriction to membrane depolarization with high potassium [63] or serotonin [64, 65] as compared to adults. Also in the ovine fetus, vasoreactivity increases in late gestation (136-146 days) compared to early gestation (94-101 days), with increased hyperoxia-induced vasodilation and pulmonary vascular blood flow [66]. In the human fetus there are similar increases in hyperoxia-induced vasodilation during the final weeks of gestation, weeks 31 through 36 [67]. This enhanced vasodilatory capacity to elevated PO<sub>2</sub> is likely due to increases in the function and expression of oxygen sensing mechanisms, including an increase in large conductance voltage- and calcium-activated potassium (BK<sub>Ca</sub>) channels [68], and is consistent with functional maturation of the pulmonary circulation in addition to structural growth and development.

Our knowledge regarding the mechanisms associated with pulmonary arterial contraction is extensive. Unfortunately, most of what we know is derived from studies performed in arteries and cells isolated from adult animals. These examinations show that many stimulatory agonists act through functional G-protein coupled receptors (GPCRs). Some of the most potent vasoconstrictors including endothelin-1 (ET-1), serotonin (5-HT) and prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>) act through Gq-coupled receptors. The signaling pathways these agonists activate are intricate and well organized (see Fig. 2). Gq-coupled receptors cause contraction through phospholipase C (PLC) and receptor tyrosine kinase pathways. In turn, these pathways act to modulate the function of Ca<sup>2+</sup>-dependent and independent signaling pathways primarily through *myo*-inositol 1,4,5-trisphosphate (InsP<sub>3</sub>), Diacylglycerol (DAG) and the Ras homolog gene family, member A (RhoA) and Rho-associated, coiled-coil containing protein kinase (ROCK) signaling pathway. Ultimately, the pathways converge to mediate their effects through phosphorylation and activity of the 20 kDa myosin light chain (MLC<sub>20</sub>).

### Membrane depolarization and Ca<sup>2+</sup> dependent contraction

Membrane depolarization is a fundamental feature of GPCR receptor activation, which is intimate to extracellular Ca<sup>2+</sup> entry. Based on studies performed in pulmonary arteries and myocytes isolated from adult animals there is still debate over the exact mechanisms involved in membrane depolarization, and even less is known regarding this process in fetal tissues and cells. However, the general consensus is that membrane depolarization is due to coordinated regulation of several ion channels. Receptor activation inhibits voltage-gated K<sup>+</sup>-channels (K<sub>v</sub>) through elevations in cytosolic Ca<sup>2+</sup>, and this causes membrane depolarization [69, 70]. Other evidence indicates receptor stimulation will activate Ca<sup>2+</sup>-dependent chloride conductances (ClCa) [71-73]. ClCa activity may depend on coordinated activation of non-selective cation channels following depletion of the sarcoplasmic reticulum Ca<sup>2+</sup> [72] or possibly through Ca<sup>2+</sup> sparks, which are due to localized Ca<sup>2+</sup>

increases mediated by ryanodine receptor (RyR) activation [74]. Together these  $K^+$ ,  $Cl^-$ , and non-selective cation conductances regulate the membrane potential, and in turn  $Ca^{2+}$  influx through L-type  $Ca^{2+}$  channel as well as non-selective cation channel pathways.

The cytosolic  $Ca^{2+}$  increase following receptor activation and coordinated membrane depolarization is attributable to interplay of activation and inactivation of  $Ca^{2+}$  permeable ion channels on the sarcoplasmic reticulum and plasma membrane. The sarcoplasmic reticulum of smooth muscle cells includes  $InsP_3$  receptors ( $InsP_3R$ ) and RyRs, which release  $Ca^{2+}$  into the cytosol when stimulated by GPCR signaling pathways. The spatial arrangement of  $InsP_3R$ s and RyRs on the sarcoplasmic reticulum and the temporal aspects of  $Ca^{2+}$  release are important to smooth muscle cell excitability [75, 76]. Our understanding of  $Ca^{2+}$  signaling in fetal pulmonary arterial myocytes is still limited, but studies performed on fetal sheep pulmonary vascular myocytes illustrate that platelet activating factor (PAF) causes  $InsP_3$  generation and  $Ca^{2+}$  responses [77, 78]. This is substantiated by our own studies showing that ATP, phenylephrine and serotonin all cause  $Ca^{2+}$  responses in pulmonary arterial myocytes from fetal sheep [64, 79]. Even still, ET-1-dependent activation of G protein coupled receptors can also induce RyR-generated  $Ca^{2+}$  sparks [80], illustrating the complexity of  $Ca^{2+}$  signaling within pulmonary vascular myocytes.

Studies performed in fetal sheep and additional models by our laboratory and by others illustrate fetal pulmonary arterial smooth muscles have several  $Ca^{2+}$  entry pathways. L-type  $Ca^{2+}$  channels are the best understood and characterized  $Ca^{2+}$  permeable ion channel. These channels are activated by membrane depolarization due to GPCR stimulation or acute hypoxia. We find L-type  $Ca^{2+}$  channels provide for roughly one-quarter of the contraction due to serotonin [64] and potassium-induced membrane depolarization in sheep fetal pulmonary arteries [63]. L-type  $Ca^{2+}$  channel blockers in piglets also dramatically reduce pulmonary arterial pressures and increase flow *in vivo* [81]. Although L-type  $Ca^{2+}$  channel activity is central to pulmonary arterial contractility, data shows that non-selective cation channels are also important to fetal pulmonary arterial  $Ca^{2+}$  signaling and arterial reactivity [64, 82]. In particular, we found that inhibition of non-selective cation channels with SKF96365 blocked about one-half of the serotonin-mediated contraction above the portion sensitive to the L-type  $Ca^{2+}$  channel inhibitor nifedipine [64]. This pharmacological evidence is imprecise, but suggests that canonical transient receptor potential channels (TRPC) are important for this  $Ca^{2+}$  influx pathway [83]. Indeed, TRPC1, TRPC3, TRPC5 and TRPC6 are expressed in fetal sheep pulmonary arteries [82]. These data illustrate that voltage-dependent and non-selective cation channels coordinate to regulate  $Ca^{2+}$  signaling and arterial contraction. Nevertheless, detailed studies are required to resolve the mechanistic changes that occur during fetal development, their importance during the transition period at birth, as well as the impact of antenatal hypoxia. Together the  $Ca^{2+}$  influx across the plasma membrane combines with  $Ca^{2+}$  release from the sarcoplasmic reticulum to cause global  $Ca^{2+}$  responses. The magnitude of this  $Ca^{2+}$  response governs the degree of myosin light chain kinase (MLCK) activation and in coordination with sensitization mechanisms this regulates the strength of pulmonary arterial contraction [84].

### Ca<sup>2+</sup> independent contraction

Sensitization of the myosin light chain to increases in cytosolic Ca<sup>2+</sup> is a fundamental feature of arterial contractility. We routinely observe this phenomenon in fetal sheep pulmonary arteries. For example, roughly one-quarter of the contractile response to 5-HT remains in sheep fetal pulmonary arteries after we remove extracellular Ca<sup>2+</sup> [64]. Sensitization is achieved largely through a convergence in signaling that reduces myosin light chain phosphatase (MLCP) activity. This allows for increased MLC<sub>20</sub> phosphorylation by MLCK, which enhances contraction. These sensitization responses are largely due to RhoA/ROCK pathways as is illustrated in our recent studies that show RhoA/ROCK contributes substantially to depolarization mediated pulmonary arterial contraction in fetal sheep [63]. The importance of sensitization is substantiated by other recent studies establishing that inhibition of RhoA/ROCK reduces pulmonary vascular resistance in fetal lambs [85, 86]. Together these studies demonstrate the significance of Rho-kinase and point to its therapeutic potential for treatment of pulmonary vascular disease in the newborn.

In addition to Rho-kinase, studies performed in newborn piglets and adult humans and rats show that Protein kinase C (PKC) also causes pulmonary artery (PA) contraction [87, 88]. Following receptor stimulation, DAG produced through PLC cleavage activates Protein Kinase C (PKC) and then the 17 kDa PKC-potentiated inhibitory protein of PP1 (CPI-17). This signaling cascade acts to reduce the function of protein phosphatase 1C (PP1C), a catalytic protein that promotes myosin light chain phosphatase activity. We were unable to find any details regarding a role of PKC in fetal pulmonary arterial contractility, although the data from the newborn piglet suggests there is a reduction in one particular PKC isoform (PKC zeta) within two weeks following birth [87]. Notably, PKC function and the influence of antenatal hypoxia have been extensively studied in the fetal sheep cerebral vasculature [89, 90], where the function of various isoforms changes substantially. Although the pulmonary and cerebral vasculatures develop at different rates and are distinct functionally these data illustrate that examinations of PKC in the fetal pulmonary vasculature are an open area for investigation.

### Pulmonary vascular transition at birth

During the transition from intra-uterine to extra-uterine life, significant changes occur. Most importantly, PVR decreases dramatically immediately after birth due to increased vessel lumen diameter and decreased wall thickness. These changes are partly mediated by cytoskeleton remodeling that occurs in both endothelial and pulmonary artery smooth muscle cells as seen in newborn piglets [91]. There is also a contribution of fetal lung fluid-removal by the alveolar epithelium as observed in newborn rabbits [92]. Interestingly, the exact mechanism that initiates pulmonary vasodilation or how the pulmonary artery endothelium or smooth muscle cells are targeted is not entirely known. The prevailing theory is that initial lung expansion and subsequent rhythmic distention as well as increased oxygen tension, trigger a series of events that result in pulmonary arterial vasodilation (Fig. 3). Endothelial cell maturation and nitric oxide (NO) production are also important, including the release of vasodilatory substances (NO, PGI<sub>2</sub>) and the inhibition of vasoconstrictive signals [93-95]. There is also the potential for release and activation of other endothelium derived hyperpolarization factors (EDHF) including carbon monoxide



and activation of endothelial K<sup>+</sup> channels that can hyperpolarize the smooth muscle through myo-endothelial gap junctions [96-100]. The close relationship between elevated oxygen tension and increased NO production [101], such as occurs when the newborn takes its first breath, will facilitate pulmonary arterial vasodilation at birth.

Oxygenation and NO are critical components to vessel dilation [93, 102-106] and the attendant reductions in pulmonary vascular resistance that occur during the transition from intra-uterine to extra-uterine life [107]. Studies performed primarily by Dr. David Cornfield show that RyRs couple to BK<sub>Ca</sub> in the fetal pulmonary vasculature and are activated by NO and oxygen signaling [68, 108-111]. The portion of the signaling cascade leading from RyR activation to vessel dilation and the decrease in pulmonary vascular resistance shown in (Fig. 3) is based on this series of studies [68, 70, 108-112]. This pathway is backed by experiments performed in the cerebral vasculature [113-116]. Collectively, these studies show that RyR activation stimulates BK<sub>Ca</sub> channels. Activation of the BK<sub>Ca</sub> channels promotes vasodilation through membrane hyperpolarization, inhibition of L-type Ca<sup>2+</sup> channels, and depression of cytosolic Ca<sup>2+</sup>, which results in vasodilation [117].

Nitric oxide also causes vasodilation through other pathways. Notably, Rho-kinase regulation by protein kinase G (PKG) downstream from nitric oxide is a fundamental regulator of vasomotor tone [118]. Indeed suppression of Rho-kinase activity contributes to PKG mediated vasorelaxation in vessels from fetal sheep [118], indicating its importance during the transition at birth.

## IMPACT OF ANTENATAL HYPOXIA ON PULMONARY FUNCTION

The respiratory system exhibits significant developmental plasticity throughout the early stages of life. This makes the lung vulnerable to threatening factors such as maternal nutritional deficiencies, placental insufficiency, smoking, and gestation at high altitude. The timing of these extrinsic stressors is important when considering the ultimate effect on lung function [119]. One common theme among these stressors, including living at high altitude, is that they can cause intrauterine growth retardation. In turn, this can have profound and long-lived consequences into adulthood.

There is a fundamental relationship between altitude and O<sub>2</sub> in the ambient air. Although the O<sub>2</sub> percentage in the air remains constant at 20.9%, the partial pressure of oxygen (PO<sub>2</sub>) falls with increasing altitude. The relationship between ambient PO<sub>2</sub> and altitude, which is governed by Boyle's law, is presented in (Fig. 4). However the inter-relationships between the inspired PO<sub>2</sub>, the mother's alveolar PO<sub>2</sub> that determines arterial PO<sub>2</sub>, and the ability to deliver oxygen to the fetus through the placenta are more important, as shown in (Fig. 5). Based on the oxyhemoglobin saturation curve of the adult, once the mother travels to high altitudes both maternal and fetal arterial partial pressure of oxygen (PaO<sub>2</sub>) falls, such that O<sub>2</sub> delivery to the fetal tissues becomes increasingly compromised as altitude increases.

Previous studies from our group demonstrate that the PaO<sub>2</sub> for fetal sheep near sea level is roughly 23 mmHg, which is substantially less than the maternal PaO<sub>2</sub> that is close to 100 mmHg [120]. Despite the marked drop in the PaO<sub>2</sub> across the placenta, fetal hemoglobin is roughly 70% saturated with oxygen, as illustrated in (Fig. 6). Despite this low oxygen

tension relative to the adult, fetal oxygen delivery is facilitated and lung growth and development requirements are met because fetal hemoglobin has a higher oxygen affinity than adult hemoglobin [121].

Fetal hemoglobin is comprised of two alpha and two gamma chains, as opposed to the two alpha and two beta chains of adult hemoglobin. Slight structural differences in the gamma subunits shift the oxyhemoglobin dissociation curve to the left relative to the adult, which facilitates the transfer of oxygen from maternal to fetal blood at the placental interface. The unloading of oxygen from fetal hemoglobin in the fetal tissues is aided by relatively low  $O_2$  tensions in the tissues [121]. Because of these lower arterial and tissue oxygen tensions the fetus is acutely sensitive to reductions in maternal  $PaO_2$ .

Decreases in environmental oxygen tension translate into even further reduced  $PaO_2$ , as illustrated in (Fig. 6). For example, reducing inspired  $PO_2$  to simulate the altitude of the Barcroft facilities at White Mountain Research Station (WMRS) in California (3,801 m) causes maternal  $PaO_2$  to equilibrate to ~60 mmHg while the fetal  $PaO_2$  decreases from 23 to 19 mmHg [120]. Importantly, although the magnitude of the decrease in maternal arterial  $PO_2$  is greater than that of the fetus, fetal oxyhemoglobin saturations are substantially more compromised. As shown in (Fig. 6), even at sea level fetal hemoglobin saturations are in the steep portion of the hemoglobin saturation curve. This means that small declines in  $PaO_2$  cause precipitous drops in hemoglobin saturation. Travel from sea level to WMRS causes maternal oxyhemoglobin saturations to decrease from ~98% to ~85% while fetal oxyhemoglobin saturations decrease from about ~70% to 40%. Despite the fall in oxyhemoglobin saturations, the arterial oxygen content in the fetus does not fall significantly because hemoglobin concentrations increase. However, the  $PO_2$  in the fetal tissues is still lower at high altitude [120], which can have detrimental effects as will be described in this section.

### **Animal models for antenatal hypoxia**

Some of the commonly used mammalian models of antenatal and perinatal hypoxia include cows [122], sheep [63, 118, 123, 124], rats [125], mice [126], piglets [127, 128], guinea pigs [129], and one group often uses llamas [124]. The etiology of PH in each model is unique. Finding consistencies and differences in the molecular mechanisms responsible for inducing PH or acclimating to the rarified environment is likely to unveil seminal pathways important to disease progression. But still, we cannot generalize the findings from one model to another, and this impacts our ability to extrapolate and translate laboratory studies to treatment of newborn patients.

### **Diversity in acclimatization**

The ability of antenatal hypoxia to induce varied responses is embodied by the dramatic differences between adapted and afflicted species, which parallels human populations that respond differently to antenatal high altitude stress (Fig. 7). This ranges from species that live and thrive at high altitudes, such as llamas and yaks, to low altitude animals somewhat less tolerant to high altitude, such as sheep, or those with an exacerbated response to high altitude, such as cows, mice, and Fawn-Hooded (FH) rats. Newborn and adult animals from

responsive species have substantial structural and functional transformations within 2 weeks of being placed in low oxygen chambers [125, 127, 130-132].

The most comprehensive comparative study thus far was by Tucker *et al.* (1975), where the muscularization of the pulmonary arteries was found to correlate highly with the degree of pulmonary hypertension. Unfortunately, these studies were performed in adolescent and adult animals and thus have limited bearing for discussions of the impact of antenatal hypoxia [132]. Animal species that have lived at high altitude for many generations including llamas [124] and yaks [133] are adapted to birth in the rarified environment. The physiological adaptations can be specific to each species, but ultimately these animals do not exhibit the pulmonary vascular remodeling or elevated pulmonary arterial pressure responses that are indicative of pulmonary vascular disease at altitude. However, one of the best *in vivo* bio-markers of animals that adapt well to high altitude is an improvement in pulmonary gas exchange, as evidenced by increased carbon monoxide diffusing capacity (DLCO). Such an increase in DLCO has been observed in guinea pigs [134], and foxhounds [135], as well as in Tibetans native to high altitude [14]. The DLCO is a marker for high altitude acclimatization because it is a rather simple clinical test that provides a surrogate measure for the surface area available for gas exchange. Augmented DLCO in these animal models and in human populations would correlate with increased gas exchange surface area and more efficient oxygen uptake and carbon dioxide removal at the alveoli. This ability to more effectively exchange gasses would be particularly helpful at high altitude, where the partial pressure of oxygen is reduced.

Most humans as well as sheep and pigs do not acclimate very well to high altitude living. Humans in particular exhibit a wide range of responses ranging from little or no PH to those who have pulmonary pressures nearly as high as the systemic vasculature. Although little is known regarding the extent of pulmonary vascular remodeling in human infants following antenatal hypoxia, affected infants can exhibit elevated pulmonary vascular pressures and growth retardation [8, 10-12, 14, 136, 137].

In contrast to animal models that are diseased, yaks are uniquely adapted to high altitude. Yaks have a blunted vasoconstrictor response, suggesting improved lung blood flow and oxygenation under hypoxic conditions [133]. However, the adaptations are complex because their pulmonary arteries are smaller and their endothelial cells have attenuated endothelium-dependent vasodilator responses to acetylcholine (ACh). There are additional structural changes that may help prevent pulmonary hypertension including longer, wider and rounder pulmonary arterial endothelial cells, thinner-walled pulmonary vessels and reduced amount of pulmonary arterial smooth muscle cells, especially in vessels > 100 microns. Although it has been suspected that similar adaptations may occur in humans who are native to high altitudes the data is, as yet, not overwhelming [138].

### **Antenatal hypoxia and airway development**

In many species, high altitude hypoxia stimulates lung growth and retards thoracic wall development (dysanaptic growth) [139]. This process is balanced, whereby at moderate levels of hypoxia thoracic wall growth retardation may predominate, while at severe levels of hypoxia lung growth dominates [140]. Although gestational hypoxia can retard

intrauterine growth of high altitude residents, Tibetan children living at altitude exhibit accelerated lung growth, with improved lung function, diffusing capacity and ventilation as compared to lowlanders [14]. These pulmonary changes are also accompanied by decreased blood viscosity and hemoglobin levels, and higher arterial oxygen saturation with rest and exercise and less loss of aerobic performance with increasing altitude [14]. These adaptations to antenatal high altitude/hypoxic gestation, birth and living may represent a coupling of environmental and genetic factors [141] and confer an advantage from a gas exchange perspective.

*In utero* hypoxia that simulates an elevation of ~ 3,800 m (maternal FiO<sub>2</sub> of 0.13) may alter alveolar development in Sprague-Dawley (SD) rats. In this animal model, alveoli are formed by septation mainly during the post-natal period resulting in larger mean alveolus volume and decreased total number of alveoli, similarly to humans [142]. These studies suggest there is a critical period of septation after birth, within the first 3 weeks of life, after which hypoxia does not alter septation and alveoli number, but may increase alveolar size [142, 143]. The latter is more consistent with the effect of high altitude hypoxia on human alveoli. Exposure of mice to hypoxia from birth to 2 weeks old, similarly impaired lung alveolarization and caused hypoxia-induced vascular remodeling and reduced lung compliance [144].

Guinea pigs and foxhounds have enhanced lung function when raised at high altitude. Weanling guinea pigs raised at high (3,800 m) compared to intermediate (1,250 m) altitudes had elevated lung volumes, increased alveolar-capillary surface areas and alveolar septal tissue volumes, with smaller alveolar duct volumes and harmonic thickness of the diffusion barrier [145]. These structural changes corresponded to the increased diffusing capacity of carbon monoxide (DLCO) mentioned earlier [134]. In a slightly different study, acclimatization of foxhound dogs for 5 months to high altitude (3,800 m) during development improved DLCO, without impacting ventilation/perfusion mismatching at 1 month, 2 years and 2.5 years after return to low altitude [135, 146], providing evidence for long-term perinatal programming of lung function.

### **Antenatal hypoxia and pulmonary vascular development**

Many newborns that suffer significant antenatal hypoxia adapt poorly to breathing air, with abnormal modulation of pulmonary vascular myocyte and fibroblast growth and function. There is increased pulmonary vessel muscularization, as well as collagen and elastin deposition that decrease internal vessel diameter and peripheral arterial density (Fig. 1). Moreover, the antenatal hypoxic stress responsible for vessel malformation pre-disposes individuals to exaggerated responses with subsequent hypoxic insults, such as occurs when someone travels repeatedly between low and high altitudes [91, 139]. For example, when preterm and neonatal SD rats were exposed to hypoxia 3 days before and after birth, re-exposure to hypoxia at 2 weeks of age resulted in elevated right ventricular systolic pressures, RV hypertrophy, vascular pruning, and decreased radial alveolar counts compared to rats not exposed to perinatal hypoxia [147]. Subjecting these rats to hypoxic stress at 3-months old still caused pulmonary artery pressure increases, and vascular remodeling that altered pulmonary artery wall tensile stress. Initially pulmonary artery wall stresses

increased, only to return to control levels in a few days after the vessel walls thickened and this correlated with changes in the elastic lamina ultrastructure [148]. Similar findings occurred in chronically hypoxic fetal sheep where the pregnant ewes were housed at 3,800 m during gestation. In these fetal sheep, the medial wall thickness was increased predominately in the distal portions of the pulmonary artery tree compared to controls [123, 149, 150]. Functionally, newborn sheep (~10 days old) born on the Andean altiplano (3,600 m) as well as WMRS (3,801 m), have PH and exacerbated hypoxic-induced pulmonary vasoconstriction [151, 224]. Although robust segmental analysis procedures to examine changes in lung structure have not been performed on either fetal or newborn sheep [152, 153], these results suggest that perinatal hypoxia causes substantial structural remodeling.

Fawn-Hooded rats are a unique strain, as newborn pups develop accelerated and severe PH when exposed to moderate hypoxemia and have spontaneous hypertension due to genetic problems with platelet aggregation [154-156]. The pulmonary hypertension response is exemplified by a series of studies that compared the effects of altitude on lung development of FH and SD rats. FH rats raised in Denver at ~1,600 m versus simulated sea-level during their gestation resulted in a decreased lung size and RV hypertrophy in the FH while the SD rats were unaffected. This was accompanied by alveolar simplification where the lungs from FH rats had larger and fewer air-spaces and thickened interstitium, without a significant difference in the level of epithelial cell maturation. These effects were not readily reversible because pulmonary arterial density was reduced in the FH rats later in life [157]. Although the malformations in FH rats are genetic and affect more than just the pulmonary vasculature [155, 158], they may be akin to human infants who appear diseased at altitude, but would be uniquely different from acclimated humans, and other species, where lung function can be enhanced. Thus, perinatal hypoxia causes a continuum of responses ranging from pathological states to enhanced functionality within human populations and among different animal species, something that also occurs in adult populations [159].

Antenatal and neonatal programming of the pulmonary vascular structures by living in hypoxic environments has been suggested for well over 40 years. In 1971 Goldberg and colleagues [160] showed that the relative medial wall thickness of small pulmonary arteries, 50-150  $\mu\text{m}$  in diameter, was increased in newborn SD rats exposed to hypoxia. Expressed as the ratio of arterial media to total external diameter, this was significantly greater in pups whose mothers were maintained in a hypoxic environment ( $\text{FiO}_2$  of 0.13, ~3,800 m equivalent altitude) during their pregnancy as compared to room air ( $\text{FiO}_2$  of 0.21) or a hyperoxic environment ( $\text{FiO}_2$  of 0.40). This difference persisted at least 13 weeks after birth and was more pronounced in arteries under 80  $\mu\text{m}$ , which are critical to PVR. Their finding supports the idea that the pulmonary vasculature undergoes development and remodeling in the fetus and newborn, and normally ceases in the young adult. From a developmental standpoint, more recent evidence suggests that branching morphogenesis, a key component of lung development, is also dependent on the environmental oxygen tension [161].

### **Antenatal hypoxia and effect on growth factors and transcriptional regulators**

There are a wide array of growth factors and transcriptional regulators that precisely orchestrate lung development. While we do not fully understand how all of the players work

together to grow a functional lung, we do know that antenatal hypoxia impinges on the function of some of them. Some of the growth factors affected by hypoxia include Hypoxia Inducible Factor (HIF) [162] and Vascular Endothelial Growth Factor (VEGF) [163], which will be discussed in a bit more detail. Other factors that are also likely to be significant include Fibroblast Growth Factor that is important during the pseudoglandular stage [164-166], Retinoic Acid whose signaling is crucial in early lung development and likely important to alveolarization [167], Bone Morphogenetic Protein (BMP) and BMP receptor 2 that have received substantial attention for their importance in familial pulmonary arterial hypertension [168], as well as TGF- $\beta$  signaling that are both important to branching morphogenesis and alveolarization [169-171] (Fig. 1).

**Hypoxia Inducible Factor**—HIF is a transcription factor with three relevant isoforms that is regulated by oxygen availability, and subsequently modulates the expression of multiple genes. It's a heterodimer consisting of HIF- $\alpha$  and HIF- $\beta$  subunits, with the former being increasingly stabilized and having higher transcriptional activity in hypoxic environments. Studies show that HIF-1 $\alpha$  levels are elevated in human fetal lung while *in utero* [172]. The HIF system is therefore key to pulmonary development [162].  $-/-$  HIF-1 $\alpha$  knockout mice do not reach full term, with enlarged vascular structures and impaired lung morphogenesis [173]. Moreover, HIF-1 $\alpha$  deficient ( $+/-$ ) adult mice are somewhat protected from hypoxia. In these mice the increase in pulmonary arterial pressure and RV hypertrophy is attenuated compared to wild-type mice, which is mainly due to reduced pulmonary vascular remodeling [174]. Similar results were noted in HIF-2 $\alpha$  deficient heterozygote mice [175].

**Vascular Endothelial Growth Factor**—VEGF and its receptor are critical mediators in pulmonary vascular remodeling under hypoxic conditions, and are dependent on HIF-1 activation [176]. In particular, hypoxia induces a more stable HIF-1 $\alpha$  subunit, which after forming a heterodimer with HIF-1 $\beta$  becomes active HIF-1. The latter translocates to the nucleus where it regulates the transcription of many genes. This includes induction of VEGF and VEGF-receptor 1 [177, 178]. The importance of VEGF to lung development is supported by studies showing that inhibiting VEGF-receptor activity with Su-5416 in newborn rats, impairs pulmonary vascular growth, reduces arterial density, causes RV hypertrophy, and increases pulmonary artery wall thickness [179]. Related to this, VEGF-receptors are primarily responsible for endothelial cell differentiation and maintenance, as well as vascular organization and permeability in addition to other functions [140]. Thus, there is a direct link between tissue hypoxia, HIF-1 and VEGF, with evidence of significant alterations in vascular morphogenesis in response to hypoxic insults.

**Nitric Oxide**—NO signaling is one potential site of acclimatization to high altitude. In adult SD rats with hypoxia-induced PH, arterial endothelial Ca<sup>2+</sup> metabolism becomes impaired resulting in endothelial NO synthase (eNOS) inactivity, decreased NO production and diminished endothelium-dependent relaxation [180]. However, in resident Tibetan populations NO production is increased through eNOS up-regulation, a response that may be part of the acclimatization process [181]. This compares with microvascular endothelial cells from fetal sheep cultured at a PO<sub>2</sub> of 30-40 mmHg to mimic high altitude (>10,000 m

equivalent altitude) [182]. Similarly, cultured human pulmonary arterial endothelial cells have significantly decreased eNOS activity, mRNA and protein expression, as well as mRNA half-life in the presence of hypoxia (PO<sub>2</sub> of ~23 mmHg, >12,000 m equivalent altitude) effects that were reversed by Rho-kinase inhibition [183]. Yet, post-natal hypoxia equivalent to 5,800 m caused complex changes in eNOS expression in newborn piglets [184]. Hypoxic exposure in the first 3 days after birth reduced eNOS density. However, exposing naïve animals on days 3-6 after birth increased eNOS density whereas later exposure, on days 14-17, did not affect eNOS density [185]. The interaction between eNOS and heat-shock protein 90 (Hsp90) was examined in these hypoxic newborn piglets, where Hsp90 binding to eNOS downregulated its activity [186]. Chronic hypoxia can therefore restrict NO production through multiple pathways causing depression of eNOS expression as well as through altered interaction with binding partners that regulate function, and the extent of these influences may depend on the altitude.

Secondarily, decreased NO levels are associated with high altitude pulmonary edema (HAPE) susceptibility in humans. This further implicates the importance of NO signaling and acclimatization to high altitude [187]. Related to this, the pathogenesis of exacerbated vascular growth during the fetal and neonatal period and development of PH in FH rats exposed to chronic hypoxia also appears to be related to eNOS deficiency [188].

**Carbon Monoxide**—Carbon monoxide (CO) signaling is similar to nitric oxide in that this gas causes vasorelaxation [189-191]. As with nitric oxide, high altitude acclimatization alters CO production, though we know very little regarding the effects of antenatal hypoxia on this process. Carbon monoxide is produced through the activity of heme oxygenase (HO-1 and HO-2) and is thought to modulate a heme moiety that is attached to the BK<sub>Ca</sub> channels. CO enhances the coupling between ryanodine receptor-generated Ca<sup>2+</sup> signals and BK<sub>Ca</sub> channels, which would accentuate vasodilation [190]. Long-term CO exposure, however, promotes vascular wall remodeling through increased production of growth factors and causes down regulation of NO production, which could impair vasodilation and promote vasoconstriction. Notably, adult llamas from the Andean altiplano have enhanced inducible heme oxygenase (HO-1) generated CO while sheep have reduced HO-1 expression and CO mediated vasodilation [99, 124]. These data illustrate that HO-1 and CO generation and function cause divergence between acclimated species and those at risk. This is certainly an important issue because HO-derived CO is likely to be important to pulmonary vascular tone as well as remodeling, and yet its importance in antenatal hypoxia-induced responses is presently unresolved.

**Prostacyclin**—PGI<sub>2</sub> is released from the endothelium and is important to vessel dilation at birth [95, 192-194]. Unfortunately, we do not know much about the effects of antenatal hypoxia on its function, though hypoxia impedes its production in pulmonary vessels from fetal sheep [195]. In addition, newborn piglets exposed to simulated high altitude (ambient PO<sub>2</sub> of 60-72 mmHg, ~6,200-7,500 m equivalent altitude) for 3 days had depressed PGI<sub>2</sub> production and arachidonic acid mediated vasodilation [196]. However, work performed in adult mice shows that prostacyclin provides protection from chronic hypoxic stress by dilating vessels and attenuating vascular smooth muscle cell proliferation. This is illustrated

with prostacyclin receptor knockout mice that exhibit a significantly greater degree of PH after exposure to hypoxia [197]. This finding also is relevant to the treatment of pulmonary arterial hypertension in humans. In these patients, remodeled pulmonary arterial smooth muscle cells may exhibit decreased PGI<sub>2</sub> synthase expression [198]. This evidence suggests that chronic hypoxia can reduce prostacyclin function, at least in the newborn and adult, which can impact both pulmonary vascular remodeling and reactivity.

**Endothelin-1**—ET-1 is a critical signaling molecule in the fetus, and as discussed, helps to maintain high vascular tone before birth. This potent vasoconstrictor, as the name implies, is predominantly secreted by endothelial cells. ET-1 mediated vascular reactivity of pulmonary arteries is preserved in fetal sheep exposed to antenatal hypoxia (3,800 m) [118]. Although antenatal hypoxia may not disrupt ET-1 efficacy in the fetal sheep lung, chronic postnatal hypoxia does alter ET-1 signaling in newborn rats and sheep. Based on one series of studies in Wistar-Kyoto rats exposed to 10% O<sub>2</sub> for 21 days (~5,800 m) ET-1 is believed to be associated with hypoxic-induced PH through pulmonary vascular remodeling, via mitogenic effects on vascular smooth muscle cells [199]. In newborn sheep born on the Andean Altiplano (3,600 m), but returned to low altitude, ET-1 potency was markedly enhanced and the efficacy was increased [100]. Moreover, hypoxia causes local ET-1 increases in the lung, and increases in pulmonary vascular pressure can elevate ET-1 levels in the serum. In piglets, chronic postnatal hypoxia (~10% O<sub>2</sub>, equivalent to ~5,800 m) causes persistently high circulating ET-1 levels, increased ET<sub>A</sub>-receptor density and binding throughout the entire pulmonary vasculature, without the normally seen transient post-natal ET<sub>B</sub> expression increase, a scenario that may impair vasodilation [200] (Fig. 3). Similarly, pulmonary arteries of chronically hypoxic piglets had as high as a 3-fold increase in contractile response to ET-1, a lack of ET<sub>B</sub>-NO-mediated vasodilatory response at 3 days and co-constriction with adjacent bronchi [201]. These findings suggest antenatal hypoxia is likely to cause complex changes in ET-1 signaling that may contribute to pulmonary vascular disease in the newborn.

**Rho-Kinase**—RhoA/Rho-kinase is important to vascular smooth muscle contraction and is activated in parallel with calcium signaling pathways by a variety of vasoactive compounds including 5-HT, norepinephrine, and ET-1 [202] (Fig. 2). There are many steps to this signaling cascade and therefore multiple points at which antenatal hypoxia could alter its function. In fetal sheep, antenatal hypoxia causes selective upregulation of ROCK II expression and activity and is important to protein kinase G dependent vasorelaxation [118]. Based on our own recent studies performed in fetal sheep we know that Rho-kinase is also important to contraction due to membrane depolarization, but that antenatal hypoxia (3,800 m) does not alter its function [63]. In a chronic hypoxia neonatal SD rat model of PH (FiO<sub>2</sub> of 0.13, ~3,800 m), RhoA/Rho-kinase activity and expression were increased. In rats with PH, Rho-kinase inhibition with Fasudil or Y-27632 reduced the elevated PVR, and yet the animals were un-responsive to inhaled NO or a systemic NO donor. In these animals, Rho-kinase inhibition failed to reverse RV dysfunction, although this was not necessarily unexpected since reversal of RV remodeling may require substantially more time once PVR is normalized [203].



### **Mast cells and high altitude pulmonary edema**

Although the above signaling molecules and their pathways have been studied to a great extent, a growing body of literature focuses on potential inducers of these molecules and their linkages to immunological components. This follows a recent trend showing that immunological dysfunctions are critical to the pathogenesis of multiple diseases not previously thought to have a significant immunological basis. The role of mast cells to vascular remodeling via VEGF related pathways is of particular interest. For example, mast cells produce IL-8 and VEGF during asthma mediated pulmonary vascular remodeling in humans [204]. Mast cells are also important in high altitude related pathology and hypoxic conditions as highlighted by their increased numbers found in HAPE human victims post-mortem [205]. Asthma and high altitude both involve reducing the alveolar oxygen tension, which implicate mast cells as one common mediator in the process of pulmonary vascular growth and differentiation under hypoxic conditions, especially considering that mast cells release VEGF.

### **Endothelial barrier function**

Most frequently when we consider the influences of high altitude the focus is on the direct effects of the rarified air on the ability to exchange gasses and the impact on vascular structure and reactivity with regards to blood flow. However, we also need to consider that the vascular structure is also critical to keeping the alveoli dry and the blood within the vessels. A characteristic example is acute respiratory distress syndrome, where the endothelial barrier is disrupted, resulting in fluid leakage from vessels to the alveoli. We actually know very little regarding the influences of antenatal hypoxia on the endothelial barrier function. However, chronic hypoxia can increase the endothelial permeability in porcine pulmonary arterial endothelial cell preparations. The increased permeability is important because the vascular leak allows growth factors and other serum products to move from the vascular lumen to the smooth muscle cell layer and into the alveoli [206]. Increased vascular permeability provides a pathway not only for causing pulmonary edema but also for the access of factors that cause vascular remodeling. Importantly, infants that are born at high altitude are at greater risk of developing high altitude pulmonary edema [141]. This suggests that children born at high altitude may have compromised endothelial cell function such that they are unable to properly maintain the endothelial barrier.

### **Antenatal hypoxia and transition at birth**

In addition to vascular remodeling and reactivity to vasoactive substances after birth, antenatal hypoxia also affects the reduction in PVR with birth. Normally, when the newborn breathes at the time of birth there is a significant increase in PaO<sub>2</sub>, which increases NO, decreases ET<sub>A</sub> receptor expression and removes the hypoxic stimulus for hypoxic pulmonary vasoconstriction (HPV), which together reduce PVR [107]. Perinatal hypoxia impedes this process as illustrated in hypoxic newborn piglets where the PVR decrease at birth is attenuated [207]. This is compounded by an enhanced HPV response in antenatal hypoxia-exposed sheep born at 3,600 m [100, 151] or 3,801 m [224]. In the newborn lamb increases in PGI<sub>2</sub> production counteract the negative effects of hypoxia [194], while newborn sheep born on the Andean altiplano have enhanced expression of eNOS,

phosphodiesterase-5, and BK<sub>Ca</sub> channels [100]. However, on balance, the factors that restrict pulmonary circulatory function at birth likely prevail. The resultant elevated PVR causes the fetal circulation to persist, induces a right to left shunt, slows closure of the ductus arteriosus, and leads to PH of the newborn [45].

### **Hypoxic induced pulmonary vasoconstriction and antenatal hypoxia**

The ability of the pulmonary vasculature to constrict in response to acute hypoxia is an intrinsic process that is thought to shunt blood away from unventilated alveoli. This process maintains the ventilation to perfusion ratio, which ultimately improves gas exchange at ventilated alveoli and ensures oxygen uptake and carbon dioxide elimination. The intracellular processes that control the HPV response have remained somewhat elusive even though this has been the focus of intense research for well over a decade. There are excellent recent reviews as well as book chapters that cover the cellular mechanisms associated with hypoxic induced pulmonary vasoconstriction [208-210].

In brief, a decrease in the alveolar oxygen levels increases the activity of pathways that cause pulmonary artery smooth muscle contraction and reduces the activity of those pathways that promote vasodilation. Presently we can only presume that the mechanisms of HPV are the same in the fetus as they are in the adult, because systematic studies regarding HPV in the fetus have not been performed. Work from many laboratories, including our own, show that the process of hypoxia-induced pulmonary vasoconstriction is intrinsic to pulmonary arterial myocytes but that it is modulated by the endothelium. We provide the core elements associated with the effects of acute hypoxia on pulmonary arterial myocytes in (Fig. 2). The depression in oxygen tension initiates an early increase in cytosolic Ca<sup>2+</sup>, which is termed Phase 1 and lasts approximately 10-15 minutes [210, 211]. The cytosolic Ca<sup>2+</sup> then decreases slightly and plateaus, maintaining an elevated level and transitions to the second phase. During this second phase there is an increase in Rho-kinase activity, which helps to sustain the contraction for a longer period. If hypoxia is maintained, as in high altitude fetuses, these and other processes regulate gene transcription that leads to vascular remodeling.

The hypoxia induced increase in cytosolic Ca<sup>2+</sup> has been well studied in pulmonary arterial myocytes from adult mice, rats, dogs, and other species [210]. Based on contraction and Ca<sup>2+</sup> imaging studies we know that the increase in cytosolic Ca<sup>2+</sup> is mediated through a highly coordinated process. Activation of ryanodine receptors on the sarcoplasmic reticulum appears to be critical to the initial response to acute hypoxia and may help coordinate other components. Tantamount to the increase in cytosolic Ca<sup>2+</sup> and ensuing contraction is entry of Ca<sup>2+</sup> from outside the cell. Many studies have illustrated that acute hypoxia leads to depolarization of the plasma membrane and that contraction is highly reliant on L-type Ca<sup>2+</sup> channels (Ca<sub>L</sub>) [212]. One component to the membrane depolarization response is its dependency on inhibition of Kv channels [213, 214]. In addition, there is also a coordinated role for Ca<sup>2+</sup> influx through a number of different cation channels including transient receptor potential channels [210, 215-218].

There is intense investigation into the oxygen sensing mechanisms that underlie HPV, and we expect these efforts to help us understand how PVR is maintained *in utero*. Substantial

focus is placed on the ability of mitochondria to sense changes in oxygen tension, and regulate the generation of reactive oxygen species that influence ion channels and other enzymes critical to the HPV process [219, 220]. However, as recently reviewed [208, 210, 221], a large range of proteins are regulated by oxygen tension and generation of reactive oxygen species is just one of several possible mechanisms. The activity of some oxygen-sensitive proteins does not change until the oxygen tension is very low, (less than ~10-15 mmHg). This includes mitochondrial cytochromes and heme-oxygenase-2, which maintain normal activity until very low PO<sub>2</sub> levels. A number of proteins, however, are sensitive to changes in oxygen tensions that may be found in arterial blood. This includes NADPH oxidase (NOX2), the activity of which changes gradually over a wide range of oxygen tensions [221]. In addition, hydrogen sulfide production in the lung and pulmonary arterial smooth muscle cells is inversely related to oxygen tension, and there is evidence that this gas is important to HPV responses [221]. Understanding how these and other oxygen sensing pathways change during the course of development and whether or not antenatal hypoxia modifies their function is likely to provide new insights into pulmonary vascular reactivity *in utero* and the development of antenatal hypoxia induced pulmonary vascular hypertension in the newborn.

As discussed throughout this review, the responses of each species to antenatal hypoxia vary dramatically and range from animals and humans that can fully acclimate to life in a rarified environment, to those who have mild dysfunction, and to those with severe disease. The results of studies that examined vessel reactivity were alluded to in previous sections because many substances that influence vessel remodeling often modulate vessel reactivity too. Therefore contractility responses to antenatal hypoxia cannot be entirely generalized across all species. This is further compounded because the neonatal pulmonary vasculature is immature and vasoactive substances have variable effect on vasomotor tone at different time periods during newborn life [222]. Vessel reactivity to various constrictor as well as dilator substances is often, but not always, attenuated in species that have chronic hypoxia induced pulmonary vascular disease. For example, chronic hypoxia *in ovo* diminishes chicken pulmonary arterial reactivity to potassium, norepinephrine, ET-1, and thromboxane A<sub>2</sub>. These animals also had higher mortality, reduced body mass and increased RV and LV wall area and thickness [223]. Yet, we also described studies illustrating that vessels from chronic hypoxic piglets have accentuated contractile responses to ET-1. Such hypoxic exposure also decreases eNOS activity in these piglets [184], which would potentiate vessel contractility.

Perinatal hypoxia-induced PH can program the lung for exaggerated HPV response. SD rats initially exposed to prenatal hypoxia (FiO<sub>2</sub> of 0.11, ~5,100 m equivalent altitude) had decreased pulmonary arterial density, though not as severe as that described for FH rats. In this study, prenatal-exposed neonatal SD rats were placed in normoxic conditions for two weeks immediately after birth and then re-exposed to hypoxia two weeks later. Re-exposure of these previously hypoxic-stressed animals accentuated the RV pressure increase compared to rats that had not undergone the initial hypoxic exposure [147]. Our data also show that newborn sheep exposed to antenatal high altitude hypoxia with gestation at 3,801 m have exaggerated HPV responses when exposed acutely to an FiO<sub>2</sub> of 0.10 [224].

Similarly, newborn sheep gestating at 3,600 m on the Andean altiplano also have exaggerated HPV responses when exposed to a  $\text{FiO}_2$  of 0.10 [100, 151]. Newborn infants born at extreme-altitudes (4,540 m Morococha, Peru), where the ambient  $\text{PO}_2$  is ~90 mmHg, exemplify the pulmonary vascular problems with birth at high altitude and illustrate that acute resolution of elevated pulmonary pressures cannot be uncoupled from remodeling of the cardio-pulmonary circuit. These infants had persistently high pulmonary arterial pressures, remaining at ~60 mmHg for the first 72 hours after birth. This contrasts with infants born at sea level where pulmonary arterial pressure falls from ~75 mmHg to ~20 mmHg over that time period. The pressure was inversely correlated to oxygen saturation, and administration of supplemental oxygen decreased pulmonary arterial pressures to sea-level values [10, 225]. Even still, the RV wall thickness is greater in infants born at high altitude throughout their first year of life compared to sea-level controls [226]. This phenomenon is indicative of persistent PH and may cause lifelong complications. Part of the problem is that when an infant is born at high altitude the change in oxygen tension from the maternal circulation to breathing air is less than in low altitude, thus minimizing the stimulus for vasodilation at birth. This may prolong pulmonary vasoconstriction in the infants pulmonary circulation, slow vessel relaxation and the reduction in pulmonary arterial pressure, contributing to increased prevalence of atrial septal defects [227].

### Antenatal hypoxia and vasodilation

Antenatal and perinatal hypoxia can also suppress endothelium-dependent vasodilation. Endothelium-dependent pulmonary arterial relaxation was reduced in newborn piglets exposed to hypobaric hypoxia (~10%  $\text{PO}_2$ , equivalent to ~5,800 m) [207]. What is more, chronic hypoxia (3-10 days at an  $\text{FiO}_2$  of 0.10 to 0.12, equivalent to ~ 4,500-5,800 m) dramatically altered pulmonary arterial responses to ACh in piglets. In this case, ACh dilated endothelium-intact vessels from control animals, compared to those exposed to chronic hypoxia, which exhibited constriction regardless of the status of the arterial endothelium. Interestingly, superoxide scavengers attenuated the vasoconstrictive response. This led the authors to suggest that chronic hypoxia increased NADPH-oxidase-produced reactive oxygen species, which are thought to be important to ACh dependent vasoconstriction [228]. In a similar series of experiments selective inhibition of cyclooxygenase-2 by NS-398, augmented dilatory responses to arachidonic acid in endothelium-denuded hypoxic pulmonary arteries, as well as diminished ACh-induced constriction and thromboxane production in denuded and non-denuded hypoxic pulmonary arteries from newborn piglets [196].

### Ion channels and antenatal hypoxia

Perinatal hypertension and hypoxia cause diverse changes in the function of ion channels expressed on the plasma membrane; channels that are critical regulators of myocyte reactivity and contraction and can contribute to remodeling responses. Previously we provided an overview of the roles of ryanodine receptors,  $\text{InsP}_3$  receptors on the sarcoplasmic reticulum and non-selective,  $\text{K}^+$ , and  $\text{Ca}^{2+}$  channels on the plasma membrane. Presently we know very little regarding the influence of high altitude gestation of ryanodine receptor and  $\text{InsP}_3\text{R}$  signaling. Our initial studies suggest antenatal hypoxia reduces the number of cells that have  $\text{Ca}^{2+}$  responses before and during 5-HT stimulation [64], but the

role of intracellular  $\text{Ca}^{2+}$  release and extracellular  $\text{Ca}^{2+}$  entry to this blunted response is unclear.

$\text{BK}_{\text{Ca}}$  activation on pulmonary arterial myocytes induces vasodilation and is important to vascular relaxation with birth [68]. However, perinatal hypertension due to ductus arteriosus compression can reduce expression of  $\text{BK}_{\text{Ca}}$  channels and their function in the ovine fetal lung, which limits the ability of the arteries to relax [112, 229]. In comparison, 1-2 week old newborn sheep born at 3,600 m have increased  $\text{BK}_{\text{Ca}}$  expression [100]. Interestingly,  $\text{BK}_{\text{Ca}}$  expression is regulated by HIF-1 and therefore this transcription factor plays a key role in hypoxic-regulation of the  $\text{O}_2$  sensitivity of the perinatal pulmonary vasculature [117].

$\text{K}_v$  channels act to maintain a negative resting membrane potential and are an important component to the vasoconstriction response to acute hypoxia [210]. Loss of expression or blocking channel activation causes membrane depolarization, calcium fluxes, and downstream pathway stimulation that lead to smooth muscle cell contraction as illustrated in (Fig. 2). In newborn piglets, extreme chronic hypoxia (ambient  $\text{PO}_2$  of 60-72 mmHg, ~6,200-7,500 m equivalent altitude) caused significant changes in pulmonary arterial myocyte membrane potential and  $\text{K}_v$  channel function [128]. Specifically, the membrane potential of hypoxia-exposed piglet pulmonary arteries was relatively depolarized when compared to their normoxic counterparts. This was accompanied by a selective decrease in  $\text{K}_v1.2$  abundance, but not  $\text{K}_v1.5$  or  $\text{K}_v2.1$  [128]. Furthermore, the generalized importance of  $\text{K}_v$  channels to pulmonary arterial myocyte proliferation [230], suggests these changes will promote pulmonary vascular remodeling [128].

L-type  $\text{Ca}^{2+}$  channel function can also be changed by chronic intrauterine hypoxia. Newborn piglets exposed to severe hypoxia ( $\text{FiO}_2$  of 0.10, ~ 5,800 m equivalent altitude) had PH and elevated  $\text{Ca}_L$  channel-dependent reactivity and vascular tone compared to normoxic piglets [127]. In these hypoxic animals, nifedipine caused a greater decrease in transpulmonary perfusion pressure. This was also associated with an increased nifedipine-sensitive  $\text{Ca}^{2+}$  current density in patch voltage-clamped pulmonary artery smooth muscle cells of hypoxic animals compared to controls [127]. This may not occur in all species however. Our studies in pulmonary arteries from fetal sheep exposed to antenatal hypoxia (3,800 m) show that the role of  $\text{Ca}_L$  channels to potassium or 5-HT-mediated contraction is maintained, suggesting channel expression is preserved [63, 64].

The preceding paragraphs illustrate that although pulmonary vascular growth and vasodilation are dependent on highly organized systems, very little is known about how antenatal hypoxia influences their function. In particular, efforts have focused on just a few of the many potential players and pathways that are important to pulmonary vascular function and growth. Systematic exploration of the various kinase- and ionic- signaling pathways integral to pulmonary vascular function are therefore warranted.

## CURRENT TREATMENT STRATEGIES

The treatment strategies for PH vary significantly based on the etiology of disease. Although there have been significant advances in the pharmacotherapy associated with PAH, most of the data available is for adult patients. Unfortunately, there are very few parallel studies for

pediatric patients. This situation has led to the practice of extrapolating treatment strategies to pediatric patients based on data obtained from adults and on expert consensus [231, 232].

The goal of current treatment for pulmonary hypertension in newborns is the same as for patients of any age and is primarily to increase blood oxygenation, with recent reviews outlining these approaches in detail [233, 234]. In brief, these strategies are based on mimicking what should occur naturally during postnatal transition and in so doing reduce PVR. Often hypoxemia can precipitate pulmonary hypertensive crisis by causing vasoconstriction and increasing PVR, such as occurs when infants are born at high altitude. Neonates with mild respiratory distress may therefore only require minimal oxygen supplementation in order to resolve the crisis. The primary rationale is that by providing the oxygen that is required for vasodilation at birth the arteries will dilate and PVR will fall. The risk of oxygen toxicity is minimal when  $FiO_2$  is increased by administering supplemental  $O_2$  via nasal cannula, but this risk must be considered when prolonged therapy is required to maintain systemic oxygenation. Unfortunately, this type of oxygen therapy may only be beneficial to select infants with very mild disease and is of limited benefit in more serious cases of PH [234, 235].

Moderate to severe hypoxemia and respiratory distress may necessitate mechanical ventilatory support, including high-frequency oscillatory (HFO) ventilation. This method of ventilation carries less risk for barotrauma and atelectrauma compared to more traditional methods of ventilation, but continues to have significant risks, including lower respiratory tract infections, hypoventilation and respiratory acidosis and the need for neuromuscular blockade. Extracorporeal Membrane Oxygenation (ECMO) is generally a last resort, when all other measures of care are exhausted. The process is extremely invasive to the infant, requiring veno-venous access with large bore venous catheters or even veno-arterial access with cannulation of major arteries. The ECMO device, which works as a gas exchange filter, is simple in concept but carries numerous risks mainly associated with the cannulation of major vessels including bleeding, infection, and vascular damage, as well as hemolysis, need for transfusions, and thrombosis.

If oxygen support fails there are a number of therapeutic options that primarily act to induce vasodilation. These include inhaled NO (iNO), phosphodiesterase-5 inhibitors (e.g. sildenafil), prostanoids (eproprostenol and iloprost), endothelin inhibitors (bosentan), and phosphodiesterase-3 inhibitors (milrinone). Although these treatments mainly target the immediate issue of high PVR, some of these (e.g. bosentan) can also suppress vascular remodeling, which is a major component of long-term health problems.

In recent years, nitric oxide inhalation has become an approved treatment for primary pulmonary hypertension of the newborn (PPHN). This therapy improves hemodynamic and oxygenation profiles in multiple pediatric PH populations [236, 237] and lowers pulmonary arterial pressure, improves infant survival, and reduces the need for ECMO [238, 239]. However, approximately 30% of the infants do not respond to iNO. The reason for non-responders is not clear, but may be due to either a dysfunction in guanylate cyclase or PKG signaling. In some cases there may be severe pulmonary hypoplasia to the extent that even when the arteries are dilated there is not an adequate number of vessels to carry the required

blood flow. Even for patients who are responsive to iNO, the high cost and technical administration challenges limit its use to mainly large urban medical centers in developed countries [240-244]. Inhibition of phosphodiesterase-5 with sildenafil works through the same signaling systems as iNO, and has fewer practical concerns, but is limited because it can also cause systemic hypotension. Additionally, it may induce non-selective pulmonary vascular relaxation, increasing shunt fraction in the lung, which would worsen oxygenation. Although this can be a significant issue in newborn infants that have low systemic arterial pressures, there is now evidence from placebo-controlled, randomized [245] and open-label, dose-escalation [246] trials in neonates with severe PPHN that phosphodiesterase-5 inhibitors can improve oxygenation index and survival.

Prostanoids such as iloprost are PGI<sub>2</sub> mimetics that stimulate PKA dependent signaling. Likewise, phosphodiesterase-3 inhibitors, including milrinone, increase cAMP associated PKA activity. As illustrated in (Fig. 3), there is substantial overlap in the targets important to vasodilation that are phosphorylated by PKA and PKG. Although these similarities between PKA and PKG signaling can be used to improve patient outcome, these overlaps also have shortcomings because therapies that target phosphodiesterase-3 carry similar risks as those that target phosphodiesterase-5. Although there is data indicating that the use of prostanoids results in decreased mortality and improved function in adults with PAH, there are few studies looking into prostanoid therapy in pediatric PH populations. Two clinical studies, showed that prostanoid therapy improved survival in 35 children with idiopathic PAH [247] and improved hemodynamics in 20 children with PH due to cardiac defects [248]. Unfortunately, prostanoid therapy is most efficacious when administered continuously intravenously, something that is accompanied by multiple risks including indwelling central venous catheter associated infections and thrombosis. Alternative routes of administration have been developed and include continuous subcutaneous injection, which can cause significant site pain and infection, as well as inhaled mist treatments, which can cause bronchospasm and other upper airway symptoms. In addition, a significant drawback of prostanoid therapy, especially when using short-half-life intravenous infusions, is a desensitization-like effect. These patients require escalating doses and can have a significant rebound effect if the prostanoids are abruptly discontinued.

Endothelin receptor blockers comprise another class of drugs and act on pathways that are unique to many of the drugs mentioned above. Endothelin-1 binds to two distinct G-protein-coupled receptors (ET<sub>A</sub> or ET<sub>B</sub>) that are predominantly expressed on pulmonary vascular smooth muscle, resulting in activation of Rho-kinase and inositol triphosphate pathways that cause vasoconstriction. Bosentan, is an FDA-approved non-specific blocker of both ET<sub>A</sub> and ET<sub>B</sub> for the treatment of adult PAH patients [249]. Although there is some data for functional improvement and exercise capacity in children with either idiopathic or associated PAH [250, 251], the drug is not yet widely used in newborns. Nevertheless there are some early indications from a recent randomized, double-blind, placebo-controlled, prospective clinical trial, that bosentan may be an option for newborns with PPHN [252].

There is now a push for combination therapies. This is based on the understanding that multiple pathways and various receptors are part of PH pathophysiology and that monotherapies often result in higher drug doses and increased side-effects. The combination

therapies are done with the use of two or three pharmacologic agents, each one from a different class of drugs. Based on studies in adults with PAH that have shown modest improvement in hemodynamics and/or exercise capacity, combination therapies are now being used to treat neonatal and pediatric patients, despite the lack of solid data for this patient population.

## FUTURE TREATMENT STRATEGIES

An important consideration in the development and use of new therapies for newborns is that adult treatments cannot always be applied even though some overlap exists. Drugs such as sildenafil, iloprost, and bosentan are of clear relevance in the treatment of PH in adults [233, 234, 253]. Still, there are fewer options for hypertensive newborns, and infants do not always respond favorably to existing treatments. As indicated, as many as 30% of patients with persistent PH of the newborn do not respond to iNO [244]. Moreover, there are few novel therapeutic modalities currently in clinical trials as of June, 2012 [254], necessitating further expansion of our treatment armamentarium.

Future strategies will also need to focus on factors important to the long-term prognosis for infants including suppressing or reversing the arterial wall thickening associated with vascular remodeling. There are practical considerations with therapeutic development because pathways that cause severe remodeling are also critical for normal lung development. For example, inhibiting VEGF in newborn rats impedes normal vascular development and worsens PH and disease [179]. Rho-kinase inhibition can achieve pronounced pulmonary arterial vasodilation but does not affect pulmonary vascular development in newborns with antenatal hypoxia induced hypertension [125]. Such studies illustrate that treatments will need to ensure that lung development continues unabated while remodeling associated with disease is restricted.

There are a number of gene products and associated signaling pathways that have received attention for the treatment of PH in adults that may also hold promise for the treatment of antenatal hypoxia-induced PH in newborns. Particularly interesting is the tyrosine kinase receptor pathway inhibitor imatinib that is in phase III clinical trials for PH in adults [255]. This drug targets the platelet derived growth factor activation pathway and reduces growth and proliferation of smooth muscle. Thus, it has the potential of reversing the course of disease, but because the drug blocks pathways important for normal development, much like VEGF and Rho-kinase, it will need to be examined much further before it can be used for neonatal patients.

Rho kinase inhibition is another pathway that is of great interest, although there are side effects. The pronounced vasodilatory properties of fasudil and other Rho-kinase inhibitors showcase their potential for treatment of PH in newborns and adults [233, 256, 257]. A number of studies show Rho-kinase inhibitors reduce pulmonary arterial pressures in adult patients with idiopathic PH [258] as well as in newborn and adult animal models with PH [85, 125, 259-263].

There are numerous other therapeutic targets for future development including ion channels that are known to regulate the membrane potential of fetal and newborn tissues such as



BK<sub>Ca</sub> [68], as well as others that have yet to be explored in fetus including calcium activated chloride channels [72, 264-266]. Channels that regulate intracellular Ca<sup>2+</sup> including TRP channels [267], ryanodine receptors and InsP<sub>3</sub> metabolism and receptors [76] are important to consider because they regulate contraction as well as cell growth and proliferation. There are also numerous transcriptional regulators that hold future therapeutic promise with HIF-1 $\alpha$  receiving the greatest attention [162]. Other transcription factors are important too, including NF $\kappa$ B that is activated by PDGF and other growth factors [268] as well as NFAT that is important for vascular remodeling [126, 269] and is stimulated by calcineurin through calcium signals downstream from TRP channel activation [270]. Gaseous mediators other than NO are also significant. Carbon monoxide signaling is upregulated in llamas at high altitude [99, 100, 271], and hydrogen sulfide (H<sub>2</sub>S) is an important mediator for HPV responses [221] and protects adult rats from hypoxic induced pulmonary arterial remodeling [272].

### Selective Drug Delivery to the Lung

Strategies that target drugs to lung tissues and cells are important for the future of therapeutics, since they will decrease undesirable systemic effects. Nebulization and aerosolization techniques are being used and developed for this purpose. Although the adaptive aerosol delivery techniques currently available remain imperfect [273], they can be used clinically in neonates to provide surfactant, induce bronchodilation, and provide diuretics [274]. These delivery techniques can be used in conjunction with nanoparticles, which are being formulated to deliver a wide range of drugs and therapies from nifedipine to gene therapy targeting VEGF [275-277]. Designer peptides using targeting residues to focus drugs with widespread systemic effects, such as Rho-kinase inhibitors, to select cells is extremely promising. Recently, researchers designed a cyclic peptide, CARSKNKDC that selectively targeted active vascular remodeling in rat lungs due to monocrotaline or the combination of SU5416 and hypoxia [278]. Such selective drug targeting and delivery is ideal, since it combines therapeutic remodeling prevention with preservation of normal lung development.

## PERSPECTIVE

In this review we provided evidence that antenatal hypoxia, which represents a significant and worldwide problem, causes prenatal programming of the lung. We presented relevant background regarding lung development and the impact antenatal hypoxia has on that process. We also provided an overview of some of the transcriptional and signaling systems of the lung that are impacted by antenatal hypoxia and are likely important for the fetal origins of hypoxia-related disease after birth. There are relatively few studies regarding the impact of antenatal stress on pulmonary vascular remodeling and reactivity, but the studies that have been performed illustrate there is a continuum of responses. Fetuses and newborns of certain species and specific human populations are well acclimated to the rarified environment. However, antenatal hypoxia causes pulmonary vascular disease in most examined mammalian species and humans, which can range from mild pulmonary vascular pressure increases, to severe vascular remodeling and dangerous elevations in pulmonary artery pressure. The effects of antenatal hypoxia are highly dependent on the timing, length,

and magnitude of the stress, but there is also a strong gene-environmental relationship that is not yet completely understood. Determining the origins of pulmonary vascular remodeling and PH and their associated effects is a formidable task, but is necessary in order to develop targeted therapies that can both treat the symptoms and curtail or reverse disease progression.

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## ABBREVIATIONS

|                                      |                                                      |
|--------------------------------------|------------------------------------------------------|
| <b>5-HT</b>                          | Serotonin                                            |
| <b>AC</b>                            | Adenylate Cyclase                                    |
| <b>ACh</b>                           | Acetylcholine                                        |
| <b>BMP</b>                           | Bone Morphogenetic Protein                           |
| <b>BK</b>                            | Bradykinin                                           |
| <b>BK<sub>Ca</sub></b>               | Calcium-and voltage-activated K <sup>+</sup> Channel |
| <b>Ca<sub>L</sub></b>                | L-type Ca <sup>2+</sup> channel                      |
| <b>CAM/CAMKII</b>                    | Calmodulin and Calmodulin kinase type II             |
| <b>ClCa</b>                          | Ca <sup>2+</sup> -dependent chloride conductance     |
| <b>CO</b>                            | Carbon monoxide                                      |
| <b>CPI-17</b>                        | 17 kDa PKC-potentiated inhibitory protein of PP1     |
| <b>DAG</b>                           | Diacylglycerol                                       |
| <b>DLCO</b>                          | Carbon monoxide diffusing capacity                   |
| <b>ECMO</b>                          | Extracorporeal Membrane Oxygenation                  |
| <b>EDHF</b>                          | Endothelium derived hyperpolarization factors        |
| <b>eNOS</b>                          | Endothelial NO synthase                              |
| <b>Epi</b>                           | Epinephrine                                          |
| <b>ET<sub>1</sub></b>                | Endothelin-1                                         |
| <b>ET<sub>A</sub>/ET<sub>B</sub></b> | Endothelin-1 receptors A and B                       |
| <b>FH</b>                            | Fawn-Hooded                                          |
| <b>Gq/Gs</b>                         | G-protein alpha-subunit                              |

|                           |                                                           |
|---------------------------|-----------------------------------------------------------|
| <b>GPCRs</b>              | G-protein coupled receptors                               |
| <b>HAPE</b>               | High altitude pulmonary edema                             |
| <b>HFO</b>                | High Frequency Oscillatory Ventilation                    |
| <b>HIF</b>                | Hypoxia Inducible Factor                                  |
| <b>HO-1 and HO-2</b>      | Heme oxygenase -1 and -2                                  |
| <b>HPV</b>                | Hypoxic pulmonary vasoconstriction                        |
| <b>Hsp90</b>              | Heat shock protein 90                                     |
| <b>INCAS</b>              | International Center for Andean Studies, Chile            |
| <b>iNO</b>                | Inhaled NO                                                |
| <b>InsP<sub>3</sub></b>   | <i>myo</i> -inositol 1,4,5-trisphosphate                  |
| <b>InsP<sub>3</sub>R</b>  | InsP <sub>3</sub> receptors                               |
| <b>K<sub>v</sub></b>      | Voltage-gated K <sup>+</sup> -channels                    |
| <b>LV</b>                 | Left Ventricle                                            |
| <b>MLC<sub>20</sub></b>   | 20 kDa myosin light chain                                 |
| <b>MLC<sub>20</sub>-P</b> | Phosphorylated isoform of 20Kd myosin light chain subunit |
| <b>MCLK</b>               | Myosin light chain kinase                                 |
| <b>MLCP</b>               | Myosin light chain phosphatase                            |
| <b>NFAT</b>               | Nuclear Factor of Activated T Cells                       |
| <b>NO</b>                 | Nitric oxide                                              |
| <b>NOX2</b>               | NADPH oxidase                                             |
| <b>NSSC</b>               | Non-selective cation channels                             |
| <b>PA</b>                 | Pulmonary artery                                          |
| <b>PAF</b>                | Platelet activating factor                                |
| <b>PAH</b>                | Pulmonary Arterial Hypertension                           |
| <b>PaO<sub>2</sub></b>    | Arterial partial pressure of oxygen                       |
| <b>PGF<sub>2α</sub></b>   | Prostaglandin F <sub>2α</sub>                             |
| <b>PGI<sub>2</sub></b>    | Prostacyclin I <sub>2</sub>                               |
| <b>PH</b>                 | Pulmonary Hypertension                                    |
| <b>PKA</b>                | Protein kinase A                                          |
| <b>PKC</b>                | Protein kinase C                                          |
| <b>PKG</b>                | Protein kinase G                                          |
| <b>PLC</b>                | Phospholipase C                                           |

|                       |                                                            |
|-----------------------|------------------------------------------------------------|
| <b>PO<sub>2</sub></b> | Partial pressure of oxygen                                 |
| <b>PP1C</b>           | Protein phosphatase 1C                                     |
| <b>PVR</b>            | Pulmonary Vascular Resistance                              |
| <b>RhoA</b>           | Ras homolog gene family member A                           |
| <b>ROCK</b>           | Rho-associated, coiled-coil containing protein kinase      |
| <b>RV</b>             | Right Ventricle                                            |
| <b>RyR</b>            | Ryanodine receptor                                         |
| <b>SD</b>             | Sprague-Dawley                                             |
| <b>SERCA</b>          | Sarcoplasmic endoplasmic reticulum Ca <sup>2+</sup> ATPase |
| <b>TGFβ</b>           | Transforming growth factor-β                               |
| <b>TRPC</b>           | Canonical transient receptor potential channels            |
| <b>VEGF</b>           | Vascular Endothelial Growth Factor                         |
| <b>WMRS</b>           | White Mountain Research Station                            |

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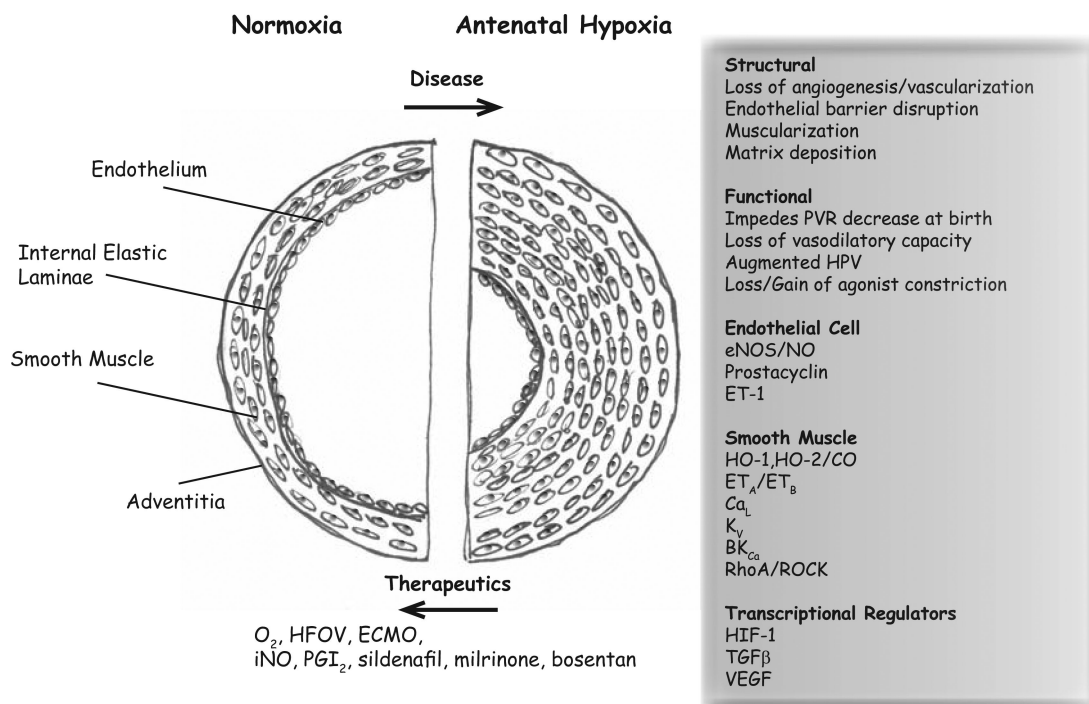
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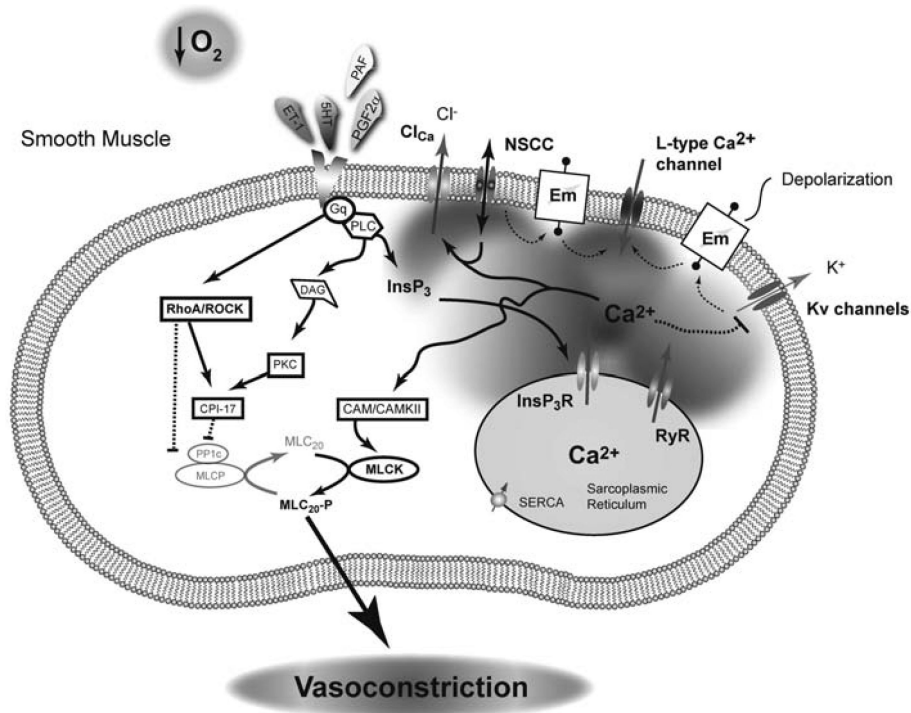
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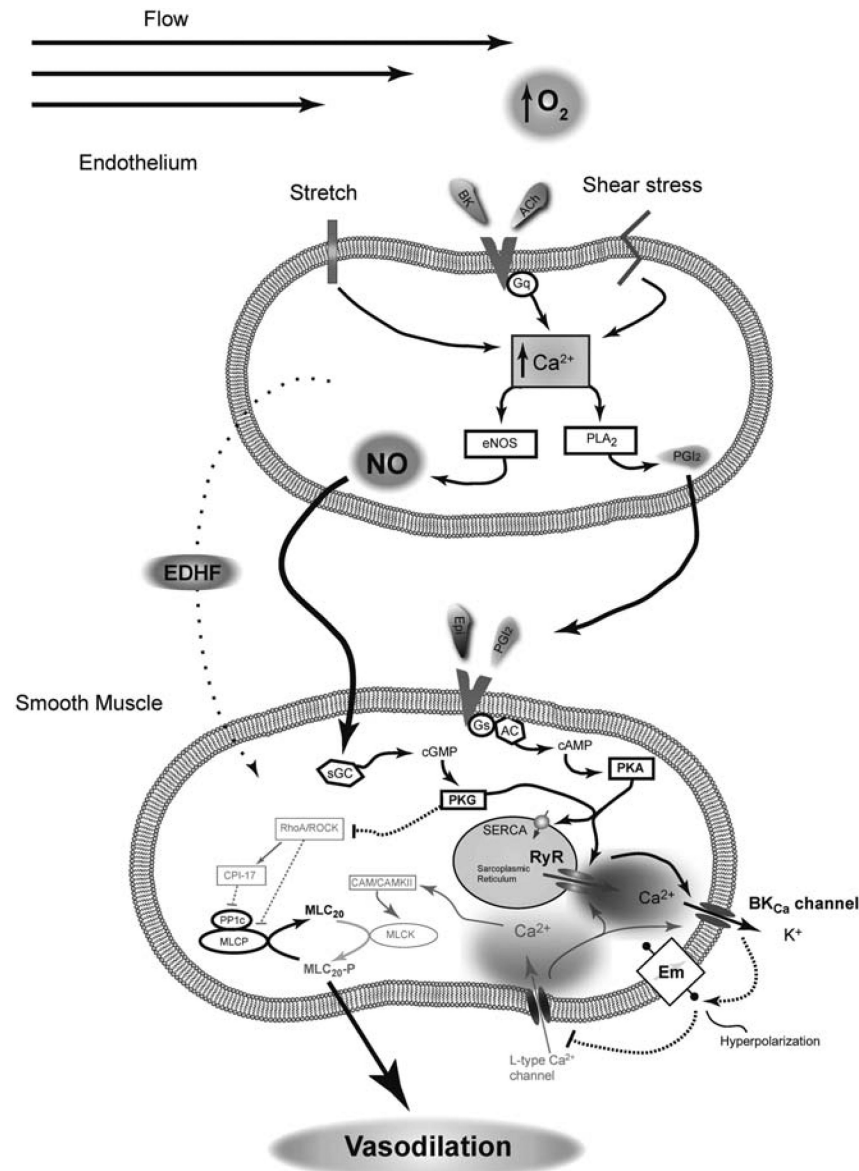
**Fig. (1). The pulmonary arterial wall in normal and antenatal hypoxia diseased lung**  
 In a normal lung the vessel wall and smooth muscle layer is thin. The endothelium lines the lumen of the artery and in distal arteries there is no smooth muscle or elastic lamina. With antenatal hypoxia there can be thickening of the smooth muscle layer that impinges on the arterial lumen along with alterations in myocyte reactivity, as well as disruption of endothelial cell structure with loss of barrier function. These changes are manifested through a number of disruptions involving transcriptional regulators, signaling pathways, and ion channels. The figure summarizes the major components that are discussed in this review.



**Fig. (2). Pulmonary vasoconstriction in the fetus is a highly coordinated process**

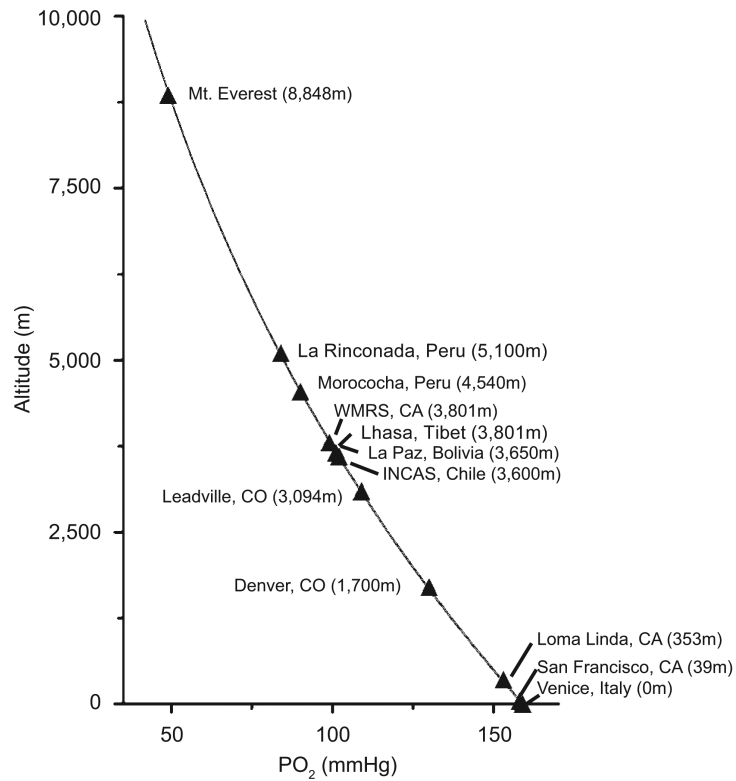
A combination of low oxygen tension and humoral mediators constrict the lung *in-utero*.

The mechanisms associated with hypoxic-induced pulmonary vasoconstriction remain controversial, but a combination of activation of L-type Ca<sup>2+</sup> channels, ryanodine receptors, rho-kinase, non-selective cation channels, and inhibition of K<sup>+</sup> channels are each important in contraction of pulmonary arteries from adult animals. Pulmonary vascular resistance of the fetal lung is thought to be maintained at a high level due to increased vasoactive agonists, including elevated ET-1 levels. The high ET-1, released from the vascular endothelium, works through a Gq coupled receptor to activate a number of intracellular signaling pathways that lead to smooth muscle cell contraction through simultaneous activation of MLCK and inhibition of MLCP. Solid line with arrow: Activation pathway, Dashed line with bar: Inhibition pathway.



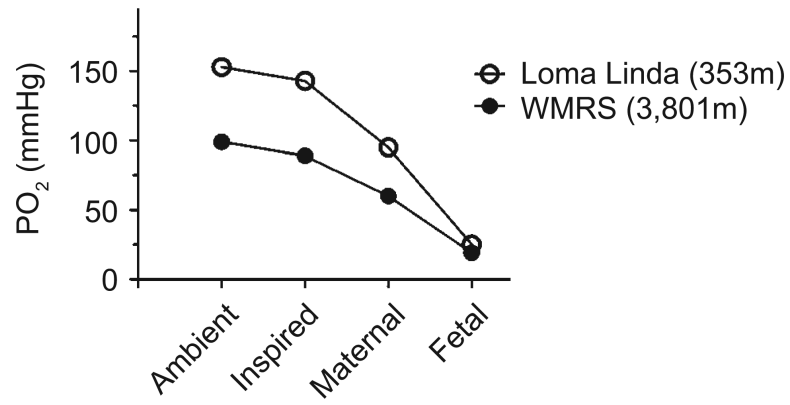
**Fig. (3). Pulmonary vasodilation at birth is orchestrated**

Mechanical forces due to breathing, combined with increases in vascular flow and blood oxygenation act to dilate vessels of the lung. Vasodilatory substances, shear stress and membrane stretch work together to increase prostacyclin (PGI<sub>2</sub>), nitric oxide (NO) production, and other pathways defined broadly as endothelial derived hyperpolarizing factors (EDHF) that have not been fully examined in the fetus. Signaling molecules released from the endothelium act in concert with epinephrine (Epi) and other neuro-humoral substances to increase Protein Kinase A and G activity (PKA and PKG). These kinases phosphorylate a wide array of different substrates to impinge on vascular contraction. As discussed, antenatal hypoxia depresses a number of these vasodilatory signals which leads to maintenance of vasoconstriction. Solid line with arrow: Activation pathway, Dashed line with bar: Inhibition pathway.



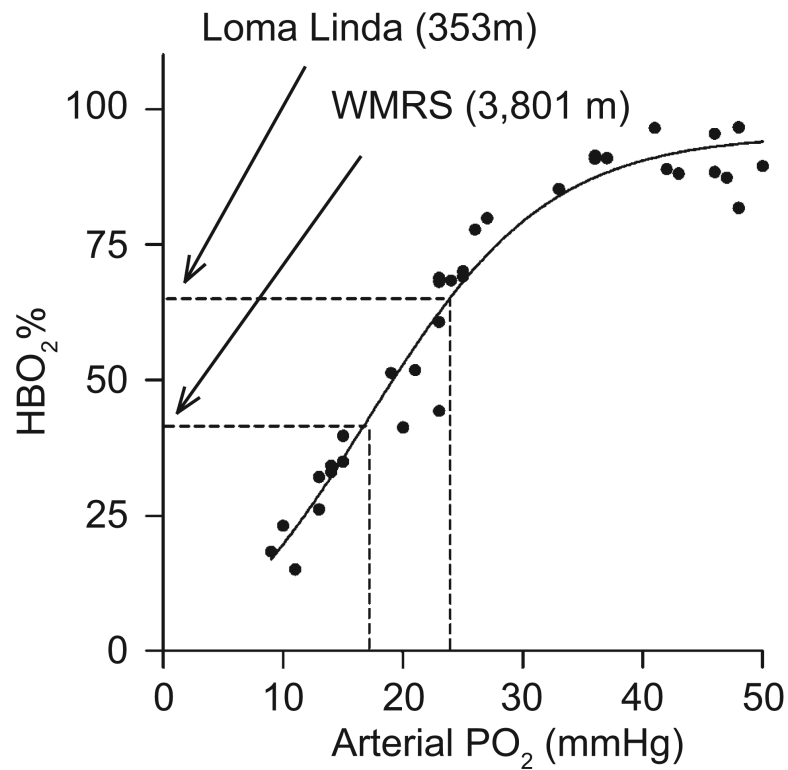
**Fig. (4). High-altitude travel is accompanied by reduced ambient PO<sub>2</sub>**

Human infants are born at altitudes ranging from sea level to about 5,100 m. We highlight work from two high altitude field stations that are at elevations similar to high-altitude cities in Tibet and Bolivia. This graph is based on Boyle's law, where the partial pressure of a gas is inversely related to the altitude.



**Fig. (5). High-altitude decreases maternal and fetal arterial PO<sub>2</sub>**

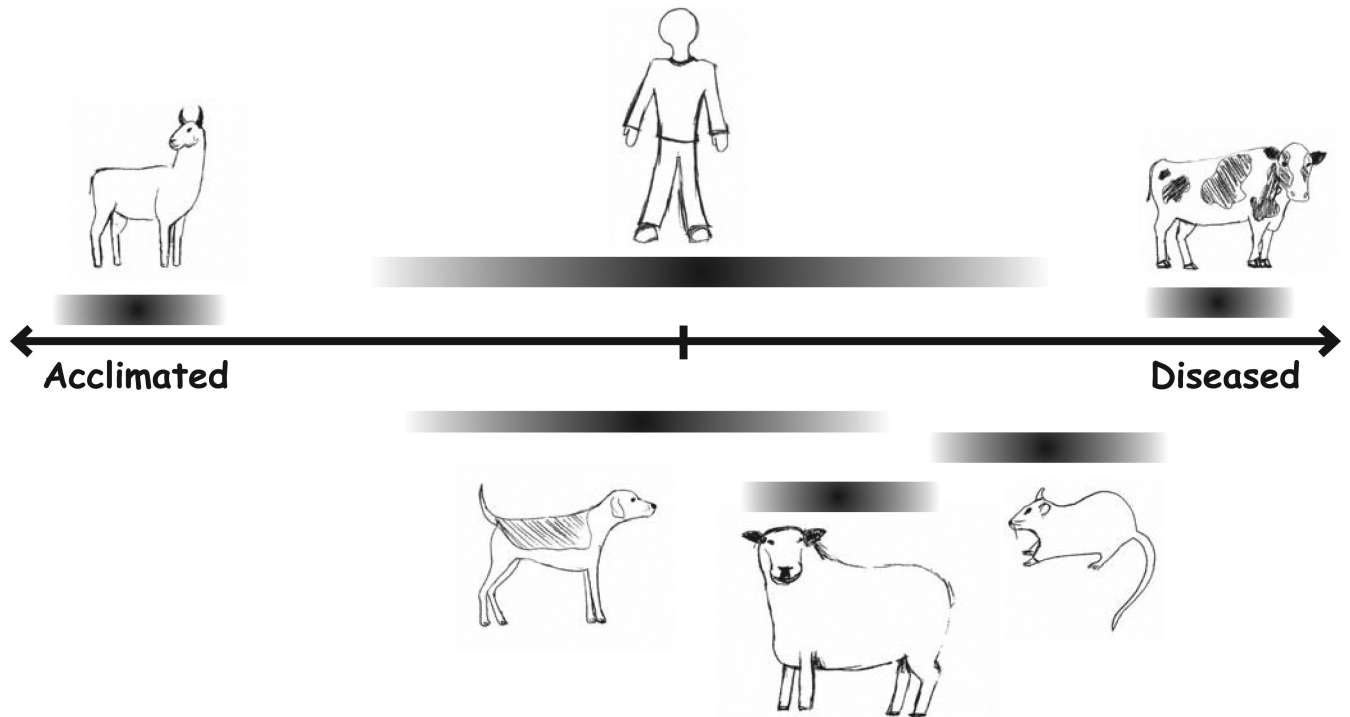
Values were obtained for sheep at altitudes simulating the ambient PO<sub>2</sub> for the Barcroft facilities at the White Mountain Research Station [WMRS]. The maternal PaO<sub>2</sub> is considerably higher than that of the fetus at low altitude and more greatly influenced by exposure to high altitude.



**Fig. (6). Relationship between fetal brachial artery PO<sub>2</sub> and oxyhemoglobin saturation (HbO<sub>2</sub>) from fetal sheep**

Blood samples were collected from chronically instrumented fetuses while the ewe was breathing an FiO<sub>2</sub> of 0.12 to 0.21, or within three hours after cesarean section followed by mechanical ventilation with FiO<sub>2</sub> adjusted to achieve PaO<sub>2</sub> ranging between 25 and 50 mmHg. All lambs had PaCO<sub>2</sub> and pH within normal range. Dashed lines denote brachial PaO<sub>2</sub> and HbO<sub>2</sub> levels for fetal lambs near sea level (Loma Linda) or at high altitude (Barcroft facilities at the White Mountain Research Station [WMRS]). Measurements were made in fetal lambs that were 127 to 130 days gestation.





**Fig. (7). Acclimatization continuum for perinatal hypoxia-induced lung structure and function responses amongst commonly studied species**

As discussed in the text, there is not only a wide degree of variance between individual species but also there can be variability within species. Llamas and cows are on opposite ends of the spectrum and based on the published studies they have lower variability than rats, dogs, or humans. The responses of newborn sheep are less severe than rodents or cows but the dysfunctions are similar to those found in human infants. The length and placement of the bars provides a subjective ranking based on the amalgamation of information provided in the text. We considered the influence of antenatal hypoxia on: decrease of lung diffusion capacity, elevation in pulmonary artery pressures and subsequent hypertrophy of the right ventricle, muscularization of the small vessels and overall vascular remodeling, increased HPV response, increases or decreases in the reactivity of the vessels to agonists as well as endothelial mediated vessel dilation, evidence of intrauterine growth restriction, and survival.

**Table 1**

## Developmental Stages of the Human Lung

| Developmental Stage              | Gestational Age (approximate)                                                | Processes                                                                                                                                                                                                                                                                                                                                                                                                                                           |
|----------------------------------|------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Embryonic period / Organogenesis | Week 1 through 7                                                             | <ul style="list-style-type: none"> <li>- Lungs appear at ~day 26</li> <li>- Early lung lobes form</li> <li>- Dichotomous divisions form the bronchial tree (branching morphogenesis) [27]</li> <li>- Pulmonary arteries [28-31] and veins [32] form via angiogenesis, vasculogenesis or both.</li> <li>- Epithelial-mesenchymal “cross-talk” is important [33-35]</li> </ul>                                                                        |
| Pseudoglandular                  | Week 5 through 17                                                            | <ul style="list-style-type: none"> <li>- Bronchial tree forms to terminal bronchiole via branching morphogenesis [36]</li> <li>- Epithelial-based acinus/alveolus forms via cell differentiation [37, 38]</li> <li>- Arterial and venous system develop in parallel to the airways [31, 32]</li> <li>- Capillary plexus forms connecting arteries and veins [39, 40]</li> <li>- Epithelial-mesenchymal “cross-talk” is important [33-35]</li> </ul> |
| Canalicular                      | Week 16 through 26                                                           | <ul style="list-style-type: none"> <li>- Respiratory epithelial growth and differentiation</li> <li>- Respiratory bronchiole formation</li> <li>- Further development of acinus/alveolus</li> <li>- Lung parenchyma “canalization” by capillaries [24, 26]</li> <li>- Air-blood barrier is formed</li> </ul>                                                                                                                                        |
| Saccular                         | Week 24 through term                                                         | <ul style="list-style-type: none"> <li>- Distal sacculle formation (lung size increases)</li> <li>- Alteration of capillary network (inter-air-space wall capillaries increase in number)</li> <li>- Interstitial elastic fibers are laid and differentiation of cells further advances</li> <li>- Surfactant production by type II epithelial cells begins</li> <li>- Larger vessels muscularize [41]</li> </ul>                                   |
| Alveolarization                  | Week 36 through 18 months post-natally                                       | <ul style="list-style-type: none"> <li>- Alveoli form via septation of the sacculles (~90% post-natally in humans) [24, 25, 42]</li> </ul>                                                                                                                                                                                                                                                                                                          |
| Microvascular Maturation         | Starts shortly after birth and continues through the first few years of life | <ul style="list-style-type: none"> <li>- Capillary network morphs from bi-layered to single-layered through capillary fusion [24, 25, 43]</li> </ul>                                                                                                                                                                                                                                                                                                |

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**Table 2**

Length of Gestation and Developmental Stages of the Lung

| Species    | Length of Gestation (days) | Pseudoglandular Stage * | Canalicular Stage * | Saccular Stage *       | Alveolar Stage *                   | References |
|------------|----------------------------|-------------------------|---------------------|------------------------|------------------------------------|------------|
| Mouse      | 19-21                      | 50 to 85%               | 85 to 90%           | 90% to postnatal day 5 | Begins postnatal day 4             | [48]       |
| Rat        | 21-23                      | 50 to 85%               | 85 to 100%          | 95% to postnatal day 4 | Begins postnatal day 4             | [20, 49]   |
| Rabbit     | 28-35                      | 30 to 80%               | 75 to 90%           | 85 to 100%             | 90 to 100%                         | [20]       |
| Guinea Pig | 58-75                      | 50 to 60%               | 60 to 75%           | 75 to 100%             | 80 to 100%                         | [50]       |
| Pig        | 101-130                    | Completed by 50%        | 45 to 80%           | 75 to 100%             | 90% to postnatal day 14            | [51]       |
| Sheep      | 145-150                    | Completed by 60%        | 50 to 80%           | 80 to 90%              | 85% to day 14                      | [20]       |
| Human      | 270                        | Completed by 50%        | 50 to 75%           | 60 to 100%             | 100% to several months postnatally | [20]       |
| Cow        | 280-290                    | Completed by 45%        | 45 to 65%           | 65 to 80%              | 80 to 90%                          | [52]       |

\* Percentages refer to the portion of gestation that has completed at the beginning and end of each respective developmental stage