Mechanisms of Right-Ventricular Dysfunction in a Rat Model of Chronic Neonatal Pulmonary Hypertension

by

Kiranjot Gosal

A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Physiology
University of Toronto

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2013

Abstract

Chronic neonatal pulmonary hypertension (PHT) frequently presents with right-ventricular (RV) dysfunction. In neonatal rats exposed to chronic hypoxia, RV dysfunction is reversed by sustained rescue treatment with a Rho-kinase (ROCK) inhibitor – the caveat being systemic hypotension. We therefore examined the reversing effects of pulmonary-selective ROCK inhibition. Rat pups were exposed to air or hypoxia from birth for 21 days and received sustained rescue treatment with aerosolized Fasudil (81 mg/ml t.i.d for 15 min) or i.p. Y27632 (15 mg/kg b.i.d) from days 14-21. Inhaled Fasudil normalized pulmonary vascular resistance, and reversed pulmonary vascular remodeling but did not improve RV systolic function. Systemic, but not pulmonary-selective, ROCK inhibition attenuated increased RV ROCK activity. Our findings indicate that RV dysfunction in chronic hypoxic PHT is not merely a result of increased afterload, but rather may be due to increased activity of ROCK in the right ventricle.
ACKNOWLEDGEMENTS

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>BPD</td>
<td>Bronchopulmonary dysplasia</td>
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<tr>
<td>C-terminus</td>
<td>Carboxyl-terminus</td>
</tr>
<tr>
<td>Ca$$^{2+}$$</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>Ca$$^{2+}$$-CaM</td>
<td>Calcium-calmodulin complex</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine 3’ ,5’-monophosphate</td>
</tr>
<tr>
<td>CO</td>
<td>Cardiac output</td>
</tr>
<tr>
<td>CPI-17</td>
<td>Protein phosphatase 1 regulatory subunit 14A</td>
</tr>
<tr>
<td>DHEA</td>
<td>Dihydroepiandrosterone</td>
</tr>
<tr>
<td>Ea</td>
<td>Arterial elastance (measure of afterload)</td>
</tr>
<tr>
<td>ECMO</td>
<td>Extracorporeal membrane oxygenation</td>
</tr>
<tr>
<td>EDV</td>
<td>End-diastolic volume</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ESP</td>
<td>End systolic pressure</td>
</tr>
<tr>
<td>ET</td>
<td>Endothelin</td>
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<tr>
<td>GAP</td>
<td>GTPase-activating proteins</td>
</tr>
<tr>
<td>GDI</td>
<td>GDP dissociation inhibitors</td>
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<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factors</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine 5’-triphosphate</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>iNO</td>
<td>Inhaled nitric oxide</td>
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<tr>
<td>LIMK1</td>
<td>LIM-kinase 1</td>
</tr>
<tr>
<td>LV</td>
<td>Left-ventricular</td>
</tr>
<tr>
<td>MLC</td>
<td>Myosin light chain</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
</tr>
<tr>
<td>MLCP</td>
<td>Myosin light chain phosphatase</td>
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<tr>
<td>MLI</td>
<td>Mean linear intercept</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>MPAP</td>
<td>Mean pulmonary arterial pressure</td>
</tr>
<tr>
<td>MSAP</td>
<td>Mean systemic arterial pressure</td>
</tr>
<tr>
<td>MYPT-1</td>
<td>Myosin light chain phosphatase targeting subunit 1</td>
</tr>
<tr>
<td>N-terminus</td>
<td>Amino-terminus</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NO-cGMP</td>
<td>Nitric oxide – cyclic guanosine monophosphate</td>
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<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>ONOO-</td>
<td>Peroxynitrite anion</td>
</tr>
<tr>
<td>PAAT</td>
<td>Pulmonary arterial acceleration time</td>
</tr>
<tr>
<td>PAP</td>
<td>Pulmonary arterial pressure</td>
</tr>
<tr>
<td>PASP</td>
<td>Pulmonary arterial systolic pressure</td>
</tr>
<tr>
<td>PAVTI</td>
<td>Pulmonary artery velocity time integral</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<tr>
<td>PDGF-R</td>
<td>Platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrein-homology</td>
</tr>
<tr>
<td>PHT</td>
<td>Pulmonary hypertension</td>
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<tr>
<td>PKG</td>
<td>Protein kinase G</td>
</tr>
<tr>
<td>PND</td>
<td>Postnatal day</td>
</tr>
<tr>
<td>PPHN</td>
<td>Persistent pulmonary hypertension of the newborn</td>
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<tr>
<td>PVR</td>
<td>Pulmonary vascular resistance</td>
</tr>
<tr>
<td>RBD</td>
<td>Rho-binding domain</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated protein kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RV</td>
<td>Right-ventricular</td>
</tr>
<tr>
<td>RVET</td>
<td>Right-ventricular ejection time</td>
</tr>
<tr>
<td>RVH</td>
<td>Right-ventricular hypertrophy</td>
</tr>
<tr>
<td>RVSP</td>
<td>Right-ventricular systolic pressure</td>
</tr>
<tr>
<td>SD</td>
<td>Sprague Dawley</td>
</tr>
<tr>
<td>sGC</td>
<td>Soluble guanylate cyclase</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>SSRI</td>
<td>Selective serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>SV</td>
<td>Stroke volume</td>
</tr>
<tr>
<td>SVR</td>
<td>Systemic vascular resistance</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
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</table>
Chapter 1

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Introduction
1.1 General Description:

Chronic pulmonary hypertension (PHT) is a rare, incurable disease with a poor prognosis. It is characterized by an increase in pulmonary arterial pressure (PAP) and pulmonary vascular resistance (PVR) (Duong-Quy et al., 2013). The resultant chronic increase in afterload is generally not well tolerated by the right ventricle, especially in infants, leading to dysfunction and, ultimately, death (Bronicki and Baden, 2010). In North America, persistent pulmonary hypertension of the newborn (PPHN) affects approximately 2% of live births and is responsible for one third of neonatal mortality. It complicates the course of approximately 10% of infants with respiratory failure (Steinhorn, 2010). In the mid-1980s, PPHN had a mortality rate of 11-34% (Hageman et al., 1984; Davis et al., 1988; John et al., 1988), which has since fallen to between 5 and 10% (Konduri et al., 2004), but remains associated with a prevalence of major neurodevelopmental impairment in survivors of at least 25% (Steinhorn, 2010). Pulmonary hypertension in early life may be characterized into one of the following three types: i) abnormally constricted pulmonary vasculature due to lung parenchymal diseases (e.g., meconium aspiration syndrome, respiratory distress syndrome or pneumonia); ii) lung with normal parenchyma but remodeled pulmonary vasculature (i.e., idiopathic PPHN); or iii) hypoplastic vasculature. Idiopathic PHT is responsible for 10-20% of all cases of PPHN in infants, while severe fatal cases are usually a result of underlying congenital parenchymal and vascular maldevelopment (Steinhorn, 2010). Newborns have a greater tendency to develop PHT when compared to the adult population (van Loon et al., 2010). This is a result of two factors: failure of the fetal to newborn vascular transition and the rapid development of structural changes in the pulmonary vasculature. The latter is believed to contribute to a ‘fixed’ increase in PVR (van Loon et al., 2010).
The primary goal of current therapies is to achieve pulmonary-selective vasodilation (Abman, 2010). The current gold-standard treatment for PPHN is inhaled nitric oxide (iNO), which is approved by Health Canada and the United States Food and Drug Administration, for use in neonates above 35 weeks of gestational age. Nitric oxide (NO) is a small gaseous molecule that is a rapid and potent vasodilator (Steinhorn, 2010) and, due to its diffusibility, can effectively be delivered as an inhalation therapy (Reeves et al., 1986; Durmowicz and Stenmark, 1999).

However, there are a number of disadvantages associated with iNO use. Approximately 40% of newborns treated with iNO are unresponsive. Patients that initially responded often rebound during weaning attempts, or show a non-sustained response (Konduri et al., 2004; Konduri et al., 2007). In large placebo-controlled trials, iNO therapy was shown to decrease the need for extracorporeal life support in newborns with PPHN (1997; Clark et al., 2000). While iNO is the most commonly used therapy for PPHN, it has not been shown to decrease mortality, length of hospitalization or to reduce the risk of neurodevelopmental abnormalities, such as cerebral palsy. Beginning iNO therapy during an earlier or milder point in the disease shows a trend towards decreased incidence of extracorporeal membrane oxygenation (ECMO) and/or death, although this has not shown to be statistically significant (Konduri and Kim, 2009). The gas and delivery system required for iNO is very costly, therefore limiting its use in developing nations (Ho and Rasa, 2007). In an experimental model of neonatal chronic PHT, prolonged iNO therapy was shown to have no effect on vascular remodeling or right-ventricular (RV) dysfunction (Jankov et al., 2010). Although the use of ECMO has been shown to decrease mortality in humans, it has also been associated with increased risk of intracranial hemorrhage.
(Steinhorn, 2010). Therefore, the investigation of novel and more effective therapies is of critical importance.

1.2 Fetal to Newborn Cardiopulmonary Transition:

For the fetus to effectively transition from the prenatal to postnatal environment, there must be dramatic circulatory changes at birth. In the fetus, pulmonary hypertension is a normal and necessary state. During fetal life, the placenta serves as the primary organ for gas exchange, with only 5-10% of the RV output passing through the pulmonary vasculature (Steinhorn, 2010). The majority of the output bypasses the pulmonary vasculature via 2 shunts – the foramen ovale and ductus arteriosus (Figure 1.1). Constriction of the muscular pulmonary arteries results in increasing PVR with gestational age. The high resistance results in equivalent pulmonary and systemic pressures \emph{in utero}. A successful cardiopulmonary transition requires elimination of placental circulation, expansion of the lungs and closure of the fetal shunts. In order to facilitate the transition to gas exchange by the lung, there is a fall in PVR and PAP, as well as a 10-fold increase in pulmonary blood flow (Steinhorn, 2010). Immediately following birth, there is a dramatic decrease in pulmonary vascular tone, resulting in dilatation of the pulmonary vasculature. This is a result of lung expansion and higher oxygen tension of ambient air. Over time, a decrease in the muscularization of pulmonary arteries, and increased number of non-muscular pulmonary arteries further contributes to the fall in PVR. PPHN results from the failure of a sustained fall in PVR (Niermeyer, 2003).
Figure 1.1: A schematic showing the fetal circulation pattern. The placenta is responsible for oxygenating blood, which travels from the umbilical vein through the ductus venosus into the inferior vena cava. It then enters the right atrium through to the right ventricle and into the pulmonary artery. Since the lungs are not responsible for gas exchange, high pulmonary vascular resistance and an open ductus arteriosus allow the majority of the blood to be shunted into the descending aorta. Blood entering the right atrium also travels through the foramen ovale into the left atrium, into the left ventricle and out into the aorta. (Source: http://www.lpch.org/DiseaseHealthInfo/HealthLibrary/hrnewborn/0181-pop.html)
1.3 Sustained Pulmonary Vasoconstriction:

Throughout the progression of pulmonary vascular disease, arteries transition from a normal physiological state to that of severe vascular remodeling. Under normal conditions, the pulmonary arterial walls are thin and compliant. The progression of chronic PHT is characterized by abnormal endothelium, non-compliant and constricted pulmonary resistance arteries and a gradual loss of microvessels.

![Diagram showing changes in pulmonary arteries](image)

**Figure 1.2:** A schematic diagram showing the changes in pulmonary arteries from a normal physiological to a severely remodeled state during pulmonary vascular disease. During the normal state, pulmonary arteries are thin walled and have healthy endothelium. During the compensatory phase, pulmonary arteries have abnormal endothelium and become constricted and stiff. This results in a mild increase in pulmonary vascular resistance (PVR) and a slight decrease in diffusion. Late in the disease, there is significant cell proliferation in the pulmonary arterial walls and vascular remodeling. This results in severely increased PVR and decreased perfusion. Image adapted from Champion HC et al., *Circulation*, 2009; 120: 992-1007.

Relaxation and contraction of smooth muscle is under the regulation of the myosin light chain (MLC)-signaling pathway. Maintenance of low PVR is dependent upon sustained vasodilatation. In chronic PHT, the function of this signaling pathway is interrupted, such that smooth muscle in the pulmonary arterial wall is no longer able to relax. This results in sustained pulmonary vasoconstriction (Somlyo and Somlyo, 1994).
1.4 Structural Changes in Vasculature:

Another factor that contributes to a higher tendency for newborns to develop PHT is the rapid development of structural changes in cardiac and pulmonary tissue. The development and persistence of structural and anatomical changes in the heart and pulmonary vasculature is termed ‘vascular remodeling’. Vascular remodeling is a pathological hallmark of PHT and is characterized by a narrowing of the vessel lumen and is associated with an exaggerated response to vasoconstrictors and decreased response to vasodilators (Guilluy et al., 2009). Pathologically, there is thickening of all three layers of pulmonary vessels: the media, intima and adventitia. There is hypertrophy and hyperplasia of many cell types, including endothelial cells, smooth muscle cells (SMC) and fibroblasts. In the vascular walls, there is accumulation of extracellular matrix components, such as collagen, elastin, fibronectin and tenascin. The imbalance of proliferative and apoptotic effects on the SMC results in medial hypertrophy, and thus a reduced vascular lumen (Jeffery and Wanstall, 2001). Extension of smooth muscle into non-muscular arteries, termed distal muscularization, further contributes to increased PVR (Kinsella and Abman, 1996). Once remodeling is established, it is believed to be irreversible with current therapies, which contributes to incomplete responses to current therapies (Durmowicz and Stenmark, 1999). These functional consequences result in a ‘fixed’ increase in PVR. This increased PVR affects the right heart by increasing RV afterload, resulting in RV hypertrophy (RVH). Pulmonary arterial muscle mass is closely related to RV weight, which makes the degree of RVH a reliable quantitative marker of the severity of PHT (Rabinovitch et al., 1979).

1.5 Right-Ventricular Dysfunction:
Under normal physiological conditions, the right ventricle is thin walled and crescent shaped. It works against a low resistance and pressure. During the compensatory phase of pulmonary vascular disease, the right ventricle has to work against an increased afterload, resulting in hypertrophy of the muscular free wall. Over time, the right heart can no longer tolerate the increased afterload, and cardiac output (CO) begins to fall. As right heart failure progresses, the ventricle becomes dilated, and is characterized by an increased end-diastolic volume (EDV). The end stage is characterized by decreased stroke volume (SV) and therefore, decreased CO, which is rapidly fatal (Bronicki and Baden, 2010).

**Figure 1.3:** A schematic diagram showing the changes in the right ventricle from a normal to failing state during pulmonary vascular disease. During a normal physiological state, the right ventricle is thin walled and has a crescent-like shape, and is associated with a normal cardiac output (CO). During the compensatory phase, the right ventricle becomes hypertrophied, while adequate CO is maintained. During failure, the right ventricle becomes dilated and there is a severe decrease in CO, which eventually proves to be fatal. Image adapted from Champion HC et al., *Circulation*, 2009; 120: 992-1007.

There is a greater understanding of the mechanisms leading to disease in the pulmonary vasculature than the right ventricle in chronic PHT. Due to this lack of knowledge, there has been an unbalanced approach in designing therapies, as current therapies primarily target the pulmonary vasculature. A better understanding of the determinants of RV dysfunction will help lead to therapies that specifically target RV dysfunction, which ultimately, is the cause of death in patients with chronic PHT (Champion et al., 2009).
1.6 Disease Etiology

As described earlier, three anatomic types of PHT exist, based on the pulmonary vascular morphology. These include underdevelopment (or hypoplasia), maldevelopment and maladaptation of the pulmonary vascular bed. PPHN can be affected by parenchymal or vascular disease, or can be referred to as idiopathic, in which there is normal lung parenchyma and pulmonary vascular remodeling (Steinhorn, 2010).

Intrauterine abnormalities may disturb normal lung development, decreasing the number of bronchial branches and number and size of alveoli, thus resulting in underdevelopment of the lung (Graves et al., 1988). In the case of underdeveloped pulmonary vasculature, increased PVR remains relatively permanent. This type of PHT is found in conditions such as congenital diaphragmatic hernia, pulmonary hypoplasia syndromes and chronic lung disease of prematurity, also known as bronchopulmonary dysplasia (BPD) (Levin et al., 1979). BPD is characterized by arrested alveolar and pulmonary vascular development and affects up to 60% of extremely premature infants of whom 25-40% develop chronic PHT, which predicts poor outcome (Khemani et al., 2007; Farquhar and Fitzgerald, 2010). Chronic PHT due to underdevelopment of the pulmonary vasculature is often irreversible and therefore patients are difficult to treat and have the worst outcomes (Graves et al., 1988).

Maldevelopment of the pulmonary vasculature is associated with excessive muscularization. Normally, muscular arteries extend no further than the terminal bronchiolus. By adulthood, these arteries may be found at the alveolar wall. In neonatal PHT, muscular arteries are found at the alveolar wall, similar to that of a normal adult. This increased muscularization reduces the vascular lumen, increasing PVR (Graves et al., 1988). The use of certain pharmaceutical agents, such as non-steroidal anti-inflammatory drugs (NSAIDs) (particularly
aspirin, ibuprofen or naproxen) (Levin et al., 1979; Alano et al., 2001) and/or selective serotonin reuptake inhibitors (SSRIs) (Chambers et al., 2006; Fornaro et al., 2007) during pregnancy can result in an increased incidence of PPHN, by promoting excessive in utero muscularization (Graves et al., 1988). Interestingly, SSRIs in adults reduce pulmonary vascular remodeling (Steinhorn, 2010). This observation further highlights the unique nature of neonatal PHT. In most cases, however, the cause of excessive muscularization is unknown.

PHT may also result from maladaptation of the fetal pulmonary vasculature, meaning that the pulmonary vascular bed is poorly equipped to adapt to extrauterine life. In these patients, the normal decrease in PVR and increase in compliance fails to occur. Hypoxia, due to airway obstruction or primary parenchymal disease, causes vasoconstriction and reduced vascular compliance (Graves et al., 1988). Maladaptation can result from conditions such as perinatal asphyxia, meconium aspiration, congenital pneumonia and congenital sepsis (Dakshinamurti, 2005).

1.7 Molecular Signaling Pathways:

The regulation of smooth muscle contraction and relaxation involves the MLC-signaling pathway, which involves both calcium-dependent and independent mechanisms. G-protein coupled receptors (GPCR), located at the plasma membrane of smooth muscle surrounding pulmonary arterioles, are activated by hypoxia, as well as GPCR ligands, such as endothelin (ET)-1 and thromboxane A_2 (Somlyo and Somlyo, 2003). Activation of GPCRs results in downstream signaling cascades, which in turn opens up calcium ion (Ca^{2+}) channels at the plasma membrane, as well as at the sarcoplasmic reticulum membranes within the SMC. Intracellular Ca^{2+} from the sarcoplasmic reticulum binds to calmodulin, leading to the formation
of the Ca\textsuperscript{2+} - calmodulin complex (Ca\textsuperscript{2+}-CaM). Ca\textsuperscript{2+}-CaM activates myosin light chain kinase (MLCK), which in turn activates MLC (also known as MLC-2 or LC-20), through phosphorylation. MLC activation activates cross-bridge cycling, and thus smooth muscle contraction. Through dephosphorylation, myosin light chain phosphatase (MLCP) inactivates MLC, leading to smooth muscle relaxation (Figure 1.4) (Somlyo and Somlyo, 1994).
Figure 1.4: A schematic diagram showing a simplified version of the myosin light chain-signaling pathway. G protein coupled-receptors are activated, opening up calcium ion (Ca^{2+}) channels. Ca^{2+} enters the cell and forms the Ca^{2+} - calmodulin (CaM) complex. Ca^{2+}-CaM activates myosin light chain kinase (MLCK), which in turn activates myosin light chain, through phosphorylation. This leads to increased smooth muscle contraction. Myosin light chain phosphatase (MLCP) is responsible for inactivating myosin light chain, through dephosphorylation, leading to smooth muscle relaxation.
1.7a RhoA/Rho-kinase Signaling Pathway:

Sustained vasoconstriction may also result from ‘calcium sensitization’. This phenomenon involves smooth muscle contraction without a change in Ca\(^{2+}\) concentration. It is thought that Ca\(^{2+}\) sensitization occurs primarily due to phosphorylation and thus deactivation of MLCP. When MLCP is phosphorylated, it cannot dephosphorylate MLC, leading to sustained muscle contraction. Deactivation of MLCP involves a small protein, RhoA, which belongs to the Rho-GTP protein family, as well as its downstream effector, Rho-associated protein kinase (ROCK). ROCK phosphorylates the MLCP targeting subunit (MYPT)-1 at threonine (Thr)-696, Thr-853 (Thr-850 in the rat) and/or Thr-855, resulting in MLCP inhibition, and increased MLC activity (Figure 1.5) (Oka et al., 2008). There are two isoforms of ROCK, ROCK I/ROCK \(\beta\) and ROCK II/ROCK \(\alpha\), both of which are ubiquitously expressed in the vasculature (Figure 1.6). ROCKs are serine-threonine kinases with a molecular mass of \(~160\) kD. ROCKs are formed by parallel homodimers, which include a catalytic (kinase) domain in its amino (N)-terminus, a coiled-coil in its middle dimerization portion, and a putative Pleckstrein-homology (PH) domain in its carboxyl (C)-terminus. An autoinhibitory region, located in the C-terminus, reduces the kinase activity of ROCKs. The Rho-binding domain (RBD) is located in the C-terminus of the coiled coil region.

ROCK activity is regulated in a variety of ways. The C-terminus of ROCK acts as a dominant-negative autoinhibitor (Amano et al., 1999). When ROCK is truncated, it becomes constitutively activated. Caspase-3 cleaves ROCK I at a conserved sequence, which removes the inhibitory C-terminus domain. This results in de-regulated and continuous kinase activity (Sebbagh et al., 2001). Similarly, cleavage of ROCK II at the C-terminus by granzyme B also leads to removal of the inhibitory region and constitutive kinase activity (Sebbagh et al., 2005).
ROCK activity is also influenced by its affinity for ATP (Doran et al., 2004). GPCRs are activated at the smooth muscle plasma membrane. This activates guanine nucleotide exchange factors (GEF), which causes GDP to be replaced by GTP on the RhoA-GTPase. When GTPase-RhoA binds to ROCK at the RBD, a conformational change is induced, which stops the autoinhibitory blockade of kinase activity. This binding is believed to stimulate ROCK’s phosphotransferase activity (Doran et al., 2004). Apart from RhoA, ROCK activity can be activated by arachidonic acid, sphingosine phosphorylcholine, caspase-3 or granzyme-B (Sebbagh et al., 2001; Shirao et al., 2002; Sebbagh et al., 2005). ROCK activity can be negatively regulated by G-binding proteins such as RhoE, Gem and Rad (Ward et al., 2002). Other negative regulators include GDP dissociation inhibitors (GDI), which inhibit the exchange of GDP by GTP, GTPase-activating proteins (GAP), which convert RhoA-GTP to RhoA-GDP, as well as protein kinases A, G and statins, all of which prevent the translocation of RhoA-GTP to the plasma membrane (Riento et al., 2003).

The potential role of ROCKs in the pathophysiology of PHT has been of considerable interest, as there is increasing evidence showing their involvement in pulmonary arterial SMC contraction and proliferation. There are various other downstream targets of RhoA/ROCK that may play a role in the pathophysiology of PHT. LIM-kinase (LIMK)-1 is activated by RhoA/ROCK by phosphorylation of Thr-508. LIMK-1 in turn phosphorylates cofilin, an actin-depolymerizing factor, which plays a role in actin cytoskeletal reorganization. LIMK-1 is also activated by PAK1, a downstream effector of Rac (Ohashi et al., 2000). Protein phosphatase 1 regulatory subunit 14A (CPI-17) is a phosphorylation-dependent protein that is expressed in smooth muscle cells. It inhibits MLCP, thus, preventing smooth muscle relaxation (Niiro et al., 2003). ROCK activates CPI-17 by phosphorylating Thr-38. Activated CPI-17 in turn leads to
smooth muscle contraction (Koyama et al., 2000). Protein kinase C (PKC) also activates CPI-17 (Mueed et al., 2005), further contributing to Ca$^{2+}$ sensitization. PKC has been shown to be upstream of ROCK as it is important in RhoA-mediated activation of ROCK (Barandier et al., 2003).

Figure 1.5: A schematic diagram showing a simplified version of the RhoA/Rho-kinase pathway. Guanine nucleotide exchange factors (GEF) convert RhoA-GDP to RhoA-GTP. RhoA-GTP activates Rho-kinase (ROCK), which inhibits the activity of myosin light chain phosphatase (MLCP). MLCP can no longer dephosphorylate myosin light chain (MLC), therefore MLC remains in its activated form. This leads to sustained vasoconstriction.
Figure 1.6: A diagram showing the molecular structure of the two ROCK isoforms, ROCK 1 and 2. The kinase domain is located in the N terminus of the protein, the coiled-coil region contains the Rho-binding domain (RBD), and the Pleckstrin-homology (PH) with a cysteine-rich domain (CRD) is in the C terminus of the protein. Image from Duong-Quy S. et al., *Pharmacol Ther* 2012; 137(3): 353-64.

1.7b NO-cGMP Signaling Pathway:

Another pathway that is important in regulating smooth muscle vascular tone is the nitric oxide – cyclic guanosine monophosphate (NO-cGMP) pathway (Figure 1.7). NO is a signaling agent that is produced from the amino acid L-arginine, in endothelial cells, by endothelial nitric oxide synthase (eNOS). NO diffuses into the smooth muscle cell and activates its receptor, soluble guanylate cyclase (sGC). sGC converts guanosine 5’-triphosphate (GTP) into cyclic guanosine 3’,5’-monophosphate (cGMP). Increased production of cGMP results in Ca$^{2+}$ desensitization and smooth muscle relaxation, through increased protein kinase G (PKG) activity (Abman, 2007).

NO can react with the oxygen radical superoxide to form the peroxynitrite anion (ONOO$^-$), limiting its bioavailability and contributing to the pathogenesis of PHT (Belik et al., 2004; Jankov et al., 2010). Additionally, low concentrations of L-arginine, an NO precursor, oxidization of sGC and an increase in phosphodiesterase (PDE) 5 activity (a cGMP degrading enzyme) are all factors that contribute to NO-cGMP pathway dysfunction, and consequently,
PHT. The RhoA/ROCK and NO-cGMP pathways interact in a reciprocal fashion, as the increased activity of one causes decreased activity of the other. For example, PKG inhibits RhoA and ROCK activation, by phosphorylating Ser-188 (Sawada et al., 2001) and Ser-695/852 of myosin phosphatase target subunit (MYPT)-1 (Gao et al., 2008), respectively, stimulation of cGMP-mediated pathways results in decreased expression of ET-1 (Kourembanas et al., 1993) and increased ROCK activity leads to down-regulation of eNOS expression and activity (Abe et al., 2006).

Figure 1.7: A schematic diagram showing a simplified version of the nitric oxide – cyclic guanosine monophosphate (NO-cGMP) pathway. Endothelial nitric oxide synthase (eNOS) converts L-arginine to NO. NO activates its receptor, soluble guanylate cyclase (sGC), which in turn converts guanosine 5’-triphosphate (GTP) into cyclic guanosine 3’,5’-monophosphate (cGMP). Increased production of cGMP results in Ca\(^{2+}\) desensitization and smooth muscle relaxation, through increased protein kinase G (PKG) activity. Phosphodiesterase (PDE) 5 degrades cGMP into guanosine monophosphate (5’-GMP).
1.8 ROCK Inhibitors as Novel Treatment for PHT:

Two pharmacological ROCK inhibitors, Y-27632 and Fasudil (HA-1077) (Figure 1.8), have been shown to have high specificity toward the two known ROCK isoforms (ROCK I and II), when compared to other kinases. Y-27632 is a pyridine derivative (Uehata et al., 1997). It inhibits the kinase activity of both ROCK I and II by competing with ATP for binding to the catalytic site (Ishizaki et al., 1996). Fasudil also inhibits ROCK by competing with ATP for binding to the kinase domain. It also inhibits other ROCK related protein kinases such as PKA, PRK2 and MSK1, to a much less lesser extent. (Davies et al., 2000). This high specificity has led to the appreciation of the role that ROCK activation may play in multiple pathways that can lead to both sustained vasoconstriction and remodeling of pulmonary resistance arteries. Other ROCK inhibitors, such as H-1152P, H89 and SLx-2119 have been more recently developed. H-1152P is a dimethylated analog of Fasudil with the same mechanism of ROCK inhibition as Fasudil and Y27632 (Breitenlechner et al., 2003). H89, a selective and potent inhibitor of PKA, has also been shown to inhibit ROCK-II to a greater extent than ROCK-I (Davies et al., 2000). Due to high amino acid sequence homology within the kinase domain, isoform specific inhibitors of ROCK I and II have been slow to develop. SLx-2119 is the only isoform-selective ROCK inhibitor that has been described, being highly selective for ROCK II (Hahmann and Schroeter, 2010). Although various ROCK inhibitors exist, Y-27632 and Fasudil are the only drugs that have thus far been tested in animal models of PHT. Out of all these agents, Fasudil is the only one approved for human use (in Japan) for post-stroke cerebral vasospasm (Tamura et al., 2005). In addition, several human studies have confirmed potent vasodilatory effects of single-dose Fasudil in patients with PHT (Duong-Quy et al., 2013).
Vascular remodeling has been recognized as a pathological hallmark of chronic PHT. This has resulted in a shift in focus away from drugs that act solely as vasodilators towards treatments that have pro-apoptotic and anti-proliferative effects on pulmonary arterial SMCs (Duong-Quy et al., 2013). While patients with chronic PHT have pulmonary vascular remodeling, as well as sustained pulmonary vasoconstriction, current therapies (such as iNO) work primarily as vasodilators. Once vascular remodeling is established, these therapies are less likely to be effective. Therapies that result in pulmonary vasodilatation, along with having pro-apoptotic and/or anti-proliferative effects on pulmonary arterial SMCs are more likely to reverse chronic PHT. ROCK inhibitors, such as Y27632 and Fasudil, have been shown to have both dilatory and pro-apoptotic effects in pulmonary vascular smooth muscle. This provides a strong rationale for the use of ROCK inhibitors as a potential treatment for chronic PHT.

1.8a Effects on Vasoconstriction in Animal Models
Y27632 has been used in experimental animal models of chronic PHT, and has been shown to decrease blood pressure in a dose-dependent manner. Treatment with Y27632 in control rats resulted in only a slight and transient drop in blood pressure (Uehata et al., 1997). These results indicated that ROCK mediated Ca\(^{2+}\) sensitization is elevated in hypertension and it plays a role in blood pressure regulation. Treatment with Y27632 has been shown to decrease elevated right-ventricular systolic pressure (RVSP). It also decreased hypoxic pulmonary vasoconstriction, as well as vasoconstriction resulting from KCl and angiotensin II (Fagan et al., 2004). Acute administration of Y27632 has been shown to acutely decrease PAP, to near-normal levels, in chronic hypoxia-exposed rats (Nagaoka et al., 2004). Oral Fasudil administration has been shown to decrease elevated PVR and mean pulmonary arterial pressure (MPAP) (Mouchaers et al., 2010) as well as decrease RVSP and improve survival in rats with monocrotaline (MCT)-induced PHT (Abe et al., 2004). ROCK inhibitors have been shown to have inhibitory effects on the pulmonary arterial myogenic response in adult rats or fetal sheep exposed to hypoxia. Fasudil treatment significantly decreased MPAP without any concurrent changes in mean systemic arterial pressure (MSAP) in rats with MCT induced PHT (Jiang et al., 2007). Treatment with a single bolus of either Y27632 or Fasudil in neonatal rats with chronic hypoxia or bleomycin-induced PHT rats completely normalized PVR, but had no effect on RV dysfunction (McNamara et al., 2008). Preventative treatment with Y27632 or Fasudil also resulted in normalized PVR, with Y27632 having a greater effect (Ziino et al., 2010). Sustained rescue treatment with Y27632 led to complete reversal of hemodynamic abnormalities and RV systolic dysfunction (Xu et al., 2010).

1.8b Effects on Vascular Remodeling in Animal Models
ROCK inhibition has been shown to have beneficial effects on remodeling of pulmonary vasculature. In pulmonary arterial SMCs, Y27632 has been shown to decrease the formation of stress fibers and attenuated Ca$^{2+}$ sensitization. It has also been shown to decrease the neomuscularization of distal pulmonary vasculature in adult mice exposed to chronic hypoxia (Fagan et al., 2004). Rats raised in Denver’s mildly hypoxic altitude develop PHT, characterized by increased arterial medial wall thickness, decreased small vessel counts and increased alveolar size. Chronic treatment with Fasudil resulted in significantly improved lung vascularization and alveolarization. This was evident through reduced arterial medial wall thickness and mean linear intercept (MLI), as well as increased small vessel counts (Nagaoka et al., 2004). Sustained rescue treatment with Y27632 was shown to cause reversal of arterial wall remodeling accompanied by increased apoptosis and a decrease in ET-1 and ET_A receptors. Treatment of primary cultures of juvenile rat pulmonary arterial SMCs with Y27632 decreased serum-stimulated ROCK activity and proliferation, and increased apoptosis (Xu et al., 2010). ROCK inhibitors have also been shown to decrease pulmonary arterial SMC proliferation, induced by 5-HT. Both preventative and long-term treatment with Fasudil prevented muscularization of pulmonary microvessels and medial wall thickening of pulmonary arteries. The decreased vascular remodeling was associated with a significant increase in vascular SMC apoptosis and increased lung eNOS expression (Abe et al., 2004). Similarly, Fasudil treatment has been shown to improve pulmonary vascular remodeling in association with decreased pulmonary arterial SMC proliferation, in high-flow induced PHT rats (Li et al., 2007). Treatment with either Y27632 or Fasudil has been shown to decrease SMC proliferation in the medial wall of pulmonary arteries of juvenile rats with chronic hypoxic PHT (Ziino et al., 2010). The effects of ROCK inhibitors on SMC proliferation and
apoptosis may explain preventative effects seen on structural abnormalities in chronic PHT models.

1.8c Inhalation of ROCK Inhibitors

While the above-mentioned studies have shown promising results in preventing or reversing chronic PHT, systemic treatment with ROCK inhibitors may lead to severe systemic hypotension, limiting the clinical translatability of this form of therapy. Treatment with inhaled ROCK inhibitors could be a means of achieving pulmonary selectivity, thus preventing adverse systemic effects. The first such study compared the effect of oral vs. inhaled Y27632 in adult rats with chronic hypoxic PHT (Nagaoka et al., 2005). Although acute administration of oral Y27632 led to a sustained reduction in MPAP, it also decreased MSAP. However, 5 minutes of inhaled Y27632 treatment decreased MPAP without causing a reduction in MSAP. Moreover, when compared with inhaled NO, the effect of inhaled Y27632 on hypoxic rat pups with PH was greater and lasted for at least 5 hours (Nagaoka et al., 2005). Inhaled Fasudil was also found to be as effective as inhaled Y-27632 in reducing MPAP without causing any significant changes in MSAP. Similarly, inhaled Fasudil treatment selectively reduced MPAP without causing any significant changes in MSAP (Nagaoka et al., 2005). In an attempt to develop a long-lasting inhalation formulation, the feasibility of polymeric microspheres, nanoparticles and liposomes as inhalable carriers has been investigated. The construction, efficiency, cellular uptake and physical properties of Fasudil liposomes in rats with PHT have been studied (Gupta et al., 2013b). It was found that using liposomal Fasudil increased the half-life of Fasudil by 10 fold, to nearly 5 hours. Administration of liposomal Fasudil resulted in sustained pulmonary vasodilation for 3 hours, whereas vasodilation by intravenous Fasudil lasted for 45 minutes. Moreover,
liposomal Fasudil had the least impact on MSAP, which suggests its potential role in producing a selective and sustained pulmonary vasodilation (Gupta et al., 2013b).

1.8d Human Studies

The acute vasodilatory effects of intravenous Fasudil on pulmonary circulation in adult patients with severe PHT were studied (Fukumoto et al., 2005). While intravenous Fasudil only decreased PAP slightly, it significantly reduced PVR by 17% in these patients. While there was an increase in cardiac index, no adverse systemic side effects, such as hypotension, were observed. In adult patients with PHT, Fasudil administration significantly decreased total peripheral resistance (TPR) and MPAP and increased cardiac index. However, it also significantly decreased systemic vascular resistance (SVR) and MSAP (Ishikura et al., 2006). The effects of acute intravenous administration of Fasudil on hemodynamic parameters in children with a mean age of 12.3 years were examined (Li et al., 2009). All patients had moderate PHT secondary to congenital heart disease. It was reported that Fasudil treatment significantly decreased pulmonary arterial systolic pressure (PASP) and PVR when compared to baseline. However, it also significantly reduced SVR, caused a slight decrease in MSAP and significantly increased cardiac index. A comparison of the acute vasodilator effect of inhaled Fasudil and NO on human adult patients with idiopathic and portal PHT, and PHT associated with congenital heart disease and connective tissue disease was studied (Fujita et al., 2010). Inhaled Fasudil had equal efficacy in reducing PVR as iNO. Importantly, the ratio of PVR to SVR, a marker of pulmonary selectivity, was significantly decreased by both treatments, indicating that inhalation of either NO or Fasudil were selective to lung tissues.
1.9 Other Treatments for PHT:

1.9a ET-1 Receptor Antagonists:

The endothelial dysfunction characteristic of PHT results in decreased production of vasodilators and anti-proliferative substances and overexpression of vasoconstrictors and proliferative agents such as thromboxane and ET-1 (Duong-Quy et al., 2013). ET has two distinct receptors, ET$_A$ and ET$_B$, both of which are part of the GPCR family. ET mediates several molecular and signaling pathways that play a role in PHT and promote lung vascular and interstitial remodelling. It stimulates proliferation of human pulmonary arterial SMCs and also stimulates human endothelial cell proliferation (Dupuis et al., 1996). Activation of the ET system has been demonstrated in nearly all pre-clinical models of PHT and all categories of human PHT (Michel et al., 2003). ET-1 is considered the most important pathophysiological isoform of ET. Lung tissue and pulmonary arteries of human subjects with PHT show an increased binding capacity of ET-1 (Davie et al., 2002) and increased lung ET-1 mRNA and protein content has been observed in a sheep model of PPHN (Abman, 2007). Increased circulating levels of ET-1 have also been found in human newborns with severe PPHN (Abman, 2010). Given the role ET-1 plays in pathophysiology of PHT, ET-1 receptor antagonists have become an important option for treatment. Bosentan (Tracleer), the first ET receptor antagonist approved in 2001, blocks both ET$_A$ and ET$_B$ receptors.

ET-1 receptor antagonists have been demonstrated to be effective in all models of PHT. They have been shown to improve survival, RVH and hemodynamics, reduce pulmonary fibrosis, improve endothelial function and have beneficial effects on pulmonary arterial remodeling (Prie et al., 1997; Prie et al., 1998; Dupuis and Prie, 1999). In patients with PHT,
intravenous Bosentan was shown to be a potent non-selective vasodilator, even in those unresponsive to iNO (Williamson et al., 2000).

Studies have found that ROCK mediates the effects of GPCR ligands, such as ET-1. ET-1 induced vasospasms occur as a result of increased Ca$^{2+}$ sensitivity, which appears to be mediated by ROCK-dependent inactivation of MLCP (Scherer et al., 2002). Further, treatment with Y27632 has resulted in attenuation of increased ET-1 production in pulmonary vascular smooth muscle cells (Yi et al., 2006).

1.9b PDGF Receptor Inhibitors:

Endothelial cell dysfunction and proliferation of vascular SMCs are evident in the pathobiology of PHT, with platelet-derived growth factor (PDGF) playing a role in both these processes (Barst, 2005). PDGF is a potent mitogen and SMC chemo-attractant. PDGFs have five isoforms, with PDGF-AA and PDGF-BB believed to play a critical role in PHT. The expression of PDGF alpha and beta receptors was shown to be up-regulated in arterial smooth muscles of newborn rats exposed to 60% O$_2$ (Jankov et al., 2005). Moreover, these receptors have also been shown to be up-regulated in a lamb model of chronic intrauterine PHT (Balasubramaniam et al., 2003). PDGF receptor inhibitor ST1571, or imatinib mesylate, has been shown to reverse pulmonary vascular changes in a both MCT-induced PHT rat model and mice with chronic hypoxic PHT (Schermuly et al., 2005). PDGF and its receptor mRNA are overexpressed in pulmonary arteries of human patients with severe PHT, localizing in pulmonary arterial SMCs and endothelial cells. In cultured human pulmonary arterial SMCs, PDGF was shown to induce proliferation and migration, which was inhibited by imatinib (Perros et al., 2008).
It has been shown that ROCK inhibition results in attenuation of up-regulated PDGF-BB and PDGF receptor (PDGF-R) β expression. Prevention of vascular remodeling as a result of ROCK inhibition occurs partly through the inhibition of the PDGF-Rβ signaling pathway (Ziino et al., 2010). ROCK inhibition may inhibit PDGF induced pulmonary arterial SMC proliferation through up-regulated activity of p27 (Liu et al., 2011).

1.9c PDE 5 Inhibitors:

PDEs are hydrolytic enzymes that catalyze cleavage of the 3’ phosphodiester bond of cyclic nucleotide second messengers, cGMP and cAMP. Hence, they control the intracellular levels of cGMP and cAMP, which play a role in signal transduction and pulmonary vascular smooth muscle relaxation. Several studies have explored the potential of PDE 5 inhibitors as a treatment for PHT, as PDE 5 is highly expressed in SMCs of pulmonary vasculature. PDE 5 inhibition leads to cGMP accumulation, resulting in pulmonary vascular relaxation.

In human neonates with PPHN, dipyridamole, a non-specific PDE 5 inhibitor, has been used to enhance the response to iNO therapy and also prevent rebound PHT during iNO weaning (al-Alaiyan et al., 1996; Ivy et al., 1998; Worwag et al., 2000). Of the PDE 5 inhibitors, Sildenafil is the most studied. In animal models, Sildenafil has been shown to be a selective pulmonary vasodilator (Weimann et al., 2000), as effective as iNO, normalizing PVR without significant adverse effects on systemic hemodynamics (Shekerdemian et al., 2002). After failed attempts to discontinue iNO in infants with PHT following surgery for congenital heart disease, Sildenafil led to a rise in cGMP level accompanied by a drop in PAP and successful weaning of iNO (Atz and Wessel, 1999). Moreover, in congruence with animal studies, there were no adverse effects on SAP (Atz and Wessel, 1999). Using oral Sildenafil for neonatal and childhood
PHT, it was reported that Sildenafil allowed for discontinuation of iNO within 4-6 hours (Humpl et al., 2011). Similarly, a single dose of Sildenafil prevented rebound PHT after cessation of iNO in ventilated infants and children (Namachivayam et al., 2006).

As discussed earlier, reciprocal effects between the RhoA/ROCK and NO-cGMP signalling pathways have been shown. PDE 5 inhibitors (thus increasing cGMP signalling) have been shown to decrease RhoA/ROCK activity in various experimental models (Guilluy et al., 2005; Hemnes et al., 2008). With the exception of Sildenafil, none of the treatments mentioned have been shown to improve RV function (Andersen et al., 2008). The signalling pathways that are targeted by various other treatments converge on the RhoA/ROCK pathway – further highlighting the importance of studying the role that ROCK may play in the pathophysiology of chronic PHT and the therapeutic potential of ROCK inhibitors.

1.10 Animal Models:

Rodents are considered to be appropriate models for neonatal lung injury, as alveolar and acinar vascular development are largely postnatal events, similar to the human (Burri, 1974). Rats are considered a preferable species for modeling PHT, as injury develops more readily than in the murine and other species (Rabinovitch et al., 1981; Chen et al., 2006). The immature rat is highly susceptible to the rapid development of pulmonary vasculopathy, which is characterized by severe pulmonary vascular remodeling and cardiac dysfunction (Rabinovitch et al., 1981; Kantores et al., 2006; McNamara et al., 2008). In humans with neonatal PHT, functional abnormalities have been described to continue into adulthood (Sartori et al., 1999). In immature rats, recovery from lung injury is often delayed or incomplete (Rabinovitch et al., 1981; King et
al., 1995; Keith et al., 2000), which contributes to impaired lung growth and a high potential for recurrence of PHT in later life (Hampl and Herget, 1990; Caslin et al., 1991; King et al., 1995).

1.10a Chronic Hypoxia Model of Neonatal PHT:

Our laboratory uses a neonatal Sprague Dawley (SD) rat model of chronic normobaric hypoxia (13% O2), from birth until postnatal day (PND) 14 to induce PHT (Kantores et al., 2006). The chronic hypoxia model of PHT produces structural and functional abnormalities that are similar to those seen in humans with neonatal PHT. Structural abnormalities include RV hypertrophy and pulmonary arterial medial wall thickening, and functional abnormalities include increased PVR and RV dysfunction (Kantores et al., 2006; Jankov et al., 2008; McNamara et al., 2008). Once injury is established (by PND 14), the injury model is unresponsive to iNO (McNamara et al., 2008), which replicates the ‘fixed’ and often fatal form of human neonatal PHT.

1.10b Previous Work in this Model Relevant to the RhoA/ROCK Pathway:

In previous work, it was shown that the RhoA/ROCK pathway is activated in pulmonary vasculature of SD rat pups with chronic hypoxia induced PHT, from birth until PND 14. A single bolus of the ROCK inhibitor, Y27632, led to potent vasodilation, shown by normalization of raised PVR (McNamara et al., 2008). Treatment with Y27632 from birth, concurrent with hypoxia exposure, resulted in a prevention of vascular remodeling by inhibiting pulmonary arterial smooth muscle proliferation (Ziino et al., 2010). It was clear from these studies that ROCK plays an important role in sustained vasoconstriction, smooth muscle proliferation and consequent arterial wall remodeling in the chronic hypoxia exposed rat. The effects of late
sustained, or rescue, treatments were studied, where ROCK inhibitor was given during the third week of life (PND 14-21), once PHT was already established. Rescue treatment resulted in a reversal of vascular remodeling, by unlocking ROCK 2-mediated anti-apoptotic effects on smooth muscle, and RV dysfunction. Despite these highly promising results, a major caveat of systemic treatment, as already discussed, is severe systemic hypotension, due to non-selective vasodilatory effects (Xu et al., 2010).

1.11 Rationale, Global Aims and Specific Hypotheses:

We postulate that there may be two mechanisms by which RV dysfunction was reversed by systemic rescue treatment with a ROCK inhibitor. Reversal of RV dysfunction may be a result of i) inhibition of ROCK activity in the lungs, leading to sustained unloading of the right heart or ii) direct inhibition of increased ROCK activity in the right ventricle. Preliminary data suggested that ROCK activity is increased specifically in the right, but not in the left ventricle of neonatal pups with chronic hypoxic PHT. Based upon these findings, the global aim of the studies upon which this thesis is based was to determine the mechanism by which RV dysfunction was reversed; would unloading of the right heart, by pulmonary-specific ROCK inhibition, reverse the structural and functional changes of chronic hypoxia induced PHT, with similar efficacy of systemic rescue treatment? We also aimed to determine whether ROCK inhibitor could be delivered with minimal systemic effects, while still leading to sustained reversal of pulmonary vasoconstriction and reversal of vascular remodeling.

Due to its much greater aqueous solubility, we chose to employ Fasudil, rather than Y-27632, for these studies. Specifically, my hypotheses were that late-sustained pulmonary-specific treatment with Fasudil in neonatal rats with established chronic PHT would:
1. Normalize raised PVR

2. Reverse structural abnormalities, including RVH and pulmonary arterial medial wall thickening

We hypothesized that ROCK activity is up regulated in the right ventricle, contributing to RV dysfunction, therefore:

3. Have no effect on RV systolic dysfunction

If ROCK activity is up regulated in the right ventricle, we hypothesized that simply unloading of the right-heart will not reverse cardiac dysfunction. Rather, up-regulated ROCK in the right ventricle, may directly contribute to the dysfunction. It would be of great interest to determine any upstream or downstream effectors of ROCK, to better understand what is contributing to RV dysfunction. One possible downstream effector is PDE 5, which has been shown to be up-regulated in the hypertrophied right ventricle (Nagendran et al., 2007). We therefore further hypothesized that PDE 5 may lie downstream ROCK, contributing to the manifestation of RV dysfunction.
**Figure 1.9:** A schematic diagram summarizing the general hypotheses. Chronic exposure to hypoxia results in pulmonary hypertension characterized by vascular remodeling and right-ventricular (RV) dysfunction. Systemic treatment with a ROCK inhibitor results in reversal of both vascular remodeling and RV dysfunction. Pulmonary-selective ROCK inhibition will provide us with insight into the mechanism by which RV dysfunction was reversed with systemic ROCK inhibition. If pulmonary-selective ROCK inhibition results in a reversal of RV dysfunction, then the dysfunction must be a result of increased pressure load on the right heart, and therefore, by reducing this pressure load, dysfunction would be reversed. If pulmonary-selective ROCK inhibition does not result in reversed RV dysfunction, we hypothesize that increased ROCK activity in the right ventricle contributes to this dysfunction, and therefore, reversal of RV dysfunction with systemic ROCK inhibition was a result of inhibition of up-regulated ROCK activity in the right ventricle.
Chapter 2

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Materials & Methods
2.1 Materials:

Fasudil was purchased from LC Laboratories (Woburn, MA, USA) and Y-27632 was purchased from Axxora (San Diego, CA, USA). Oxygen exposure chambers and automated controllers (OxyCycler model A84XOV) were from Biospherix (Lacona, NY, USA). A customized mass-dosing nebulization chamber (cat no. PLY 5000) was from Buxco Research Systems (Wilmington, NC, USA). An Aeroneb Pro nebulizer (pore size, 2-4 microns) was purchased from Aerogen (Galway, Ireland). Tris-glycine precast gels and PVDF membranes were from Thermo Scientific (Rockford, IL, USA). Phosphastase and protease inhibitors were purchased from Sigma Life Science (St. Louis, MO, USA) and Calbiochem (San Diego, CA, USA), respectively. A PDE-V activity assay was purchased from BIOMOL (Brockville, ON, Canada). Acids, alcohols, organic solvents, paraformaldehyde, Permount and Superfrost/Plus microscope slides were purchased from Fisher Scientific (Whitby, ON, Canada).

Goat anti-rabbit and anti-mouse IgG peroxidase antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Primary antibody sources and concentrations used for Western blot (WB) are shown below (Table 2.1):

<table>
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<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
<th>Catalogue #</th>
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<td>Sc-5560</td>
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<tr>
<td>cGB phosphodiesterase (PDE) V</td>
<td>BD Biosciences</td>
<td>1:1000</td>
<td>611498</td>
</tr>
<tr>
<td>Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH)</td>
<td>Santa Cruz Biotechnology</td>
<td>1:5000</td>
<td>Sc-25778</td>
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</tbody>
</table>
2.2 Institutional Review:

All procedures involving animals were performed in accordance with the standards of the Canadian Council on Animal Care. Approval for studies was obtained from the Animal Care Committee of the Hospital for Sick Children Research Institute.

2.3 Hypoxia Exposure System:

Timed-pregnant Sprague-Dawley rats were obtained from Taconic Farms (Germantown, NY, U.S.A.). Experiments were conducted as paired exposures, where one litter received 21% O₂ (air) and the other received 13% O₂ (hypoxia) up to postnatal day 17 (for acute studies) or 21 (for sustained rescue studies). O₂ and CO₂ levels, temperature and humidity were continuously monitored, recorded and regulated using customized computer software (AnaWin Run-Time, Version 2.2.18, Watlow-Anafaze, St. Louis, MO, USA). Gas delivery was automatically adjusted to maintain O₂ level within 0.1% of the set point, and sensors were calibrated weekly. On the day following delivery, dams were placed in a Plexiglass chamber (12 hour/12 hour light-dark cycles), with the temperature maintained at 25±1°C, humidity ≈ 50% and CO₂ concentration <0.5%. Litter sizes were culled to 10-12 pups and equal sex distribution was maintained throughout the exposure period. Food and water were available ad libitum. Dams were exchanged daily between paired air and hypoxia chambers in order to prevent maternal toxicity and consequentially any detrimental effects on the pups. At the end of each exposure period, pups were sacrificed either by exsanguination after anesthesia or by pentobarbital sodium overdose.
2.4 Aerosolized Fasudil Exposure System:

Rat pups were placed in a custom Plexiglass chamber and Fasudil was administered using a fine-mist nebulizer for 15 minutes. At the end of exposure, rat pups were allowed to recover for 15 minutes.
2.5 Interventional Studies:

Injections (Y27632, 15 mg/kg body weight in saline) were given intraperitoneally (i.p.) via a 30-gauge needle, twice daily, from PNDs 14-21 as previously described (Xu et al., 2010). Aerosolized treatment (Fasudil, 54 mg/kg body weight = 150 mM, 81 mg/kg body weight = 225 mM or 108 mg/kg body weight = 300 mM in saline) was given on PND 17 once 30 minutes prior to 2D echocardiographic/pulse wave Doppler studies for initial dose-ranging studies or 3 times daily (81 mg/kg body weight), for 15 minutes each from PNDs 14-21. Appropriate controls (saline-injected or aerosolized saline-exposed) were also included.

2.6 Measurement of Blood Pressure:

DBP, SBP and MBP were measured noninvasively using a tail cuff Doppler device (LE5002, Harvard Apparatus; Holliston, MA, USA). Rat pups were anesthetized with i.p. ketamine hydrochloride (40 mg/kg) and xylazine hydrochloride (6 mg/kg), laid supine, and measurements were conducted. Due to technical limitations of the device in smaller animals, measurements were only possible in animals 17 days or older (approximately 40 grams), which was the rationale for performing the acute studies on PND 17.

2.7 Two-dimensional Echocardiography and Pulse Wave Doppler Ultrasound:

Two-dimensional (2D) echocardiography and pulsed wave Doppler ultrasound was used as a non-invasive method for assessment of pulmonary hemodynamics. A Vivid 7 Advantage
(GE Medical Systems, Milwaukee, WI) cardiovascular ultrasound system was used with a small high frequency linear probe (I13L). Due to small size of the animals and high heart rate, an ultra-high frame rate was used, providing high image quality. Rat pups were anesthetized with i.p. ketamine hydrochloride (40 mg/kg) and xylazine hydrochloride (6 mg/kg) and then laid supine. The probe was gently applied to the chest while the pups were spontaneously breathing room air. A short axis view at the level of the aortic valve was obtained, and the pulmonary artery was identified using colour flow Doppler. Analysis was undertaken in a blinded fashion using an offline analysis system (ECHOPac, GE Medical Systems).

2.7a Pulmonary Vascular Resistance:

The pulsed Doppler gate was placed proximal to the pulmonary valve leaflets and aligned, with an angle of insonation < 20°, maximizing laminar flow. The right-ventricular ejection time (RVET) and pulmonary arterial acceleration time (PAAT) were estimated using the pulmonary Doppler profile. RVET was measured as the time from onset of systolic flow to completion of systolic pulmonary flow, and PAAT was measured as the time from onset to peak pulmonary outflow velocity. A surrogate measure of PVR was calculated as a ratio of RVET:PAAT (Figure 2.1). Measurements from 3-5 heartbeats were averaged to account for beat-to-beat variability.
Figure 2.1: A) Diagram showing a typical trace in animals with pulmonary hypertension. RVET = right-ventricular ejection time; PAVTI= pulmonary artery velocity time integral; PAAT = pulmonary arterial acceleration time; arrow pointing to mid-systolic notch, which represents rapid deceleration during mid-systole. B) 2D echo/Pulse Wave Doppler trace showing differences in RVET and PAAT between normoxia- or hypoxia-exposed animals.
2.7b Right-ventricular Output:

RV output was used as a measure of RV systolic performance, which is known to be decreased in chronic hypoxia-exposed neonatal rats (Kantores et al., 2006). Colour flow Doppler was used to measure the diameter of pulmonary artery (PAD) at the hinge-point of the pulmonary valve leaflets. The pulmonary artery velocity time integral (PAVTI) was measured by tracing the leading edge of the velocity time graph from the onset to completion of systolic pulmonary flow. From the same Doppler trace used to measure RVET and PAAT, RV stroke volume was estimated using the formula: \( \frac{(PAD/2) \times \pi \times PAVTI}{2} \). RV stroke volume was corrected for heart rate (HR) and body weight (kg) to derive a RV index (RVI; ml/kg/min).

2.8 Right-heart Catheterization:

2.8a Validation of Model:

In order to validate hemodynamic and RV systolic performance data obtained by 2D echo studies, measurements were taken by right-heart catheterization and the results were compared to echo-derived indices of PVR, RVO and RVSV.

2.8b Hemodynamic Measurements:

Rat pups were anesthetized by exposure to 2% isoflurane, intubated using an 18-guage catheter and ventilated using a volume controlled ventilator (Hugo Sachs Elektronik, Harvard
Apparatus, Type 845, Holliston, MA, USA). Tidal volume \( V_t \) and respiration rate (RR) were calculated and set using the following formulas: 
\[
V_t, \text{ ml} = 6.2 \times M^{1.01} \quad (M = \text{animal mass, kg})
\]
\[
RR, \text{ min}^{-1} = 53.5 \times M^{0.26} \quad (\text{Pacher et al., 2008})
\]
Proper intubation was confirmed by observation of chest expansion and retraction during ventilated breaths. Ventilation parameters were set to maintain normocapnia.

Rats were laid supine on a water circulating heating pad, with the temperature set to 38 °C. Pressure-volume (PV) loops were generated by inserting a 1.2F Scisense PV impedance catheter (FTS-1212B-4518) into the right jugular vein and advancing it into the right ventricle. Data was acquired (Labscribe 2.2, iWorx Systems Inc., Dover, NH, USA) under steady-state conditions and following inferior vena cava occlusion (preload reduction). Signals were monitored continuously using an Advantage PV system (Model FY897B, Scisense, Ontario, Canada). The following RV parameters were then calculated: The end systolic pressure (ESP), \( E_a \), RVO and RVSV (Pacher et al., 2008).

**2.9 Right-ventricular Hypertrophy:**

Measurement of right-ventricular hypertrophy using the Fulton index is a well-established marker of PHT (Fulton et al., 1952). RV hypertrophy has been shown to correlate closely with thickening of distal pulmonary arteries in hypoxia-exposed rats (Rabinovitch et al., 1979). After sacrificing the pups, the thoracic contents were removed en bloc. The heart and lungs were separated, and the atria were removed inferior to the atrio-ventricular valves. The right ventricle was separated from the left ventricle and septum. Each component was freeze-
dried and weighed separately. RVH was measured using a ratio of the right ventricle: left ventricle + septum (RV:LV+S).

2.10 Hart’s Elastin Stain:

Four randomly selected animals from each group were anesthetized using i.p. ketamine (80 mg/kg) and xylazine (20 mg/kg). Following opening of the thoracic cavity and trachea cannulation, the pulmonary veins were divided. The pulmonary circulation was flushed with PBS + heparin, clearing the lungs of blood. The lungs were then perfusion fixed with paraformaldehyde while air-inflated at a constant pressure (20 cm of H$_2$O). Paraffin embedded tissues were cut into 5 µm sections and mounted onto Superfrost slides, allowed to air dry and bake overnight (43° C). Sections were de-waxed by immersing them in xylene, rehydrated in ethanol, rinsed in several washes of distilled water and then left overnight for 13 hours in Weigert’s resorcin-fuchsin stain. Slides were then washed with distilled water and counterstained with tartrazine. Slides were dehydrated and mounted with a coverslip using a 70% Permount/30% xylene solution.

2.11 Percent Medial Wall Area:

Percent medial wall area was used as a marker of pulmonary vascular remodeling. Pulmonary arteries were identified by the presence of an inner and outer elastic lamina using Hart’s elastin stain. Images were digitally captured and analysis was performed in a blinded fashion. Vessels from 20-100 µm in size were included, with a minimum of 20 vessels per group,
from a total of 4 sections per group. Obliquely sectioned vessels that were greater than three times longer than they were wide were excluded. Adobe Photoshop CS5 (Adobe Systems Incorporated, San Jose, CA) was used to calculate the medial wall area of each vessel. Using the ‘Quick Selection’ tool, the area of the inner lumen and of the whole vessel was determined. The following formula was used to determine the percent medial wall area: \[
\frac{\text{whole vessel area} - \text{inner lumen area}}{\text{whole vessel area}} \times 100
\] (Figure 2.2).
Figure 2.2: A schematic representation of pulmonary arteries and a simplified description of the method used to determine medial wall area. The top panel shows the various components of pulmonary arteries: external elastic lamina, tunica media, internal elastic lamina and the lumen. The bottom panel describes how medial wall area is calculated. Areas to be calculated are represented by the shaded pink region. The luminal area is subtracted from the total vessel area, which equals the medial wall area. The medial wall area consists of the inner and outer elastic lamina, as well as the tunica media.
2.12 Western Blot Analyses:

Whole lung, right or left-ventricular free wall tissues (flash frozen, 4 samples per group), were homogenized and sonicated (40 W for 30 seconds) in RIPA cell lysis buffer (10 mM NaPO4, 0.3 M NaCl, 0.1% (w/v) sodium dodecyl sulphate (SDS), 1% (v/v) Nonidet P-40, 1% (v/v) sodium deoxycholate, 2 mM EDTA, pH 7.2) containing protease and phosphatase inhibitors. The homogenate was left on ice for 10 minutes before centrifugation (8500 x g for 10 min). The supernatant was collected and protein concentration was measured by a commercially available spectrophotometric assay (Bradford, 1976). Samples were stored at -80 °C until further analysis. Tissue containing 50-100 µg of protein was boiled for 5 minutes in SDS sample buffer (60 mM Tris-HCl, 10% (w/v) SDS, 5% (v/v) glycerol, 2 mM β-mercaptoethanol, pH 6.8) and separated under reducing conditions by SDS polyacrylamide gel electrophoresis for 1.5 hours at 130 V. Following electrophoresis, proteins were transferred to PVDF membranes. All membranes were blocked with 5% skim milk or 6% BSA for 1 hour at room temperature, followed by incubation with primary antibody overnight at 4 °C. Blots were then washed with TBST and placed in secondary antibody for 1 hour at room temperature. Following blotting, bands were imaged using an enhanced chemiluminescence kit (SuperSignal West Dura Chemiluminescent Substrate, Thermo Scientific, Rockford, IL, USA). Blots were electronically captured using a chemiluminescent camera (MicroChemi, DNR Bioimaging Systems, Jerusalem, Israel) and band density was quantified using Image J software (NIH, Bethesda, MD, USA). Any differences in protein loading were controlled for by re-blotting for GAPDH, and results expressed after normalization to the GAPDH band.
2.13 Phosphodiesterase 5 Activity Assay:

Cardiac tissue (flash frozen, 3 hearts pooled to form n=1, minimum n=4 per group) was homogenized in lysis buffer and treated with protease and phosphatase inhibitors. Samples were placed on ice and assayed the same day. This assay is dependent upon free phosphate; therefore endogenous phosphates were removed by passage through a Centri-Spin 10 column (Princeton Separations, Adelphia, NJ, USA). In order to determine PDE 5-specific cGMP hydrolytic activity, total protein was assayed for cGMP hydrolytic activity using a commercially available colorimetric cyclic nucleotide PDE assay kit (Biomol, Plymouth Meeting, PA, USA) in the presence of absence of Sildenafil (200 nM), a PDE 5-specific inhibitor, or IBMX (500 mM), a non-specific PDE inhibitor. Results are presented as a percentage of the total PDE ([PDE 5/total PDE] * 100 = % PDE 5 activity).

2.14 Exercise Tolerance Studies:

Adult rats were placed on a treadmill (Columbus Instruments, Exer-3/6 Treadmill, Columbus, OH, USA) in order to determine their exercise tolerance. The treadmill speed was initially set to 25 metres/minute with a 10% grade. The speed was then increased by 5 metres/minute, every 5 minutes, until the animal was no longer able or willing to maintain pace with the treadmill belt (ie. unable to come off the back of the treadmill for > 3 seconds), despite encouragement to do so by application of up to 3 manual busts of high pressure air to the hind legs (no electrical stimuli used). It was observed that fatigue is often preceded by lowering of the hindquarters and a raised snout, resulting in a significantly altered gait. Once the animal was
removed from the treadmill, fatigue was confirmed by placing the animal on its back and observing a delayed (> 2 seconds) righting reflex, which occurred instantly in non-fatigued rats. If the animal was found not to be fatigued, the exercise test was repeated on the following day. Otherwise, the time to fatigue was recorded to the nearest second and the maximum total distance covered was calculated.

2.15 Data Presentation and Statistical Analyses:

All values are expressed as the mean ± standard error of the mean (SEM). Statistical significance (p<0.05) was determined using SigmaPlot Software (Version 12.5, Chicago, IL, USA) using one-way analysis of variance (ANOVA) followed by pair-wise multiple comparisons using the Tukey’s test when significant differences were found (Snedecor and Cochran, 1968; Glantz, 2002). Where only 2 groups were compared, the Student’s t-test was used.
Chapter 3

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Results
3.1 Acute Experiments:
3.1a Determining the Optimal Dose of Aerosolized Fasudil

In order to determine an optimal dose of aerosolized Fasudil that was appropriate for sustained rescue treatment, we looked at the effects of 3 doses, ranging from 150 – 300 mM, on: blood pressure (diastolic, systolic, mean), PVR and lung and cardiac ROCK activity. The ideal dose would have minimal effects on DBP, normalize PVR, and have inhibitory effects on lung, but not cardiac, ROCK activity. A dose range of 150 – 300 mM was chosen based on preliminary experiments, employing 50 and 100 mM, which showed minimal acute effects on raised PVR secondary to chronic hypoxia (data not shown). For the purpose of these studies, a dose of 150 mM was arbitrarily labeled as “low dose”, 225 mM as “intermediate dose” and 300 mM as “high dose”.

i) Blood Pressure:

A single bolus of i.p. Y27632 (15 mg/kg) resulted in a significant drop in systolic (p<0.01) and mean blood pressure (p<0.001) (approximately 60 mm Hg) (SBP or MBP, respectively) and a single bolus of i.p. Y27632 (15 mg/kg) or Fasudil (30 mg/kg) resulted in a significant (p<0.01) drop in diastolic blood pressure (DBP) (approximately 60 mm Hg), when compared to air-exposed controls. While to a lesser extent, treatment with aerosolized Fasudil at the high dose (108 mg/mL, 300 mM) also resulted in a significant (p<0.05) drop in DBP (approximately 30 mm Hg), while treatment with aerosolized Fasudil at the intermediate (81 mg/mL, 225 mM) or high dose resulted in a significant (p<0.05) drop in SBP (approximately 30 mm Hg) and MBP (approximately 25 mm Hg). Treatment with aerosolized Fasudil at the low (54 mg/mL, 150 mM) or intermediate dose did not lead to any significant decreases in DBP.
(Figure 3.1), while treatment with aerosolized Fasudil at the low dose did not lead to any significant decreases in SBP (Figure 3.2) and MBP (Figure 3.3).

**ii) Pulmonary Vascular Resistance:**

Hypoxia-exposed, inhaled saline treated animals had significantly higher (p<0.01) PVR values when compared to the air-exposed controls. A single bolus of aerosolized Fasudil at any of the three doses was effective at normalizing PVR (p<0.01) (Figure 3.4).

**iii) ROCK Activity:**

ROCK activity was measured by Western blot analysis of the ratio of phosphorylated Thr 850: pan MYPT1, as previously described (Xu et al., 2010). Hypoxia-exposed, inhaled saline treated animals had significantly higher ROCK activity in lungs and RV tissue, when compared to air controls. A single bolus of aerosolized Fasudil at any of the three doses was effective at significantly (p<0.05) inhibiting ROCK activity in the lungs. A single bolus of systemic treatment with Y27632 was also effective at significantly (p<0.05) inhibiting ROCK activity in lung tissue (Figure 3.5). Treatment with a single bolus of aerosolized Fasudil at the high dose significantly (p<0.05) inhibited ROCK activity in the right ventricle. Treatment with aerosolized Fasudil at the low or intermediate dose did not cause significant inhibitory effects on ROCK activity in the right ventricle (Figure 3.6).

From these experiments, it was concluded that treatment with aerosolized Fasudil at the intermediate dose would be appropriate for pulmonary-selective ROCK inhibitory treatment.
This dose was found to be effective at normalizing PVR and had the greatest effect on pulmonary ROCK activity while having no significant effects on DBP or RV ROCK activity.
Figure 3.1: High dose of aerosolized Fasudil and systemic ROCK inhibition causes significant drop in diastolic blood pressure (DBP). Changes in diastolic blood pressure in d21 hypoxia-exposed rat pups treated with a single bolus of inhaled normal saline or Fasudil at the low, intermediate or high dose, or with systemic Fasudil or Y27632. *p<0.05 by one-way ANOVA, when compared to the inhaled saline group. #p<0.01, by one-way ANOVA, when compared to all other groups.
Figure 3.2: Intermediate or high dose of aerosolized Fasudil and systemic ROCK inhibition causes significant drop in systolic blood pressure (SBP). Changes in systolic blood pressure in d21 hypoxia-exposed rat pups treated with a single bolus of inhaled normal saline or Fasudil at the low, intermediate or high dose, or with systemic Y27632. *p<0.05 by one-way ANOVA, when compared to the inhaled saline group. #p<0.01, by one-way ANOVA, when compared to all other groups.
Figure 3.3: Intermediate or high dose of aerosolized Fasudil and systemic ROCK inhibition causes significant drop in mean blood pressure (MBP). Changes in mean blood pressure in d21 hypoxia-exposed rat pups treated with a single bolus of inhaled normal saline or Fasudil at the low, intermediate or high dose, or with systemic Y27632. *p<0.05 by one-way ANOVA, when compared to the inhaled saline group. #p<0.001, by one-way ANOVA, when compared to all other groups.
Figure 3.4: Aerosolized Fasudil treatment results in normalized PVR. Changes in pulmonary vascular resistance (PVR) in d21 hypoxia-exposed rat pups at baseline, or following a single bolus of aerosolized treatment with normal saline, or Fasudil at the low, intermediate or high dose. PVR is estimated by a ratio of the right-ventricular ejection time to the pulmonary arterial acceleration time. *p<0.01, by one-way ANOVA, when compared to hypoxia-exposed baseline and inhaled saline groups.
**Figure 3.5**: Aerosolized Fasudil treatment inhibits lung ROCK activity. Changes in lung tissue ROCK activity in d21 hypoxia-exposed rat pups treated with a single bolus of aerosolized normal saline, Fasudil at the low, intermediate or high dose, or systemically with Y27632. ROCK activity is measured as the ratio of phosphorylated MYPT1: total MYPT1. Representative blots shown. *p<0.05, by one-way ANOVA, when compared to the hypoxia-exposed inhaled saline group. Bars are shown as multiples/fractions of the air-exposed control.
Figure 3.6: High dose of aerosolized Fasudil treatment inhibits right-ventricular (RV) ROCK activity. Changes in RV ROCK activity in d21 hypoxia-exposed rat pups treated with aerosolized normal saline or Fasudil at the low, intermediate or high dose. ROCK activity is measured as the ratio of phosphorylated MYPT1: total MYPT1. *p<0.05 by one-way ANOVA, when compared to the hypoxia-exposed inhaled saline group. Bars are shown as multiples/fractions of the air-exposed control.
3.1 Acute Experiments:
3.1b Determining the Duration of Activity of Aerosolized Fasudil

Once the intermediate dose of Fasudil was chosen as an appropriate dose for pulmonary-selective ROCK inhibitory treatment, we sought to determine the duration of activity following a single treatment. A time course experiment was therefore conducted, examining longevity of effects on PVR and lung ROCK activity.

i) Pulmonary Vascular Resistance:

In order to determine the duration of effect of treatment with aerosolized ROCK inhibitor, we examined PVR at intervals of 1, 3 and 5 hours following treatment. A single treatment with aerosolized Fasudil was effective at normalizing PVR levels 1 and 3 hours post treatment. Five hours following treatment, PVR had returned to pre-treatment levels (Figure 3.7).

ii) ROCK Activity:

A single treatment with aerosolized Fasudil at the intermediate dose did not have any inhibitory effects on lung ROCK activity 5 hours post-treatment. ROCK activity was significantly (p<0.05) higher, 5 hours post-treatment, when compared to the baseline group (Figure 3.8). It was concluded that treatment with aerosolized Fasudil at the intermediate dose had duration of effect of between 3-5 hours.
Figure 3.7: Aerosolized Fasudil treatment normalizes PVR for up to 3 hours following treatment. Changes in pulmonary vascular resistance (PVR) in d17 hypoxia-exposed rat pups treated with a single bolus of aerosolized Fasudil at the intermediate dose, over time. Data is shown at baseline, 0, 1, 3 and 5 hours post-treatment. PVR is estimated by a ratio of the right-ventricular ejection time to the pulmonary arterial acceleration time. *p<0.05, by one-way ANOVA, when compared to the baseline group.
Figure 3.8: Aerosolized Fasudil is no longer effective 5 hours following treatment. Changes in ROCK activity in d17 hypoxia-exposed rat pups treated with a single bolus of aerosolized Fasudil at the intermediate dose. Data is shown at baseline and 5 hours post-treatment. ROCK activity is measured as the ratio of phosphorylated MYPT1: total MYPT1. Representative blots shown. *p<0.05, by t-test, when compared to the baseline group.
3.2 Chronic Experiments

Once the appropriate dose and treatment schedule were determined, the effects of chronic treatment with aerosolized Fasudil were studied. Based on the previous dose-response and time course experiments, we chose to use an intermediate dose of Fasudil (81 mg/mL; 225 mM) 3 times daily. Following 7 days of treatment (from PND 14–21), we examined effects on body and lung weight, RVH, pulmonary arterial wall remodeling and RV function.

3.2a Body and Lung weight:

Hypoxia-exposed animals had lower (p<0.001) body weights when compared to air-exposed controls. Treatment with aerosolized Fasudil did not have any effect on body weights in either group (Figure 3.9). Lung weight was greater (p<0.05) in the air-exposed group treated with aerosolized Fasudil, when compared to all other groups (Figure 3.10). Lung weight: Body weight ratio was higher (p<0.05) in the hypoxia-exposed, inhaled saline and air-exposed, inhaled Fasudil groups, when compared to the air-exposed inhaled saline group. Body weight was also higher (p<0.05) in the hypoxia-exposed inhaled Fasudil group, when compared to air-exposed groups (Figure 3.11).

3.2b Right-ventricular Hypertrophy:

Hypoxia-exposed, inhaled saline treated animals had significantly (p<0.001) greater RV hypertrophy. Sustained treatment with aerosolized Fasudil resulted in a partial, but significant (p<0.001) decrease in RV hypertrophy (Figure 3.12).
3.2c Percent Medial Wall Area:

Hypoxia-exposed, inhaled saline treated animals had significantly (p<0.05) greater percent medial wall area when compared to air-exposed controls. Sustained treatment with aerosolized Fasudil resulted in a partial, but significant (p<0.05) decrease in percent medial wall area in small pulmonary arteries (20-60 µm) (Figure 3.13), while it resulted in complete normalization in medium sized pulmonary arteries (60-100 µm) (Figure 3.14 and 3.15).

3.2d Pulmonary Vascular Resistance:

As mentioned previously, hypoxia-exposed inhaled saline animals had significantly (p<0.05) greater PVR, when compared to air-exposed controls. Sustained treatment with aerosolized Fasudil was effective at normalizing PVR levels, within 1 hour following the last dose. Greater than 12 hours following the last dose, sustained treatment with aerosolized Fasudil was no longer effective at normalizing PVR (p<0.05), although PVR was less than measured in hypoxia-exposed animals treated with saline (Figure 3.16).

3.2e Right-ventricular Systolic Pressure:

Hypoxia-exposed, inhaled saline treated animals had significantly (p<0.001) higher RV systolic pressure when compared to air-exposed controls. Sustained treatment with either aerosolized Fasudil or systemic Y27632 resulted in normalization of RV systolic pressure (Figure 3.17).

3.2f Right-ventricular Performance:
Hypoxia-exposed, inhaled saline treated animals had significantly (p<0.05) lower RVI when compared to the air-exposed controls. Treatment with aerosolized Fasudil did not result in improved RVI either within 1 hour or more than 12 hours following the last dose of treatment (Figure 3.18).

3.2g Heart Rate:

Heart rates of hypoxia-exposed, inhaled saline treated animals were not significantly different than air-exposed controls. Sustained treatment with aerosolized Fasudil did not have any effect on heart rates in both air- or hypoxia-exposed animals (Figure 3.19).

3.2h Right-ventricular Stroke Volume:

Hypoxia-exposed, inhaled saline treated animals had significantly (p<0.001) lower RV stroke volumes, when compared to air-exposed controls. Sustained treatment with aerosolized Fasudil did not improve impaired stroke volume, either within 1 hour or more than 12 hours following the last dose of treatment (Figure 3.20).

3.2i Validation of Echocardiography-derived Measurements:

As mentioned previously, it was found that PVR, estimated by 2D echo, was significantly (p<0.001) higher in hypoxia-exposed animals. This correlated with a significantly increased afterload (p<0.001), measured by right-heart catheter, in hypoxia-exposed animals (Figure 3.21). Both 2D echo and catheter measurements showed a significantly decreased (p<0.05 and p<0.001, respectively) RV stroke volume in hypoxia-exposed animals, when compared to air-exposed controls (Figure 3.22). 2D echo and catheter measurements also showed significantly decreased
RV output (p<0.05 and p<0.001, respectively) in hypoxia-exposed animals, when compared to air-exposed controls (Figure 3.23).

### 3.2j PKC Activity

High concentrations of Fasudil have been shown to have inhibitory effects on other kinases (ex. PKC) (Shimomura et al., 2004). In order to determine the selectivity of aerosolized Fasudil treatment for ROCK, we examined phospho-Thr38-CPI-17, as a marker of PKC activity, by Western blot analysis. No differences in CPI-17 activity were observed with aerosolized Fasudil treatment, when compared to saline controls (data not shown).

From the chronic experiments, it was determined that sustained treatment with aerosolized Fasudil was effective at decreasing RVH, reversing vascular remodeling and normalizing PVR. Sustained treatment with either aerosolized Fasudil or systemic Y27632 normalized RVSP. Although sustained treatment with aerosolized Fasudil had beneficial effects on cardiac and vascular remodeling, it did not result in improvements in impaired RVI or RVSV. This observation suggests that reversal of RV systolic dysfunction secondary to treatment with systemic Y-27632 (Xu et al., 2010) was not simply the result of reduced afterload.
Figure 3.9: Hypoxia exposed rat pups have decreased body weight. Body weights of d21 air- or hypoxia-exposed rat pups given sustained treatment with aerosolized normal saline or Fasudil. *p<0.001, by one-way ANOVA when compared to air-exposed groups.
Figure 3.10: Lung weights of d21 air- or hypoxia-exposed rat pups given sustained treatment with aerosolized normal saline or Fasudil. *p<0.05, by one-way ANOVA, when compared to all other groups.
**Figure 3.11:** Lung weight: Body weight ratio of d21 air- or hypoxia-exposed rat pups given sustained treatment with aerosolized normal saline or Fasudil. *p<0.05, by one-way ANOVA when compared to air-exposed groups. #p<0.05, by one-way ANOVA, when compared to the air-exposed inhaled saline group.
Figure 3.12: Sustained aerosolized Fasudil treatment results in decreased right-ventricular hypertrophy (RVH). A) Changes in RVH in d21 air- or hypoxia-exposed rat pups given sustained treatment with aerosolized normal saline or Fasudil. *p<0.001, by one-way ANOVA, when compared to all other groups. B) Cross-sections of d21 rat hearts stained with Hematoxylin & Eosin, highlighting changes in RVH.
Figure 3.13: Sustained treatment with aerosolized Fasudil results in a partial decrease in medial wall area in small vessels. Medial wall area in small vessels (diameter 20-60 µm) in d21 air- or hypoxia-exposed rat pups given sustained treatment with aerosolized normal saline or Fasudil. *p<0.05, by one-way ANOVA, when compared to all other groups.
Figure 3.14: Sustained treatment with aerosolized Fasudil results in completely normalized medial wall area in medium-sized vessels. Medial wall area in medium vessels (diameter 60-100 µm) in d21 air- or hypoxia-exposed rat pups given sustained treatment with aerosolized normal saline or Fasudil. *p<0.05, by one-way ANOVA, when compared to all other groups.
Figure 3.15: Sustained treatment with aerosolized Fasudil reverses vascular remodeling. Pulmonary arteries from d21 air- or hypoxia-exposed rat pups given sustained treatment with aerosolized normal saline or Fasudil, stained for Hart’s elastin. Increased elastin staining in hypoxia-exposed inhaled saline pulmonary arteries demonstrates vascular remodeling, which is attenuated with aerosolized Fasudil treatment.
Figure 3.16: Sustained treatment with aerosolized Fasudil acutely normalizes pulmonary vascular resistance (PVR). PVR in d21 air- or hypoxia-exposed rat pups given sustained treatment with aerosolized normal saline or Fasudil, shown within 1 hour and greater than 12 hours following treatment. PVR is estimated by a ratio of the right ventricular ejection time to the pulmonary arterial acceleration time. *p<0.05, by one-way ANOVA, when compared to the air-exposed control group. #p<0.05, by one-way ANOVA, when compared to all other groups.
**Figure 3.17**: Sustained treatment with aerosolized Fasudil or systemic Y27632 decreases elevated right-ventricular end systolic pressure (RVESP). RVESP in d21 air-exposed rat pups or hypoxia-exposed rat pups given sustained treatment with vehicle, aerosolized Fasudil or systemic Y27632. *p<0.001, by one-way ANOVA, when compared to all other groups.
Figure 3.18: Sustained treatment with aerosolized Fasudil does not improve impaired right-ventricular index (RVI). Changes in RVI in d21 air- or hypoxia-exposed rat pups given sustained treatment with aerosolized normal saline or Fasudil, shown within 1 hour and greater than 12 hours following treatment. *p<0.05, by one-way ANOVA, when compared to air-exposed controls. Bars are shown as fractions of the air control.
Figure 3.19: Sustained treatment with aerosolized Fasudil has no effect on heart rate (HR). HR in d21 air- or hypoxia-exposed rat pups given sustained treatment with aerosolized normal saline or Fasudil, shown within 1 hour and greater than 12 hours following treatment.
**Figure 3.20:** Sustained treatment with aerosolized Fasudil does not improve impaired right-ventricular stroke volume (RVSV). Changes in RVSV in d21 air- or hypoxia-exposed rat pups given sustained treatment with aerosolized normal saline or Fasudil, shown within 1 hour and greater than 12 hours following treatment. *p<0.001, by one-way ANOVA, when compared to air-exposed controls.
Figure 3.21: Hypoxia exposure results in increased pulmonary vascular resistance (PVR) and afterload (Ea), measured by 2D echo or right-heart catheter, respectively. Comparison of PVR estimated by 2D echo and Ea measured by right-heart catheter in d21 air- or hypoxia-exposed rat pups treated with aerosolized normal saline. *p<0.001, by t-test, compared to air-exposed control. Bars are shown as multiples of the air control.
Figure 3.22: Hypoxia exposure results in impaired right-ventricular stroke volume (RVSV), measured by either 2D echo or right-heart catheter. Comparison of RVSV measured by 2D echo or right-heart catheter in d21 air- or hypoxia-exposed rat pups treated with aerosolized normal saline. *p<0.05, by t-test, when compared to air-exposed control. #p<0.001, by t-test, when compared to air-exposed control. Bars are shown as fractions of the air control.
Figure 3.23: Hypoxia exposure results in impaired right-ventricular output (RVO), measured by either 2D echo or right-heart catheter. Comparison of right-ventricular output (RVO) measured by 2D echo or right-heart catheter in d21 air- or hypoxia-exposed rat pups treated with aerosolized normal saline. *p<0.05, by t-test, when compared to air-exposed control. #p<0.001, by t-test, when compared to air-exposed control. Bars are shown as fractions of the air control.
3.3 Investigating the Mechanism Underlying RV Dysfunction

As sustained treatment with aerosolized Fasudil did not result in reversal of RV dysfunction and previous data has shown up-regulation of PDE 5 in the hypertrophied right ventricle (Nagendran et al., 2007), we investigated a potential link between up-regulated ROCK activity and increased expression/activity of PDE 5.

3.3a ROCK Activity:

Hypoxia-exposed, vehicle treated animals had significantly (p<0.05) higher ROCK activity in the right ventricle, when compared to air-exposed animals. There was no difference seen in ROCK activity in the left ventricle between air- and hypoxia-exposed animals (Figure 3.24).

3.3b ROCK I and II Content:

Hypoxia-exposed, inhaled saline treated animals showed no difference in RV ROCK I or ROCK II content, when compared to air-exposed controls. Sustained treatment with aerosolized Fasudil did not affect RV ROCK I or ROCK II content in air- or hypoxia-exposed animals (Figure 3.25 and 3.26). LV ROCK I content was similar across all groups, except for air-exposed, inhaled Fasudil treated animals, who had lower (p<0.05) ROCK I content when compared to air-exposed, inhaled saline and hypoxia-exposed, inhaled Fasudil groups. Hypoxia-exposed, inhaled saline treated animals showed no difference in LV ROCK II content, when compared to air-exposed controls. Sustained treatment with aerosolized Fasudil did not affect LV ROCK II content in air- or hypoxia-exposed animals.
3.3c Phosphodiesterase 5 Content:

Hypoxia-exposed, vehicle treated animals had significantly (p<0.001) higher RV PDE 5 content, when compared to air-exposed controls. Sustained systemic treatment with Y27632 resulted in partial, but significantly (p<0.05) decreased PDE 5 content. Sustained treatment with aerosolized Fasudil did not have any effect on PDE 5 content (Figure 3.27). Hypoxia-exposed, vehicle treated animals had significantly (p<0.05) higher LV PDE 5 expression (while to a lesser extent than seen in RV tissue), when compared to air-exposed controls. Sustained systemic treatment with Y27632 resulted in partial, but significantly (p<0.05) decreased PDE 5 expression.

3.3d Phosphodiesterase 5 Activity:

Hypoxia-exposed, vehicle treated animals had significantly (p<0.05) higher PDE 5 activity in the right ventricle, when compared to air-exposed controls. Sustained systemic treatment with Y27632 resulted in a complete normalization of PDE 5 activity, while sustained treatment with aerosolized Fasudil had no effect (Figure 3.28). Hypoxia-exposed, vehicle treated animals showed no difference in LV PDE 5 activity, when compared to air-exposed controls. Sustained treatment with systemic Y27632 or aerosolized Fasudil did not affect LV PDE 5 activity in air- or hypoxia-exposed animals.

3.3e Exercise Tolerance:

Hypoxia-exposed, inhaled saline treated animals had significantly (p<0.001) lower exercise tolerance when compared to air-exposed controls. Sustained treatment with aerosolized Fasudil or systemic Y27632 did not lead to improved exercise tolerance (Figure 3.29).
From these experiments, it was found that ROCK activity and PDE 5 content and activity are up regulated in the right ventricle, which may be important in RV dysfunction. Sustained systemic treatment with Y27632 resulted in decreased PDE 5 content and activity, while sustained treatment with aerosolized Fasudil had no effect on PDE 5 content and activity in the RV. This suggests that PDE 5 expression and activity is regulated by ROCK.
Figure 3.24: ROCK activity is increased in the right ventricle, but not the left ventricle. Changes in right-ventricular (RV) and left-ventricular (LV) ROCK activity in d21 air- and hypoxia-exposed rat pups. ROCK activity is measured as the ratio of phosphorylated MYPT1: total MYPT1. Representative blots shown. *p<0.05 by one-way ANOVA, when compared to the air group. Bars are shown as multiples/fractions of the air control.
**Figure 3.25:** Hypoxia exposure or sustained treatment with aerosolized Fasudil has no effect on ROCK I content in the right ventricle. ROCK I content in right-ventricular tissue from d21 air- or hypoxia-exposed rat pups given sustained treatment with aerosolized normal saline or Fasudil, shown as a ratio of ROCK I: GAPDH content. Representative blots shown. Bars are shown as multiples of the air control.
Figure 3.26: Hypoxia exposure or sustained treatment with aerosolized Fasudil has no effect on ROCK II content in the right ventricle. ROCK II content in right-ventricular tissue from d21 air- or hypoxia-exposed rat pups given sustained treatment with aerosolized normal saline or Fasudil, shown as a ratio of ROCK II: GAPDH content. Representative blots shown. Bars are shown as multiples/fractions of the air control.
Figure 3.27: Sustained treatment with systemic Y27632 decreases elevated phosphodiesterase (PDE) 5 content in the right ventricle, while sustained treatment with aerosolized Fasudil has no effect. PDE 5 content in right-ventricular (RV) tissue from d21 air- or hypoxia-exposed rat pups given sustained systemic treatment with vehicle or Y27632, or aerosolized treatment with Fasudil, shown as a ratio of PDE 5: GAPDH content. Representative blots shown. *p<0.001, by one-way ANOVA, when compared to all other groups. #p<0.05, by one-way ANOVA, when compared to all other groups. Bars are shown as multiples of the air control.
**Figure 3.28:** Sustained treatment with systemic Y27632 decreases elevated phosphodiesterase (PDE) 5 activity in the right ventricle, while aerosolized treatment with Fasudil has no effect. PDE 5 activity in right-ventricular (RV) tissue from d21 air- or hypoxia-exposed rat pups given sustained systemic treatment with vehicle or Y27632, or aerosolized treatment with Fasudil. *p<0.05, by one-way ANOVA, when compared to all groups. Bars represent % PDE 5/ Total PDE activity.
**Figure 3.29:** Hypoxia exposure results in decreased exercise tolerance in adult rats, and sustained treatment with either systemic Y27632 or aerosolized Fasudil has no effect. Exercise tolerance in d21 air- or hypoxia-exposed rat pups given sustained treatment with vehicle, aerosolized Fasudil or systemic Y27632. *p<0.001, by one-way ANOVA, when compared to all other groups. Bars are shown as fractions of the air-exposed control.
Chapter 4

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Discussion
4.1 Discussion of Results

The aims of this project were to examine the effects of sustained pulmonary-specific treatment with a ROCK inhibitor. We sought to determine whether sustained treatment with aerosolized Fasudil would reverse the structural and functional changes of chronic PHT, with similar efficacy to systemic rescue treatment (with Y27632) (Xu et al., 2010), while avoiding severe systemic effects. We hypothesized that sustained pulmonary-specific ROCK inhibitory treatment would normalize PVR and reverse structural abnormalities, including RVH and pulmonary arterial wall remodeling, as seen with systemic rescue treatment. We also hypothesized that up-regulated ROCK activity in the right ventricle is critical to RV dysfunction and that therefore, pulmonary-specific ROCK inhibition would have no effect on RV systolic dysfunction.

Our findings were that late sustained treatment with aerosolized Fasudil resulted in completely normalized PVR in neonatal rat pups with well-established chronic PHT. It also resulted in the reversal of vascular remodeling, with a greater effect seen in medium sized pulmonary arteries, and a decrease in RVH. Sustained treatment with either aerosolized Fasudil or systemic Y27632 resulted in normalized RVSP. Aerosolized Fasudil treatment did not result in improved RV dysfunction.

Normalization of PVR occurred as a result of the vasodilatory effects of Fasudil and its inhibition of the RhoA/ROCK signaling pathway. Ca\(^{2+}\) sensitization results in sustained vasoconstriction as a result of MLCP deactivation, through the increased activity of ROCK. Inhibition of the RhoA/ROCK signaling pathway, through the activity of Fasudil, prevented the phosphorylation and therefore, deactivation of MLCP, allowing for smooth muscle relaxation.
(Duong-Quy et al., 2013). Similar to previous observations with systemic treatment (Xu et al., 2010), normalization of PVR was dependent upon ongoing ROCK inhibition, though PVR did not return to values observed in vehicle-treated controls. This observation supports the contention that increased PVR in chronic PHT occurs through ROCK-mediated pulmonary vasoconstriction whether or not the vasculature is remodeled (Stenmark and McMurtry, 2005; McNamara et al., 2008). As mentioned previously, activation of the RhoA/ROCK pathway can result in decreased NO/cGMP signaling. It has been shown that ROCK inhibition augments NO/cGMP signaling by increasing eNOS expression and activity (Shimokawa, 2002; Abe et al., 2004; Fagan et al., 2004) and by enhancing the vasodilatory effects of cGMP (Gao et al., 2007). Our lab has shown that sustained rescue treatment with Y27632 in chronic hypoxia-exposed animals results in increase pulmonary arterial eNOS expression and lung NOx content (Peng et al., 2012). Therefore, vasodilatory effects seen with sustained ROCK inhibition may in part be due to increased NO/cGMP signaling.

Chronic exposure to hypoxia results in pulmonary vascular remodeling, which is a hallmark characteristic of chronic PHT in neonates, characterized by a ‘fixed’ increase in PVR (van Loon et al., 2010). Vascular SMC proliferation involves the activation of various pro-mitogenic signals, which interfere with cell cycle regulation (Assoian and Marcantonio, 1996). ROCK is known to be involved in various cellular processes, including SMC mitogenesis (Liu et al., 2004). The cyclin-dependent kinase inhibitor, p27, is an important negative regulator of vascular SMC proliferation. There is evidence suggesting that increased ROCK activity results in decreased p27 expression, resulting in increased vascular SMC proliferation (Laufs et al., 1999; Sawada et al., 2000; Croft and Olson, 2006). Inhibition of the RhoA/ROCK signaling pathway may thus result in a decreased number of vascular SMCs or decreased proliferation of these cells,
possibly through the up-regulation of p27 expression. There is evidence that ROCK inhibition promotes vascular SMC apoptosis. In previous work in our lab, we have shown that sustained systemic treatment with Y27632 results in attenuated proliferation and increased apoptosis of pulmonary arterial SMCs (Xu et al., 2010). Although there are no isoform specific ROCK inhibitors currently available, *in vitro* studies conducted in our laboratory suggested that ROCK II may be the dominant anti-apoptotic isoform of ROCK (Xu et al., 2010). Pro-apoptotic effects seen with ROCK inhibition may also be related to subsequent attenuation of ET-1 expression. Our lab has previously shown that 8-isoprostane increased ET-1 expression and ROCK activity in pulmonary arterial SMCs, and that treatment with Y27632 or Fasudil completely attenuated increased ET-1 production and expression (Yi et al., 2006). ET-1 has been shown to be of critical importance in smooth muscle vasoconstriction and pulmonary arterial remodeling, by promoting proliferation and having anti-apoptotic effects. Therefore, reversal of vascular remodeling with sustained ROCK inhibition may occur partly through attenuation of up-regulated ET-1 expression in lung tissue (Yi et al., 2006; Xu et al., 2010).

Sustained pulmonary-specific ROCK inhibition resulted in decreased RVH. This was similar to effects seen with sustained systemic ROCK inhibitory treatment. Our observation of decreased RVH provided us with insight into the mechanism by which the right ventricle becomes hypertrophied. Our results suggest that RVH occurs as a direct result of increased pressure load on the right ventricle.

Previous dogma, now challenged, held that RV dysfunction in chronic PHT was predominantly the result of increased pressure load on the heart (Kjaergaard et al., 2006; Voelkel et al., 2006; Bronicki and Baden, 2010). In previous work, sustained systemic treatment with Y27632 resulted in complete normalization of RV dysfunction (Xu et al., 2010). In the present
thesis, we inhibited ROCK activity specifically in the lung – thus decreasing the pressure load on the heart, without affecting ROCK activity in other organs. Pulmonary-specific ROCK inhibition did not result in improved RV dysfunction. This observation is highly novel, as it reveals that afterload may not play as big a role in the pathogenesis of RV dysfunction as once thought. In our chronic hypoxia model, RV output was impaired as a result of impaired systolic function (manifesting as decreased RVSV), which was not improved with aerosolized Fasudil treatment. In our initial hypothesis, we questioned whether normalization of RV dysfunction with systemic ROCK inhibition was a result of decreasing the afterload, or whether it occurred as a result of an RV specific mechanism. The observation that ROCK activity is increased in the right ventricle, but not the left ventricle, suggests that the RhoA/ROCK signaling pathway may not only be important in terms of pulmonary vasoconstriction and arterial remodeling, but may also play a large role specifically in the right ventricle. Furthermore, for the first time, we have described a potential relationship between ROCK and PDE 5, which may be an important downstream effector of ROCK contributing to RV dysfunction. PDE 5 has been shown to be highly expressed in the hypertrophied right ventricle (Nagendran et al., 2007; Shan and Margulies, 2011). Treatment with Sildenafil has also been shown to have positive inotropic effects on the right ventricle, resulting in increased cardiac output (Nagendran et al., 2007). Together with these previous observations, our results suggest that both ROCK and PDE 5 may play a role in RV dysfunction.
4.2 Limitations and Future Work

While we were able to avoid severe systemic effects seen with systemic ROCK inhibition, the present study still had limitations. Although the chronic hypoxia model was able to provide us with valuable endpoints of interest (structural and functional abnormalities), it still does not replicate the human disease. There is no one animal model that replicates the human disease; therefore, it would be worthwhile to carry out the study in various other animal models. Pulmonary inflammation, which is an important feature in human neonates and infants with chronic PHT, is absent in the chronic hypoxia model (McNamara et al., 2008). Sustained pulmonary-specific ROCK inhibitory treatment should be studied in the bleomycin-sulfate model (McNamara et al., 2008), in which pulmonary inflammation, characterized by macrophage influx, is central to pathogenesis (Moore et al., 2001; Jankov et al., 2008; Sewing et al., 2012). Additionally, chronic hypoxia does not morphologically resemble chronic lung disease in human neonates, since abnormal lung structure completely normalizes by adulthood (unpublished observation), despite there being persistently decreased exercise tolerance. Therefore, it would be worthwhile to look at chronic neonatal lung injury with PHT, in a model that manifests sustained abnormalities in lung structure. Our lab has recently developed a chronic hypoxia model coupled with a single dose of VEGF receptor inhibitor (SU5416) given on PND 1, which more closely resembles chronic lung disease in humans. Study of the effects of ROCK inhibition would also be worthwhile in this model. We observed that chronic hypoxia exposure resulted in decreased exercise tolerance in adulthood, and that treatment with systemic or pulmonary-specific ROCK inhibition had no effect on decreased exercise tolerance. It would be interesting to see whether ROCK inhibitory treatment, in combination with existing treatments, such as Sildenafil, would
have any beneficial effects. It would also be important to confirm in future studies whether poor exercise tolerance was the result of recurrent RV dysfunction in adulthood, which is likely to be the case. We recognize that the duration of treatment may not have been long enough, and that treatment beyond the 1-week period could possibly have beneficial effects on RV dysfunction. While systemic rescue treatment resulted in reversal of RV dysfunction, it also led to severe systemic hypotension. It is possible that the severe systemic vasodilation observed may explain the reversal of RV dysfunction, and the lack of systemic vasodilation with pulmonary-specific treatment explains the lack of reversing effects on RV systolic dysfunction.

While pulmonary-specific ROCK inhibition avoided adverse systemic effects, making it more clinically applicable, its short duration of activity may present a challenge to clinical use, requiring constant or frequent inhalations, which can prove costly in terms of time, equipment and the amount of drug required. While inhalation of drugs with short half has been successfully applied to patients with PHT, such as prostacyclin and NO (Zwissler et al., 1996; Olschewski et al., 2002; Ivy et al., 2008), it would be worthwhile to investigate other preparations that may extend its duration of activity. Recently, aerosolized liposomal Fasudil has been investigated as a non-invasive method of administration, and was found to result in prolonged vasodilation in distal pulmonary arterioles (Gupta et al., 2013a). It would be interesting to see whether sustained treatment using liposomal Fasudil would have the same effects seen with aerosolized treatment, as this new method of delivery would be extremely useful in a clinical setting.

While these studies have provided us with the very important mechanistic insight that RV dysfunction does not occur as a result of increased pressure load on the heart, much remains to be investigated with regards to the pathogenesis of cardiac dysfunction in chronic PHT. We have described a potential relationship between ROCK and PDE 5 in the right heart; therefore, it
would be worthwhile to investigate the effects of treatment with Sildenafil on RV dysfunction. If PDE 5 lies downstream of ROCK, as our results suggest, treatment with Sildenafil should result in improved RV function in animals with chronic PHT. It would also be important to look at potential upstream mediators of ROCK, in the right ventricle, which likely includes oxidative stress (Redout et al., 2007). Treatment with dehydroepiandrosterone (DHEA) in rats with severe PHT resulted in a reduction in elevated RVSP and improvement of cardiac function. DHEA treatment inhibited oxidative stress, by decreasing NADPH levels in the right ventricle, as well as decreased ROCK activity (Alzoubi et al., 2013). Indeed, there is data suggesting that reactive oxygen species (ROS) mediated up-regulation of PDE 5 plays an important role in cardiac dysfunction (Lu et al., 2010), suggesting that oxidative stress may lie upstream of both ROCK and PDE 5. Should we find that oxidative stress is increased, it would be worthwhile to look at the effects of Tempol (Chatterjee et al., 2000), a superoxide scavenger, on RV dysfunction. It would be interesting to further examine potential sources of reactive oxygen species (ROS) in the right ventricle that may contribute to dysfunction (Ungvari et al., 2005). Microarray or PCR array studies would also prove useful in identifying other potential downstream mediators of ROCK, such as cytokines and chemokines, which may play a role in RV dysfunction.

Comparing effects of inhaled to systemic treatment with ROCK inhibitors provides a valuable opportunity to study the potential role these mediators may play in the mechanism of RV dysfunction.

While use of ROCK-selective inhibitors has provided us with interesting insights, determining the exact cellular mechanism of action along with the contribution of each isoform of ROCK is not possible with the use of these inhibitors alone. Investigation of the cellular mechanism of action further requires more sophisticated molecular approaches, such as siRNA-
mediated knockdown or conditional ROCK 1/2-knockout mice (Massaro et al., 2004; Rikitake et al., 2005; Zhang et al., 2006). These methods would provide us with further insight into the role that ROCK may play in various organs and the importance of each isoform in various cellular processes.

Though much remains to be understood about the role that ROCK may play in the pathogenesis of RV dysfunction, these studies have provided us with important mechanistic insight that will drive future research. We have confirmed that aerosolized Fasudil is a feasible and non-invasive mode of delivery that confers pulmonary-selectivity and avoids adverse systemic effects. We have also further highlighted the importance of the RhoA/ROCK signaling pathway in chronic PHT and the manifestation of RV dysfunction. Also, for the first time, we have identified a potential relationship between ROCK and PDE 5 that may be therapeutically exploited. All of these contributions form the basis of future work aimed at understanding the underlying mechanism of RV dysfunction in chronic neonatal PHT.
References


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