Simvastatin Prevents and Reverses Chronic Pulmonary Hypertension in Newborn Rats via Pleiotropic Inhibition of RhoA Signaling

By

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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Master’s of Science in Physiology
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2016

Abstract
Chronic neonatal pulmonary hypertension (PHT) frequently results in early death. Systemically administered Rho-kinase (ROCK) inhibitors prevent and reverse chronic PHT in neonatal rats, but with side effects including systemic hypotension and growth restriction. Simvastatin has pleiotropic inhibitory effects on isoprenoids that may limit activity of RhoA, which signals ROCK. We examined the preventive and rescue effects of simvastatin on chronic hypoxia-mediated chronic PHT. Newborn rats were exposed to normoxia (room air) or moderate hypoxia (13% O₂) from postnatal day 1 and received simvastatin (2 mg/kg/day i.p.) from postnatal days 1-14 (prevention) or from postnatal days 14-21 (rescue). Simvastatin decreased RhoA/ROCK signaling in the hypoxia-exposed lung. Treatment of chronic hypoxia-exposed animals with simvastatin decreased pulmonary vascular resistance, right ventricular hypertrophy, and pulmonary arterial remodeling. We conclude that simvastatin limits RhoA/ROCK activity in the chronic hypoxia-exposed lung, thus ameliorating hemodynamic and structural markers of chronic PHT, without causing adverse systemic effects.
Table of Contents

1.1 General Overview of PHT .......................................................... 2
1.2 Etiology of PHT in Neonates .................................................. 2
1.3 Right Ventricular Dysfunction and Failure ................................ 5
1.4 Current PHT therapy ............................................................... 7
1.5 Role of ROCK in PHT ............................................................. 8
  1.5.1 Sustained pulmonary vasoconstriction mediated by ROCK signaling .......... 10
  1.5.2 Vascular compliance in the pulmonary circuit .......................... 11
  1.5.3 Cell migration and proliferation mediated by ROCK signaling ............ 12
  1.5.4 ROCK-mediated anti-apoptotic signaling in smooth muscle ............. 13
  1.5.5 ROCK inhibitors ............................................................. 15
1.6 Hypoxia and HIF-1α regulation ............................................... 15
1.7 eNOS-cGMP signaling pathway ............................................... 16
1.8 Statins and cholesterol reduction ............................................. 17
1.9 Pleiotropic effects of statins on isoprenoid intermediates ................. 18
1.10 Evidence for pleiotropic effects of statins .................................. 21
1.11 Effects of statins in experimental pulmonary hypertension ............... 22
1.12 Clinical trials of statins for adult pulmonary hypertension ............... 24
1.13 Prospects for statins in the pediatric population .......................... 26
1.14 Potential side effects of statins .............................................. 26
1.15 Neurological impact of statins ................................................ 28
1.16 Rationale, Global Aims, Specific Hypotheses .............................. 30

Chapter 2 ..................................................................................... 31

2.1 Materials .................................................................................. 32
2.2 Institutional Review .................................................................. 34
2.3 In Vivo Experiments .................................................................. 34
2.4 In Vitro Experiments .................................................................. 37
2.5 Simvastatin preparation and delivery ......................................... 37
2.6 Hypoxia Exposure System ....................................................... 38
2.7 Serum collection ........................................................................ 38
2.8 Two-Dimensional Echocardiography and Pulse Wave Doppler Ultrasound .... 38
3.1 Determining the optimal dose of simvastatin in the newborn rat ........................................... 50
3.2 Lung FPP content and RhoA/ROCK activity were attenuated by simvastatin .......................... 50
3.3 Pulmonary arterial smooth muscle cell ROCK activity was attenuated by simvastatin .......... 53
3.4 Simvastatin prevented chronic PHT ...................................................................................... 54
3.5 Chronic hypoxia-induced pulmonary arterial remodeling was attenuated by simvastatin .... 56
3.6 Chronic hypoxia-induced PHT was reversed by rescue treatment with simvastatin ......... 59
3.8 Treatment with simvastatin improved weight gain and did not alter systemic blood pressure .................................................................................................................. 65
3.9 Preventive treatment with simvastatin did not decrease cholesterol levels in newborn rat serum .................................................................................................................. 67
3.10 Preventive treatment with simvastatin did not cause adverse effects on the developing brain ......................................................................................................................... 68
3.11 Cleaved ROCK I, but not ROCK I or II content was decreased by preventive treatment with simvastatin ...................................................................................................................... 70
3.12 Preventive simvastatin decreased HIF-1α content, and increased lung eNOS content

3.13 Preventive treatment with simvastatin did not increase serum markers of muscle or liver toxicity

Chapter 4

4.1 Discussion of Results

4.2 Limitations and Future Directions

References

List of Abbreviations
Chapter 1

Introduction
1.1 General Overview of PHT

Chronic pulmonary arterial hypertension (PHT) arising in the neonatal period is frequently lethal. Extremely premature birth (earlier than 29 completed weeks of gestation) and associated chronic lung injury, known as bronchopulmonary dysplasia (BPD) (16), is frequently complicated by PHT (11). Chronic PHT is characterized by increased pulmonary arterial pressure (PAP) and pulmonary vascular resistance (PVR) (23, 49). With progression of PHT, the right ventricle (RV) must contract against an increased afterload, which leads to compensatory RV hypertrophy (RVH) and potentially maladaptive chamber dilatation, which precedes RV failure and eventual death (24, 26). PHT has been reported to complicate up to 20% of all preterm births, and to be present in up to 60% of newborns with severe chronic lung disease (11, 158, 159). Newborns with chronic PHT associated with severe BPD, have a high mortality rate, with one study reporting a two-year survival rate of 52% (11, 158). Moreover, the long-term impact for newborn PHT survivors is an increased risk for impaired neurological development (57, 87, 116, 177, 190), and potential recurrence of PHT later in life (11, 196). Currently, there are no treatments of proven efficacy for chronic PHT and RV failure in neonates (115, 121, 221).

1.2 Etiology of PHT in Neonates

Clinically, PHT is defined by PAP (measured by catheterisation) being equal or greater than 25 mmHg at rest (59). Pulmonary vascular disease arising in the neonatal period can be broadly categorized into conditions leading to acute or chronic PHT based on clinical presentation and underlying etiology.
Figure 1.1: A schematic flowchart illustrating the consequences of increased pulmonary vascular resistance on the RV. Chronically increased afterload frequently results in RV dysfunction and reduced cardiac output, leading to systemic hypotension. Image from Jain, A., and McNamara, P.J., Semin Fetal Neonatal Med., 2015; 20(4):262-71, reproduced with permission from Avery’s neonatology: pathophysiology and management of the newborn, 7th edition.

The acute category includes newborns who develop persistent pulmonary hypertension (PPHN) during the transition from fetal to postnatal life (232). It is estimated to affect 2 per 1000 live births, and accounts for 10% of cases of respiratory failure in neonates (222). Of the known neonatal forms of PHT, PPHN is the best understood, leading to oxygenation failure during the first few days of life (77, 78). Mortality is estimated at 5-10% (135, 243), and generally related to the underlying precipitant, such as birth asphyxia or sepsis.
The chronic progressive PHT category presents the greatest challenge in terms of prevalence, survival, and effects on quality of life of newborns and infants. The most common underlying disorder in this category is moderate-severe BPD (>800 new cases per year in Canada (207)), because of extremely premature birth (20, 154). BPD is the most common complication of premature birth, being most frequent among newborns born less than 29 weeks gestation and weighing less than 1000 g (223). The advent of exogenous surfactant and other advances in neonatal care (56) have extended the limits of viability to gestational periods as short as 23 weeks (176). The unintended consequence for premature, extremely low birth weight (ELBW), or small for gestational age (SGA) newborns is a greater risk of developing BPD and PHT due to arrested lung development, characterized by reduced angiogenesis, alveolar simplification, and inflammation (23, 49, 68, 176).

A common feature of BPD is vascular remodeling and thickening of the pulmonary arteries which contributes to elevated PAP and PVR, and resultant PHT (11). The variability of presentations in newborns may range from being asymptomatic/clinically silent, to requiring prolonged ventilator or oxygenation support, to shock secondary to a low cardiac output state. Furthermore, the respiratory function may deteriorate at different rates (gradual or rapid), and pulmonary hypertensive crises may occur when precipitated by interventions such as inappropriately applied mechanical ventilation, sedation or anaesthesia, or by an intercurrent infection (11). Due to its enigmatic nature, routine echocardiographic screening of BPD infants is recommended to detect and diagnose PHT (54). The incidence of BPD in preterm infants may be as high as 50% (157), and the risk of developing PHT with moderate-
severe BPD ranges from 25-43% (12, 108). Moderate-severe BPD newborns with PHT presents the greatest challenge for quality of life (160), and a poor survival rate after diagnosis with a 52% survival after 2 years (108).

1.3 Right Ventricular Dysfunction and Failure

In a healthy newborn, the RV is thin-walled and crescent shaped relative to the left ventricle (LV). This is ideal for the low resistance, low pressure, and high volume pulmonary circulation. With the progression of PHT and chronic pressure overload, the RV compensates through hypertrophy in order to reduce wall tension and generate enough contractile force against an increased afterload. When RVH is insufficient, the RV chamber dilates to increase stroke volume by the Frank-Starling mechanism. The exact physiologic mechanisms that contribute to the transition from hypertrophy to dilatation are not understood, but changes in cardiomyocyte protein composition and extra-cellular matrix (ECM) have been reported (242). As the wall tension increases, cardiomyocyte oxygen and metabolic demand increases, which also reduces contractility, leading to a vicious cycle of exacerbated dilatation in an attempt to compensate (8). Chronic maladaptive compensation results in decreased stroke volume, decreased cardiac output (CO), and eventual death (24, 26, 82). RV failure is the major cause of death in PHT (16, 20, 24, 26, 240). This is crucial as the newborn cannot adapt as well as the adult, and experience a rapid progression from RV adaptation to failure, for reasons that are not well understood (254, 268).
Figure 1.2: A diagram illustrating the changes in pulmonary arteries and the right ventricle from a healthy state to a severely remodeled state during pulmonary vascular disease. In the healthy state, pulmonary arteries are thin walled and have a functional endothelium. The right ventricle is thin walled, crescent shaped chamber with a normal cardiac output. During the compensatory phase, pulmonary arteries have an abnormal endothelium and start to become constricted and stiff. This leads to a small increase to pulmonary vascular resistance, where the right ventricle becomes hypertrophied in order to maintain an adequate cardiac output. Late stage pulmonary hypertension can be exemplified by vascular remodeling and the significantly elevated cell proliferation and of the arterial walls. This results in a severely increased pulmonary vascular resistance, where the right ventricle becomes dilated, leading to a decreased cardiac output and fatal right ventricle failure. Image from Champion et al., Circulation, 2009; 120:992-1007.
1.4 Current PHT therapy

The current therapeutic focus for PHT is pulmonary-specific vasodilation. Of the available therapies, inhaled nitric oxide (iNO) is the current gold standard of therapy for newborn BPD and PHT patients (111). iNO for term newborns is approved in North America by both Health Canada and the US Food and Drug Administration (41). While iNO has reduced the need for extracorporeal membrane oxygenation (ECMO), there is no evidence that iNO improves survival in patients with PPHN or chronic neonatal PHT (37, 115). Premature newborns may require respiratory support through assisted ventilation, and newborns with BPD-associated PHT require prolonged ventilator support, which further contributes to injury (22, 246). Neonates with BPD-associated PHT have four times the mortality rate compared to BPD cases without PHT (215). Supplementary O₂ therapy is also used as respiratory support, but hyperoxic conditions may interfere with iNO therapy (120). O₂ can generate reactive oxygen species (ROS), diverting NO to produce peroxynitrite, which stimulates vasoconstriction (91, 122).

ECMO and/or lung transplantation are a last resort for severe oxygenation failure due to PHT. However, ECMO is not always possible due to the small size of many neonates with PHT (129). Furthermore, infants frequently do not meet the criteria for transplant or do not find suitable donors (72, 112). A new line of drug therapy would be necessary as the use of iNO, ECMO, or transplantation is not always feasible or readily available. Studies for treatments such as phosphodiesterase (PDE) 3/5 inhibitors and endothelin 1 (ET-1) receptor antagonists have demonstrated therapeutic potential in the pediatric and newborn population (152, 229, 237). However, whether or not this off-label
use of pulmonary vasodilators reduces mortality and morbidity for chronic neonatal PHT remains unknown (39).

1.5 Role of ROCK in PHT

Rho-associated coiled-coil containing kinases (ROCK) are known to have two isoforms (ROCK I/ROCK β and ROCK II/ROCK α) which are both expressed in the heart and lung vasculature (13). The structure of ROCK is comprised of an N-terminal kinase domain, followed by a coiled-coil region with the Rho-binding domain (RBD), and pleckstrin homology domain (PHD) bisected by a cysteine-rich zinc finger-like motif domain (CRD) at the C-terminal (238). Both of the ROCK homologs are regulated by two distinct modes of activation. Rho-independent activation is mediated by caspase-3 (ROCK I) or granzyme B (ROCK II), which cleaves the autoinhibitory PHD of ROCK (99). The Rho-dependent mode of activation is initiated by attachment of ras-homolog gene family member A (RhoA) to the RBD of ROCK, inducing a conformational change, releasing the PHD from the kinase domain (99). Either cleavage or conformational change of the PHD exposes the active kinase region, where it can modulate numerous downstream substrates (9, 10). Complete ROCK I or II knockout mice appear to be embryonic lethal or are very short lived, therefore ROCK is vital to growth and development (13). ROCK is crucial to the pathogenesis of PHT as ROCK II*−/− knockout mice were resistant to the pulmonary vascular effects chronic hypoxia, while transgenic overexpression of ROCK II mice had augmented development of PHT (212). We have previously reported that ROCK signaling is increased in both chronic hypoxia and bleomycin-injured newborn rat lungs, which led to dysregulation of pulmonary vascular tone and pulmonary arterial remodeling (75, 151). ROCK mediates arterial remodeling
via regulation of anti-apoptotic, pro-proliferative, and pro-differentiation signaling in pulmonary arterial endothelial cells, vascular smooth muscle cells (VSMC) and fibroblasts (79, 201, 254).

Figure 1.3: A schematic diagram illustrating the involvement of the RhoA/ROCK signaling pathway in pulmonary hypertension. Image from Connolly et al., *Pulm Pharmacol Ther*, 2011; 24:1-14.
Figure 1.4: An illustration of the molecular structure and activation pathways of ROCK. The Kinase Domain is located on the N-terminus of the protein. The coiled region contains the Rho-binding domain (RBD). The autoinhibitory is comprised of the Pleckstrin-homology (PH) and cysteine-rich domain (CRD), at the C-terminus of the protein chain. Inactive ROCK can be activated by a Rho protein binding to the RBD or have the autoinhibitory region cleaved by Caspase-3 (ROCK I) or Granzyme B (ROCK II). Image from Julian L. and Olson M.F., 2014, Small GTPases, 5:E29846 1-12.

1.5.1 Sustained pulmonary vasoconstriction mediated by ROCK signaling

Pulmonary vasoconstriction is a key factor in elevation of PVR as a contributor of PHT. The RhoA/ROCK pathway is a major regulator of vascular tone and is upregulated in patients with PHT (80, 218). In vascular smooth muscle cells (VSMC), vasoconstriction is regulated by phosphorylation of myosin light chain (MLC) through myosin light chain kinase (MLCK), and dephosphorylation by myosin light chain phosphatase (MLCP) for vasorelaxation (52). MLCK is regulated by Ca$^{2+}$ signaling, and MLCP by the NO/PKG signaling pathway (52). ROCK acts by phosphorylating the Thr853 (Thr850 in rats) site of myosin phosphatase target subunit 1 (MYPT-1) of MLCP, which inhibits MLCP activity, thus favoring a contractile state known as Ca$^{2+}$
sensitization (197, 218). Inhibiting ROCK restores the regulatory status of MLC through MLCP activity and relaxation of the vascular smooth muscle (75, 151).

1.5.2 Vascular compliance in the pulmonary circuit

In clinical practice, diagnosis of PHT is most often described in terms of PVR, and treatment is vasodilation-oriented (123). Therapeutic goals should also address the stiffness of the pulmonary vasculature, as vascular remodeling has an additional detrimental effect on vascular compliance (201). Compliance is a measure of arterial distensibility, or the elastic properties of the arteries (69). The normal pulmonary circulation is a low resistance, high compliance vascular system designed to accommodate the entire cardiac output. In PHT patients, increased thickening of pulmonary arteries from smooth muscle and ECM deposition contributes to an increased pulmonary arterial stiffness or reduced compliance (123). There is an inverse hyperbolic relationship between increased PVR and arterial compliance such that resistance changes little until compliance decreases to a critical level; thereafter PVR (and RV afterload) increases rapidly (124, 163). The role of compliant pulmonary arteries during systole is to store a portion of the ejected stroke volume. During diastole, the stored blood is released. The compliance of pulmonary arteries reduces the RV stroke volume and ejection pressure, sparing excess energy expenditure (35, 249). In practice, assessment of arterial compliance is difficult but potentially achievable through catheterization combined with magnetic resonance imaging (69). Therefore, optimal PHT management requires a multi-faceted approach for alleviating RV afterload, through reduction in PVR and prevention or reversal of arterial remodeling.
Figure 1.5: Baseline measurements of patient A (mild PHT) and patient B (severe PHT). Curve demonstrates relationship of pulmonary resistance-compliance to RV afterload. Figure from Lankhaar et al., 2008; Eur Heart J., 29(13):1688-1695.

1.5.3 Cell migration and proliferation mediated by ROCK signaling

As previously mentioned, vascular remodeling decreases pulmonary arterial compliance and increases resistance. Remodeling is characterised by a narrow blood vessel lumen and thickened wall as a consequence of intimal, medial, and adventitial layer proliferation and an increase in extracellular matrix deposition (180, 181). The etiology of arterial thickening is multifaceted with the hypertrophy and hyperplasia of smooth muscle cells and fibroblasts, and the loss of endothelial cells through apoptosis (201). Arterial remodeling appears to originate from altered crosstalk between the endothelium, local immune cells, and smooth muscle (79). Arterial thickening is further
enhanced by accumulation of extracellular matrix components such as elastin and collagen (21, 201). Pulmonary arterial smooth muscle cells (PASMC) and VSMC migration and proliferation may be stimulated by ROCK activity through substrates such as cyclophilin A, platelet-activating factor (PAF), extra-cellular signal-regulated kinases (ERK), and platelet-derived growth factor (PDGF) (185, 198, 241, 266).

RhoA activation is required for nuclear translocation of myocardin-related transcription factors (MRTF), which mediates smooth muscle cell differentiation (141). Activated ROCK regulates the cytoskeleton structure by acting on LIM domain kinase (LIMK), to stabilize the formation of actin filaments by inhibiting cofilin, which normally functions by binding to actin for promotion of disassembly (167). An additional consequence of inhibited phosphorylation of MYPT1 is promotion of actomyosin stress fiber formation (217). The vasoconstriction phenotype is also augmented by excitation-contraction coupling, increasing transcription for genes of smooth muscle cell proteins such as α-smooth muscle actin (171, 244). Downstream substrates of ROCK, such as LIMK, adducin, and ERM (ezrin, radixin, moesin) family proteins, also promote actin cytoskeleton assembly (133, 148, 168, 225).

1.5.4 ROCK-mediated anti-apoptotic signaling in smooth muscle

ROCK activity is linked to pro- and anti-apoptotic processes in the pulmonary vasculature depending upon the cell type (153, 224). During apoptosis, cells shrink due to actin-myosin generated contractile force. This drives membrane blebbing, nuclear disintegration, and apoptotic fragmentation. The apoptotic process is driven from caspase-3 induced cleavage of the autoinhibitory region of ROCK I to provide the sustained contractile force needed for blebbing (38, 99, 204). Caspase-3 inhibitors
prevented ROCK I cleavage, limiting apoptosis (34). In experimental PHT, ROCK inhibitors stimulated apoptosis in PASMC by reducing ET-1 (254). ROCK appears to enhance cell survival of VSMC thus contributing to medial wall thickening. Transgenic mice with overexpression of cleaved ROCK I in cardiomyocytes had increased cell death and fibrosis (257). Similarly, ouabain-induced cleaved ROCK I activity led to the apoptosis of endothelial cells (15). As shown below, ROCK activity promotes the survival of PASMCs, whereas in other cell types such as endothelial cells and cardiomyocytes, it promotes apoptosis; all of which contribute to PHT pathogenesis.

**Figure 1.6:** A schematic diagram illustrating the involvement of the ROCK isoforms and cell type-specific functions. Image from Hartmann et al., *Front Pharmacol*, 2015; 6:276-292.
1.5.5 ROCK inhibitors

Experimental work with ROCK inhibitors such as Fasudil and Y-27632, has demonstrated that the activity of RhoA and its downstream mediator ROCK is critical to the pathogenesis of experimental chronic neonatal PHT, by prevention and reversal of either chronic hypoxic- or bleomycin-induced PHT in neonatal rats (75, 130, 209, 254, 268). Our laboratory had previously reported that ROCK inhibitors normalized PVR, attenuated RVH, and prevented or reversed pulmonary arterial remodeling in experimental models of PHT (130, 151, 254, 268). However, there are serious side effects with use of systemic ROCK-specific inhibitors as they have been shown to induce severe systemic hypotension (75, 81). Treatment with Y-27632 or Fasudil also led to significant growth restriction in neonatal rats (268).

1.6 Hypoxia and HIF-1α regulation

Hypoxia-inducible factor-1 alpha (HIF-1α) is well-established as a master regulator of gene transcription in hypoxic environments. HIF-1α structure is stabilized in the absence of oxygen, and may increase the expression and activity of RhoA and ROCK (70, 182). In human PHT, HIF-1α is highly expressed in pulmonary endothelial and smooth muscle cells (14, 61). HIF-1α contributes to pulmonary remodeling as HIF-1α−/− knockout mice exposed to chronic hypoxia did not have the characteristic pulmonary vascular remodeling of PHT. This would suggest that HIF-1α activation is required for subsequent RhoA-mediated PHT remodeling (17). In human PHT, HIF-1α downregulates miR-223 expression, enhancing poly(ADP-ribose) polymerase I (PARP-1) mediated cell proliferation in distal pulmonary arteries and PASMCs (153). Exoenzyme C3 transferase, a Rho inhibitor, partially blocked HIF-1α stabilization,
demonstrating that RhoA could create a positive feedback loop by enhancing stabilization of HIF-1α in cardiomyocytes (74). In human umbilical vein endothelial cells (HUVEC), the ROCK inhibitor Fasudil decreased HIF-1α expression in hypoxic conditions (228). The role of statins are unclear as dose-dependent positive and negative effects on HIF-1α content have been reported (97, 165, 234). Nitric oxide (NO) is another known regulator and has been shown to inhibit HIF-1α stabilization (96).

1.7 eNOS-cGMP signaling pathway

Endothelial dysfunction is a critical contributor to PHT (113). The main function of NO in the endothelium is vasodilation through stimulation of soluble guanylate cyclase (sGC), which induces cyclic guanosine monophosphotase (cGMP) formation in VSMC. cGMP activates protein kinase G (PKG), which promotes Ca^{2+} reuptake by the sarcoplasmic reticulum. Low intracellular Ca^{2+} decreases MLCK activity, reducing phosphorylation of MLC (267). The protective effect of NO is evident in eNOS⁻/⁻ (endothelial nitric oxide synthase) knockout mice, which experience accelerated RV remodeling and a greater degree of RVH secondary to chronic hypoxia (162). Reduced NO formation has been observed in humans with PHT (3, 32, 62, 63). In PHT, the RhoA/ROCK pathway negatively regulates eNOS by inhibition of cGMP-dependent protein kinase Iα (103). ROCK can also disrupt the stability of eNOS mRNA, further limiting bioavailability (186). The eNOS homodimer is uncoupled in hypoxia, and shifts in production from NO to superoxide formation. This impairs NO signaling by reducing overall production and increasing formation of ROS through the oxidation of NO (62, 109). In vivo studies have shown that eNOS transcription and subsequent expression is downregulated in hypoxia and PHT (60, 86). However, this issue remains under debate.
as other investigators have demonstrated that the total eNOS expression was upregulated in the arteriolar endothelium and media in PPHN neonates, which may be a compensatory response (219).

**Figure 1.7**: A schematic diagram illustrating the interaction between vascular endothelium and smooth muscle. Activated ROCK inhibits eNOS content. Image from Wirth, A., *Biochim Biophys Acta*, 2010; 1802(12):1276-84.

### 1.8 Statins and cholesterol reduction

Statins [3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase inhibitors] prevent conversion of HMG-CoA to mevalonate, which is the rate-limiting step in cholesterol biosynthesis (25, 55). The Framingham Heart Study and more recent clinical trials such as: JUPITER, ASCOT-LLA, and METEOR, have demonstrated the effectiveness of statins in reducing both LDL cholesterol and cardiovascular related death (83, 101, 161, 256). Based on pleiotropic effects described below, statins could
be an ideal means of achieving RhoA/ROCK inhibition, as there is a large body of data spanning several decades documenting the mechanisms of action and relative safety in adult treatment of cholesterol-related cardiovascular disease. There is an urgent need for a novel treatment of PHT beyond pulmonary vasodilators, and based on past studies of ROCK inhibitors in PHT, statins may have potential as a new treatment option for newborns (253).

**Figure 1.8:** Molecular structures of HMG-CoA and simvastatin. Image modified from Sirtori, C.R., *Pharmacol Res*, 2014; 88:3-11.

![](image)

**1.9 Pleiotropic effects of statins on isoprenoid intermediates**

The pleiotropic effects of statins are an additional treatment benefit due to inhibition of mevalonate synthesis, which subsequently decreases the synthesis of the isoprenoid intermediate pathway (73). The isoprenoid intermediates of interest in PHT are farnesyl-pyrophosphate (FPP) and geranylgeranyl-pyrophosphate (GGPP) (71, 73, 187, 265). Of the isoprenoid intermediates, it is known that FPP interacts with the Ras family of proteins, while GGPP binds to the Rho family. The small GTP-binding protein RhoA cycles between an inactive GDP-bound and active GTP-bound state by translocation and activation at the membrane (126). An inactive reservoir of GDP-RhoA
remains in the cytosol bound to Rho-specific guanine nucleotide dissociation inhibitor (RhoGDI) (66). Lipid attachment to a protein, a process known as prenylation, enables intracellular trafficking. GGPP-mediated prenylation of RhoA is required for intracellular cytosol-membrane trafficking (245). In addition, GGPP-mediated prenylation of RhoA may prevent the binding action of RhoGDI to GDP-RhoA, directing membrane translocation and preventing extraction when membrane-bound (50). Once localized to the membrane, RhoA is activated by guanine nucleotide exchange factors (GEF) catalyzing the exchange of bound GDP for GTP. Activated GTP-RhoA binds to the Rho-binding domain of ROCK, inducing an active conformational change (213). GTP-RhoA is inactivated by GTPase activating proteins (GAP) through hydrolysis of the phosphate bond (125). Therefore, by limiting the synthesis of GGPP and possible prenylation of RhoA, statins may reduce RhoA activation and inhibit downstream ROCK activation (245).
Figure 1.9: A schematic diagram illustrating the cholesterol biosynthesis pathway and isoprenoid intermediate branch. Statins (HMG-CoA Reductase inhibitors) prevent conversion of HMG-CoA to mevalonate, the rate limiting step of cholesterol and isoprenoid synthesis. This reduces overall bioavailability of cholesterol, farnesyl pyrophosphate, and geranylgeranyl pyrophosphate. Image from Sirtori, C.R., Pharmacol Res, 2014; 88:3-11.
1.10 Evidence for pleiotropic effects of statins

Protein prenylation is an essential post-translational modification (PTM) for many proteins and the pleiotropic, or cholesterol-independent effects of statins have been studied in many organ systems and disease. At first, the benefits of statin therapy in vascular health were generally accepted to be solely related to the reduction of cholesterol levels. Meta-analyses from large-scale clinical trials such as WOSCOP and CARE indicated a much lower incidence of coronary artery disease and myocardial infarctions for patients on statin therapy compared to placebo controls, despite having comparable serum cholesterol levels (27, 146, 174, 193, 210). Since then, it has been suggested that cholesterol-independent effects may only account for some of the observed positive outcomes with statin therapy. For example, dysregulation of geranylgeranylation may contribute to diseases of the cardiovascular system, to neurological impairment, and to some forms of cancer (255).

Statins have also been shown to increase the bioavailability of eNOS and NO, limiting or preventing myocardial infarction, diabetes, and epilepsy in animal models (7, 145, 206). The mechanism is believed to be a PTM to increase the mRNA half-life of eNOS. Statins may protect the eNOS mRNA by lengthening the 3’ polyadenylation tail to prevent enzyme degradation (117). Statins and eNOS expression is reversed by GGPP supplementation and is not affected by addition of FPP or LDL cholesterol (126). Both in vitro and in vivo studies with farnesyl transferase inhibitors (FTIs) and geranylgeranyl transferase inhibitors (GGTIs) support the role of prenylation by GGPP in RhoA activity (156, 164, 236). Furthermore, supplementing FPP or GGPP after the
specific transferase inhibitor or genetic transferase knockdown re-introduces the activity of RhoA and promotion of cardiomyocyte hypertrophy (258, 259).

1.11 Effects of statins in experimental pulmonary hypertension

Experimental studies have shown that statin pleiotropy protects adult animals against PHT. The pathogenesis of PHT is complex and there is no single pathway implicated. Adult rats chronically exposed to 10% O2 and treated with simvastatin (20 mg/kg) had reduced mPAP, RVH, medial wall thickening, and ROCK I/II protein content. Rats receiving simvastatin treatment and supplemented with mevalonate were no different from the hypoxia-exposed controls (71). In the bleomycin model of PHT, adult C57B1 mice were treated with 5 or 20 mg/kg of simvastatin. Simvastatin had a dose-response reduction to markers of pulmonary inflammation and fibrosis through reduction of lung transforming growth factor beta 1 (TGF-β1), connective tissue growth factor (CTGF), collagen III mRNA expression and protein. Additionally, simvastatin decreased pro-fibrotic differentiation proteins such as p-38, RhoA, and p-Smad2 (170). Adult rats given bleomycin then treated with simvastatin (2 mg/kg) had less fibrosis, improved lung compliance, attenuated RVH, and had improved exercise capacity (203). In a monocrotaline model of PHT in adult rats, simvastatin (2 mg/kg) downregulated nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB) expression in the pulmonary arteries, which suggests inhibition of the inflammatory pathogenesis of PHT (138). A separate study demonstrated that monocrotaline-exposed rats treated with simvastatin (2 mg/kg) can activate p38 to increase activity of heme oxygenase-1 (HO-1), an anti-proliferative, anti-inflammatory modulator, to reduce medial wall area and downregulate IL-6 (264). Similarly, other statins have demonstrated efficacy in
experimental PHT (28, 132, 149). Additionally, combination therapy with statins and the 
PDE-5 inhibitor, sildenafil, has been shown to have synergistic effects in limiting chronic 
PHT (94, 118, 119, 143, 265).

**Figure 1.10:** A schematic representation of the RhoA activation and ROCK-mediated 
myosin light chain signaling pathway. RhoA is localized to the membrane by the 
isoprenoid geranylgeranyl pyrophosphate. RhoA is switched to the active form by 
guanine nucleotide exchange factor by exchanging GDP for GTP. Activity is regulated 
by GTPase activating proteins hydrolyze the phosphate bond, returning to a GDP-
bound state. RhoA activates ROCK, which in turn phosphorylates myosin light chain 
(MLC) and myosin phosphatase target subunit 1 (MYPT1). Phosphorylation of Thr850 
on MYPT1 inactivates myosin light chain phosphatase (MLCP), the enzyme responsible 
for dephosphorylation of MLC and vasorelaxation. Image from Shimokawa, H., et al. 
1.12 Clinical trials of statins for adult pulmonary hypertension

Use of simvastatin for adults with PHT was first published just over a decade ago from a series of 16 individual case studies. The observational studies showed some promise for statin therapy as there were some small improvements to RVSP, PVR, and 6MWD; together with no evidence of toxicity (102). The promising effects from the initial publication and well-documented safety profile of statins in cholesterol management prompted further investigation through double-blinded randomized clinical trials. An exploratory trial examined 60 patients receiving either Rosuvastatin (10 mg/d) or a placebo. Rosuvastatin decreased P-selectin (as a biomarker for platelet adhesion) and improved 6 minute walk distance (6MWD) after 6 months (18). In 53 adult chronic obstructive pulmonary disease (COPD) patients with PHT, there was a significant increase in duration of exercise time, improvement in Borg dyspnoea score, and decreases in systolic PAP and urinary ET-1 after 6 months of Pravastatin (40 mg/d) therapy. It was suggested that the mechanism may be due to inhibition of vasoconstriction by reduced ET-1 synthesis (131). The ASA-STAT trial placed 65 patients in: aspirin (81 mg), simvastatin (40 mg), combined aspirin-simvastatin, or placebo groups. None of the treatment groups displayed improvements of 6MWD, Borg dyspnoea score or secondary biological markers of PHT progression. Aspirin lowered thromboxane (TxB₂) and simvastatin lowered LDL cholesterol as expected. The study was terminated early based on a lack of expected improvement in 6MWD and Borg Dyspnoea scores (105, 106). Another study examined 42 patients receiving simvastatin (40 mg) or a placebo for 24 weeks. There was a non-significant increase to the 6MWD, but significant decrease to RV mass change and to circulating N-terminal prohormone
of brain natriuretic peptide (NT-proBNP) over 6 months. The study described a need for a larger study powered to determine if the change to 6MWD was statistically significant and for a longer duration to evaluate simvastatin’s sustained effect as a treatment for PHT (250). The most recent and largest human study to date was the APATH study. 220 patients were divided into groups receiving either atorvastatin (10 mg) or a placebo for 24 weeks. There was no significant change to 6MWD, cardio-pulmonary hemodynamics, or overall survival. Based on their findings, the authors did not recommend prescribing statins for treating adults with PHT (263).

There are a number of limitations related to the adult PHT and statin clinical trials. 6MWD is not an ideal marker for drug efficacy and does not apply to the target population of present interest (infants and children). The patients in the trials were all given the statin or placebo in addition to conventional PHT therapy (PDE-5 inhibitors, ET-1 receptor antagonists, diuretics, anti-coagulants, calcium channel antagonists, and/or digoxin), which may have masked the efficacy of statin therapy. All trials involved patients who had a variety of forms of PHT (heritable, idiopathic, co-morbidity of Eisenmenger’s syndrome, chronic thromboembolic PHT, and PHT-associated with a connective tissue disease) and degrees of severity as indicated by the WHO PHT classifications. Many patients may have also had co-existing conditions such as: liver cirrhosis, chronic thromboembolisms, and arrhythmias, which may confound the results. In addition, if statins have potential to reverse the progression of PHT, their use may need to be prolonged. In newborns, the pathology progresses much more quickly when compared to adults and older children and new treatments may act differently during postnatal development. Lastly, it is difficult to assess the efficacy of statins as there is
no standard prescription or dosage for cholesterol management, let alone for isoprenoid inhibition.

1.13 Prospects for statins in the pediatric population

Statins have been used in adults for decades but there are no set guidelines for use in children, which is a problem for most therapies adopted from adults (58). A small observational study with 12 children (aged 4-15 years old) with PHT, were treated with simvastatin (mean dose equivalent of 0.17 mg/kg) for a duration ranging 3-36 months. The study did not find any benefit of simvastatin, as determined by the presence and velocity of tricuspid regurgitation, which was used as a surrogate for PHT. The authors did not report any adverse effects from simvastatin (110). An Australian study retrospectively examined atorvastatin, pravastatin, simvastatin, and rosuvastatin (0.2 mg/kg) for familial hyperlipidemic children receiving transplants. The authors concluded that longer term studies for determining safety were required, as a limitation to existing reports was the absence of consistently-reported side effects (67). A literature review supports the use of statins for pediatric patients with elevated cholesterol. However, a long-term concern was raised as it is not known if statin therapy will affect steroid and hormone production (188). These pediatric studies exclude the neonatal population of interest, but does suggest that low-dose statins are not toxic to younger subjects.

1.14 Potential side effects of statins

Neonates are particularly susceptible to adverse and off-target effects of pharmacological agents (6). As statins have been on the market for the past 3 decades, their toxicity profile in adults is well-documented, with muscle aches, liver damage, and memory loss being most frequently reported. A study examining toxicity in simvastatin
treated rabbits had found elevated serum creatine kinase (CK) and alanine transaminase (ALT) levels after 14 days of 50 mg/kg treatment but did not significantly increase aspartate transaminase (AST) or troponin I levels (95). Liver toxicity may be affected by the lipophilicity or hydrophilicity of the statin, and by the rate of drug metabolism and elimination. Individual statins act on different CYP family liver enzymes which may also effect excretion and toxicity (227). In three separate analyses, patients prescribed statins at a dose ranging from 5-40 mg/d, had no indications of elevated ALT or hepatotoxicity. Cases of liver toxicity were rare, mild-moderate in severity, and improved upon cessation of therapy. Furthermore, the studies support the safety of statins and suggested that prospective routine hepatic monitoring is not necessary (42, 100, 191).

Clinically, it is difficult to assess the myopathies in patients as muscle biopsies are too invasive for routine monitoring. Serum or urine CK has been used as a diagnostic surrogate, but alone it is not entirely predictive of rhabdomyolysis as patients within normal serum CK values can still present with skeletal muscle damage or myalgia (36, 44). Self-reported qualitative symptoms such as muscle soreness, cramping, general weakness, and fatigue are therefore most commonly monitored to assess statin tolerance (84). Advanced age may be an important risk factor for adverse effects of statins on muscle mass and strength. A study showed that patients receiving statins over the age of 65 are four times as likely to be hospitalized for rhabdomyolysis than those under 65 (202).
1.15 Neurological impact of statins

It is not known how statins might impact neurological development in the neonatal population. Cholesterol and isoprenoid intermediates were found to be required for myelination when larval zebrafish were treated with atorvastatin (2 µM) and Ro 48-8071, a downstream inhibitor of cholesterol synthesis (147). Circulating systemic cholesterol does not play a role in mammalian brain development due to the impermeability of the BBB to cholesterol, as the brain is capable of de novo cholesterol production (46, 47, 233). In early development, cholesterol is synthesized through the action of HMG-CoA reductase and the mevalonate pathway (1, 195). Presumably, the carbon source for the precursor intermediate acetyl-CoA arises from acetate and glucose (48, 175).

Lipid soluble statins such as simvastatin can be detected in the mouse cerebral cortex, demonstrating their ability to cross the BBB by lactonization (98, 194). However, statins do not accumulate in the brain, being rapidly eliminated via the P-glycoprotein (P-gp) transporter (169). Furthermore, CYP3A4 the enzyme responsible for simvastatin metabolism and activation, is absent from the rodent brain (200, 233), and has only been detected in the human brain at one-tenth the level of hepatic CYP3A4 (252). High doses of statins have not been found to alter brain cholesterol levels in patients with elevated cholesterol levels (233). Additional evidence for the inactivity of transient simvastatin can be demonstrated from findings of serum and brain total cholesterol in guinea pigs, which had revealed no change in brain cholesterol between controls and administration of 150 mg/d of simvastatin mixed in chow after 2 weeks (139).
Both the cholesterol lowering and the pleiotropic effects of statins have been examined for therapeutic potential in neurodegenerative diseases (Alzheimer’s Disease, Parkinson’s Disease, multiple sclerosis, stroke, epilepsy) and neurodevelopmental diseases (Neurofibromatosis type 1, Rett Syndrome, Fragile X syndrome) (134, 150). Premature neonates have an increased risk for long term neurodevelopmental disability and are especially prone to white matter injury. In a preterm mouse model, statins prevented fetal brain abnormalities by reducing C5a complement system activated inflammation (173). Statins are also protective of the developing rat brain when receiving glucocorticoid therapy. Glucocorticoids are used in the clinic to manage inflammation in BPD, but are known to have adverse effects on motor skills and cognition, possibly due to a decrease in NO bioavailability associated with cerebral endothelial dysfunction and white matter damage. Co-administration of glucocorticoids and pravastatin (10 mg/kg/d), maintained plasma NOx, restored brain weight, and prevented loss in white matter volume (235). Maternally administered prenatal pravastatin (5 mg/kg/d) in a preeclampsia mouse model prevented fetal brain abnormalities in the offspring. The mode of action is believed to be linked to an increased vascular endothelial growth factor (VEGF) and placental growth factor (PLGF) (pro-angiogenic), concurrent with reduction of soluble fms-like tyrosine kinase-1 (sFlt-1), an anti-angiogenic factor (30). Simvastatin (5, 10, 20 mg/kg) can protect against hypoxic-ischemic injury in neonatal rat brains by increasing PI3K-Akt signaling (29). Additionally, Akt phosphorylation and activation triggers LTP induction and memory retention. RhoA is a negative regulator of Akt. The active, membrane-bound RhoA content in amygdala and hippocampus was reduced with simvastatin treatment,
therefore sustaining Akt activity. Supplementation of GGPP returned the phosphorylation of Akt to same levels as the vehicle rats (144, 205).

1.16 Rationale, Global Aims, Specific Hypotheses

Current therapeutic options for chronic neonatal pulmonary hypertension are limited and the utility of current therapies is unknown. Previous studies from our laboratory have demonstrated the importance of upregulated RhoA activity and its downstream mediator Rho-Kinase (ROCK) in the structural and functional maladaptation in PHT. Previous observations suggest that statins, a pleiotropic RhoA inhibitor, may prevent and reverse chronic PHT in neonatal rats by downregulation of ROCK signaling. Statins have promise as a potential new treatment for PHT and a globally safer alternative to ROCK inhibitors in the newborn.

This work comprises three aims:

1) To investigate effects of simvastatin on isoprenoid intermediate metabolism and on RhoA and ROCK activity.

2) To determine the effects of statin therapy on prevention and rescue of experimental chronic neonatal PHT.

3) To examine safety of simvastatin in the newborn rat.

We hypothesized that by downregulating RhoA activity, statins may inhibit pathological downstream ROCK activation and prevent or reverse experimental chronic neonatal PHT.
Chapter 2
Materials and Methods
2.1 Materials

Simvastatin was from Cayman Chemical (catalog no. 10010344, Ann Arbor, Michigan, USA). Oxygen exposure chambers and automated controllers, OxyCycler model A84XOV, were from Biospherix (Parish, NY, USA). Tris-glycine precast gels (catalog no. XP04205BOX, 15 well, 4-20%) and PVDF membranes were from Thermo Scientific (Rockford, IL, USA). Phosphatase and protease inhibitors were from Sigma Life Science (catalog no. P5726-5ML, St. Louis, MO, USA) and Calbiochem (catalog no. 53913-10VL, San Diego, CA, USA), respectively. Acids, alcohols, organic solvents, paraformaldehyde, Permount and Superfrost/Plus microscope slides were from Fisher Scientific (Whitby, Ontario, Canada). Weigert’s resorcin-fuchsia stain was from Rowley Biochemical (catalog no. F-370-1, Danvers, MA, USA). Luxol Fast Blue stain solutions were from Electron Microscopy Sciences (catalog no. 2668101, Hatfield, PA, USA). ELISA kits for Alanine Transaminase (ALT; catalog no. ab105134), Creatine Kinase (CK; catalog no. ab155901), High-density lipoprotein (HDL) and Low-density/Very-low density lipoprotein (LDL/VLDL) Cholesterol (catalog no. ab65390) were purchased from Abcam (Toronto, Ontario, Canada). A Free Cholesterol assay kit (catalog no. 10007640) was from Cayman Chemical. DMEM, trypsin, and heat-inactivated FBS were from Gibco (Burlington, Ontario, Canada).
Antibody sources and concentrations for Western Blot are shown in the table below (Table 2.1):

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<th>Antibody</th>
<th>Molecular Weight</th>
<th>Source</th>
<th>Dilution</th>
<th>Catalogue #</th>
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<tbody>
<tr>
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<td>SC-5560</td>
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<tr>
<td>Anti-ROCK II</td>
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<td>BD Biosciences (San Jose, California, USA)</td>
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<td>610624</td>
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<tr>
<td>Anti-Cleaved ROCK I</td>
<td>30 kDa</td>
<td>Santa Cruz Biotechnology</td>
<td>1:1000</td>
<td>SC-52953</td>
</tr>
<tr>
<td>Anti-phospho-threonine 850 myosin phosphatase target (MYPT1)</td>
<td>80 kDa</td>
<td>Millipore</td>
<td>1:1000</td>
<td>36-003</td>
</tr>
<tr>
<td>Anti-pan-MYPT1</td>
<td>135 kDa</td>
<td>BD Biosciences</td>
<td>1:1000</td>
<td>612164</td>
</tr>
<tr>
<td>Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH)</td>
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<td>Santa Cruz Biotechnology</td>
<td>1:5000</td>
<td>SC-25778</td>
</tr>
<tr>
<td>Anti-HIF-1α</td>
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<td>GeneTex, Inc. (Irvine, California, USA)</td>
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<td>821503130</td>
</tr>
<tr>
<td>Anti-pan-eNOS</td>
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<td>BD BioSciences</td>
<td>1:1000</td>
<td>61098</td>
</tr>
<tr>
<td>Anti-GTP-RhoA</td>
<td>21 kDa</td>
<td>NewEast Biosciences</td>
<td>1μL/sample</td>
<td>26904</td>
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</table>
### Antibody Table

<table>
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<th>Source</th>
<th>Dilution</th>
<th>Code</th>
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<td>14709</td>
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<tr>
<td>Anti-rabbit IgG, Biotinylated Antibody</td>
<td>Cell Signaling Technologies</td>
<td>1:10000</td>
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</tbody>
</table>

### 2.2 Institutional Review

All procedures involving animals were approved by the Animal Care Committee of the Hospital of Sick Children Research Institute and conformed to the guidelines of the Canadian Council on Animal Care.

### 2.3 In Vivo Experiments

Timed-pregnant Sprague-Dawley rats were obtained from Taconic Farms (Germantown, New York, USA). Commencing on the day after birth (postnatal day 1, (PND 1)), Sprague-Dawley rat pups (equalized sex distribution) were chronically exposed to normobaric hypoxia (13% O₂) or normoxia (21% O₂) until PND 14 (prevention study). Pups receiving rescue simvastatin were exposed to hypoxia or normoxia until PND 21. Concurrent with normoxia or hypoxia exposure, rat pups received daily intraperitoneal simvastatin (2 mg/kg) or vehicle (20% DMSO in PBS), from PND 1-14 (prevention study) or from PND 14 to PND 21 (rescue study) (Fig. 2.1). Simvastatin was selected as it was the most frequently studied statin in clinical and experimental PHT literature during the conception of the study design and with a well-defined dose range in neonatal rats (29, 71, 110, 173, 203, 250). Initial dose-response
studies (0.1, 2, 5, 10 and 20 mg/kg) were conducted to determine the lowest effective dose in preventing PHT, as guided by echocardiographic estimation of PVR. The highest dose (20 mg/kg) adversely affected weight gain and the lowest dose (0.1 mg/kg) had a minimal effect on PVR (Fig. 2.2); therefore 2 mg/kg was the dose chosen for all subsequent studies. Each litter was maintained at n=10-12 pups, to control for nutritional effects. At the end of each 14- or 21-day exposure period, pups were either killed by pentobarbital overdose or were exsanguinated after anesthesia. Upon completion of the rescue protocol, a subgroup of animals was housed in room air until 10 weeks of age for exercise testing.

**Figure 2.1:** A diagram summarizing the experimental design for prevention and rescue protocols.
Figure 2.2: Pups were exposed to chronic hypoxia (13% O₂) or normoxia (21% O₂) from postnatal day 1 until day 14. Pups were treated by daily intraperitoneal injections of simvastatin (0.1, 2, or 10 mg/kg) or 20% DMSO in PBS (vehicle control) from days 1-14. Pulmonary vascular resistance (PVR) index, as estimated by the right-ventricular ejection time (RVET)–to–pulmonary arterial acceleration time (PAAT) ratio (values represent means ± SEM for n=8-12 animals/group. *p<0.001, by one-way ANOVA compared to all other groups. *p<0.05, by one-way ANOVA compared to hypoxia vehicle group. 

Figure 2.2: Pups were exposed to chronic hypoxia (13% O₂) or normoxia (21% O₂) from postnatal day 1 until day 14. Pups were treated by daily intraperitoneal injections of simvastatin (0.1, 2, or 10 mg/kg) or 20% DMSO in PBS (vehicle control) from days 1-14. Pulmonary vascular resistance (PVR) index, as estimated by the right-ventricular ejection time (RVET)–to–pulmonary arterial acceleration time (PAAT) ratio (values represent means ± SEM for n=8-12 animals/group. *p<0.001, by one-way ANOVA compared to all other groups. *p<0.05, by one-way ANOVA compared to hypoxia vehicle group.
2.4 *In Vitro* Experiments

Pulmonary artery smooth muscle cells (PASMC) were isolated by an explant technique using pooled pulmonary arteries from PND 14 Sprague-Dawley rats, as previously described (90). All experiments using PASMC from the same litter were performed in quadruplicate using cells from the first passage only. Cells were passaged by trypsinization using 0.05% (wt/vol) trypsin/EDTA and centrifugation at 300 g for 5 min, followed by reseeding in 96-well plates. Equal numbers of cells (105/well) were seeded in 48-well plates and grown to semiconfluence with 10% (vol/vol) fetal bovine serum (FBS), were seeded per well, allowed to attach, and grown to subconfluence (~70%), after which they were serum starved for 24 h in Dulbecco’s Modified Eagle’s Medium (DMEM) and 0.1% (vol/vol) FBS. Before assays of ROCK activity, PASMC were incubated with 20% FBS (positive control), 1% FBS (negative control) or with 20% FBS with 1 µM of simvastatin for 24 h at a gas phase of 21% O₂, 5% CO₂, and 74% N₂.

2.5 Simvastatin preparation and delivery.

Simvastatin was provided as a crystallized solid and stored in aliquots as a 25 µg/µL solution in DMSO at -20°C. As simvastatin is not soluble in saline, a 0.4 mg/mL solution in 20% DMSO in PBS was heated to 37°C in a temperature regulated shaker just prior to delivery to ensure solubility. Injections were given intraperitoneally (5 µL per gram body weight) via a 27-gauge needle, once daily. Vehicle controls received an equivalent volume of 20% DMSO in PBS.
2.6 Hypoxia Exposure System

O₂ and CO₂ levels, temperature, and humidity were all continuously monitored, recorded, and regulated using customized computer software (AnaWin Run-Time, Version 2.2.18, Watlow-Anafaze, St. Louis, Missouri, 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 light/dark cycles), with temperature maintained at 25 ±1°C, humidity ~50% and CO₂ concentration <0.5%. Litters were equal in sex distribution and sizes were maintained at 12 pups throughout the exposure period. Food and water were available ad libitum. Dams were exchanged daily between paired air and hypoxia chambers to prevent maternal toxicity and consequentially any detrimental effects on the pups. At the end of each exposure period, the pups were sacrificed by pentobarbital sodium overdose.

2.7 Serum collection

Following sacrifice of animal for tissue collection, the thoracic cavity was exposed and blood was drawn by direct LV cardiac puncture with a 21-gauge needle. Samples of blood were kept at room temperature for 15 minutes to coagulate then spun down at 2000 g for 10 min to separate serum and cellular components. Serum was collected by Pasteur pipette and stored at -80°C until assay.

2.8 Two-Dimensional Echocardiography and Pulse Wave Doppler Ultrasound

Two-dimensional (2D) echocardiography and pulsed wave Doppler ultrasound was performed as a non-invasive method to assess pulmonary hemodynamics. A Vivid 7 Advantage (GE Medical Systems, Milwaukee, Wisconsin, USA) cardiovascular
ultrasound machine was used with a small high frequency probe (113L). Due to the small size of the animals and high heart rate, an ultra-high frame rate was used, providing a high image quality. Rat pups were anesthetized with 5% (v/v) isoflurane, the animal was laid supine while spontaneously breathing 2-3% (v/v) isoflurane through a modified face mask. The pups were kept warm with a heat lamp. The probe was gently applied to the chest while the pups were 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).

2.9 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 flow, and PAAT was measured from onset to peak pulmonary outflow velocity. A surrogate measure of PVR was calculated as a ratio of RVET: PAAT. To determine heart rate, measurements from 3 intersystolic intervals were averaged to account for beat-to-beat variability.

2.10 Measurement of Systemic Blood Pressure

Systemic blood pressure was measured non-invasively using a tail cuff Doppler device (LE5002, Harvard Apparatus, Holliston, MA, USA), as previously described (254). Rats were anesthetized with 5% (v/v) isoflurane, the animal was laid supine while spontaneously breathing 2-3% (v/v) isoflurane through a modified face mask, and had
the tail cuff attached to take the measurement. Due to technical limitations of the device in smaller animals, measurements were carried out between PND21 and PND26.

2.11 Right-Ventricular Hypertrophy

Measurement of right-ventricular hypertrophy using the Fulton index (RV/LV+S) is a well-established marker of PHT. RVH has been shown to correlate closely with thickening of distal pulmonary arteries in hypoxia-exposed rats. After sacrificing pups, the thoracic contents were removed. The heart and lungs were separated, and the atria were removed inferior to the atrio-ventricular valves. The RV was separated from the LV and septum. Each component was freeze dried overnight and weighed separately, as previously described (92).

2.12 Tissue homogenate preparation

Right lower lobes (6 per group) were flash frozen, homogenized and sonicated (40W for 30s) 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 (Calbiochem) and phosphatase (Sigma Life Science) inhibitors. Left lung lobes (6 per group) were flash frozen, homogenized in lysis buffer (250 mM Tris-HCl, pH 8, 750 mM NaCl, 50 mM MgCl2, 5 mM EDTA, 5% Triton X-100) containing protease and phosphatase inhibitors for immunoprecipitation. The lung homogenates were left on ice for 10 minutes before centrifugation (8,500 g for 10 min). The supernatant was collected and protein concentration was measured by a commercially available spectrophotometric assay. Samples were stored at -80°C until further analysis.
2.13 Protein analysis

Lung homogenates from 6 animals per group (equal sex distribution) stored in RIPA buffer containing protease and phosphatase inhibitors. 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 1-2 hours at 100-150 V depending on protein of interest. Following electrophoresis, proteins were transferred to PVDF membranes. All membranes were blocked with 5% 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, Illinois, USA). Blots were electronically captured using a MicroChemi chemiluminescent camera (DNR Bioimaging Systems, Jerusalem, Israel) and band density was quantified using ImageJ software (NIH, Bethesda, Maryland, USA). Bands were quantified by digital densitometry of non-saturated images with background density removed. Any differences in protein loading were controlled for by re-blotting for GAPDH or the pan-protein, and results expressed after normalization to the control band, as previously described (89).

2.14 RhoA activity assay

The concentration of lung tissue homogenates were equalized to 2 mg/mL in 1 mL lysis buffer. Anti-active RhoA monoclonal antibody (1 µL) was added to the tube followed by 30 µL of re-suspended A/G agarose bead slurry. The tubes were incubated
at 4°C for 1.5 hours with gentle agitation following which beads were pelleted by centrifugation for 2 min at 6,500 g at 4°C. The supernatant was removed and stored for measurement of non-GTP-bound RhoA. The beads were washed 3 times with 0.5 mL 1X lysis buffer, centrifuging, aspirating, and discarding the supernatant each time. After the last wash, all supernatant was removed and discarded. The pellet was re-suspended in 20 µL 2X reducing SDS-PAGE sample buffer. Samples were boiled for 5 minutes then centrifuged for 10 seconds at 6,500 g. Two Western blots were run in parallel with samples from the pellet or the supernatant. The membranes were probed with anti-RhoA primary antibody. The proportion of active RhoA was determined by band densities of active RhoA normalized to total RhoA [GTP-RhoA/(GTP-RhoA+non-GTP-RhoA)].

2.15 Histological studies

Two randomly selected males and females, from each of the experimental groups were sacrificed by sodium pentobarbital overdose. Following the opening of the thoracic cavity and cannulation of the trachea, the pulmonary veins were divided. The pulmonary circulation was flushed with PBS and heparin, in order to clear the lungs of blood. The lungs were then perfusion fixed with paraformaldehyde while air inflated at a constant pressure (20 cm of H₂O). Percentage medial wall area (%MWA) of pulmonary arteries (20-100 µm external diameter) was measured on elastin-stained paraffin-embedded lung sections, as a marker of vascular remodeling, as previously described (130). Degree of muscularization of pulmonary resistance arteries (20-100 µm external diameter) was measured on paraffin-embedded lung sections immunostained with α-smooth muscle actin as a marker of pulmonary arterial smooth muscle content. Images
were captured digitally and analysis was performed in a blinded fashion. Identification of arteries was based on proximity to pulmonary airway and size (range of 20-100 µm in diameter), with a minimum of 40 vessels per section of lung, from a total of 4 unique sections (equal sex distribution) per experimental group.

2.16 Hart’s Elastin Stain

Paraffin embedded tissues were cut into 5 µm sections and mounted onto Superfrost slides, allowed to air dry and bake overnight at 43°C. Sections were dewaxed by immersion in xylene, rehydrated in ethanol, rinsed in several washes of distilled water and then left overnight for 17 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.

2.17 Hemotoxylin and Eosin

Paraffin embedded hearts embedded on the short axis were cut into 5 µm sections and mounted onto Superfrost slides, allowed to air dry and bake overnight at 43°C. Slides were dewaxed and rehydrated. Sections were stained with hematoxylin, washed in distilled water and partially dehydrated before counterstaining with eosin. Slides were dehydrated and mounted with a coverslip using a 70% permount/30% xylene solution.

2.18 Luxol Fast Blue

Paraffin embedded sagittal sections of a brain hemisphere were cut into 5 µm sections and mounted onto Superfrost slides, allowed to air dry and bake overnight at
43°C. Slides were dewaxed and rehydrated. Sections were stained with Luxol fast blue solution overnight for 18 hours at 56°C, washed in distilled water, placed in lithium carbonate solution for 30 seconds, and partially dehydrated before counterstaining with cresyl violet blue solution. Slides were dehydrated and mounted with a coverslip using a 70% permount/30% xylene solution.

2.19 Whole-section imaging

Whole sections were scanned by the Imaging Facility at the Hospital for Sick Children Research Institute, using a 3DHistech Pannoramic 250 Flash II Slide Scanner and Pannoramic Viewer 1.15.4 (3DHISTECH, Budapest, Hungary) software.

2.20 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 the Hart’s elastin stained slides. Images were digitally captured (Pixera Penguin 600CL, San Jose, California, USA) and analysis was performed in a blinded fashion. Obliquely sectioned oblong vessels that were greater than three times longer than wide were excluded. Adobe Photoshop CS5 (Adobe Systems Incorporated, San Jose, California, USA) was used to calculate the medial wall area of each vessel. Using the “Quick Selection” tool, the area of the inner lumen and the whole vessel were determined. The measurement conversion was set so 50 pixels was equal to 234 microns in length. The following formula was used to determine the percent medial wall area: 

\[
\frac{(\text{Whole Vessel Area} - \text{Inner Lumenal Area})}{\text{Whole Vessel Area}} \times 100.
\]
2.21 Immunohistochemical staining of α-smooth muscle actin

Paraffin embedded tissues were cut into 5 µm sections and mounted onto Superfrost slides, allowed to air dry and bake overnight at 43°C. Sections were de-waxed by immersion in xylene, rehydrated in ethanol, and washed with several rinses of PBS. Concentration of primary antibody for α-smooth muscle actin used was 1:1500 and secondary goat anti-mouse antibody was 1:200. Slides were then counterstained with Carazzi hematoxylin. Slides were washed in tap water, dehydrated, and mounted with a coverslip using a 70% permount/30% xylene solution, as previously described (92, 254).

2.22 Evaluation of Vessel Muscularization

Degree of vessel muscularization was divided into 3 categories: non-muscular, partially muscular, or fully muscular. Vessels were assessed based on the amount of stain visible (none, partially or completely) surrounding the lumen. Pulmonary arteries were identified based on their proximity to respiratory bronchioles and were categorized by the presence and degree of smooth muscle in the tunica media: completely surrounding the vessel (muscularized), no smooth muscle (non-muscularized) and partially surrounding the lumen (partially muscularized). Vessels below 20 μm or exceeding 100 μm external diameter were excluded. Category of muscularity was expressed as a fraction of the total number of vessels examined.
2.23 Analysis of Periventricular White Matter Myelination

Histological studies were performed on scanned brain sections stained with Luxol Fast Blue, which identifies myelin. Quantification was performed with Image-Pro Plus 7.0 (Media Cybernetics Inc., Rockville, MD, USA). Pixel counting was performed using the “Count” tool, excluding the cerebellum and brain stem regions.

2.24 LC-MS/MS

Tissue samples weighing approximately 200 mg were homogenized (750 mg/3mL) in 1:1 2-propanol:100 mM ammonium bicarbonate. The equivalent of 225 mg (900 µL of homogenate) was added to a 1.7 mL plastic tube containing internal standards (Sigma-Aldrich, Oakville, Ontario, Canada) and briefly vortexed. A standard curve was generated (0.1 – 200 ng) for each analyte (mevalonic acid, FPP, and GGPP) and was treated in the same way as the tissue samples and had 900 µL of 1:1 2-propanol:100 mM ammonium bicarbonate added instead of sample. Each tube was processed as follows: 900 µL of acetonitrile added, vortex, chilled on ice, and then centrifuged for 10 min at 4°C at 20,000 g. The supernatant was transferred to a clean tube and the pellet re-extracted with 1 mL acetonitrile, vortexed, chilled on ice, and centrifuged for 10 min at 4°C at 20,000 g. The supernatants were combined and taken to dryness under a gentle flow of nitrogen gas. The residues were reconstituted in 50 µL of 7:3 methanol:ammonium hydroxide, transferred to 1.5 mL plastic tubes, and centrifuged for 10 min at 4°C at 20,000 g. The supernatant was transferred to a 200 µL insert in a 1 mL autosampler vial and injected for LC-MS/MS analysis.
Samples were analyzed by LC-MS/MS using an Agilent 1290 HPLC with QTRAP 5500 mass spectrometer (AB Sciex, Concord, Ontario, Canada) in negative ion mode. 1 µL of sample extract was injected into a Kinetex XB-C18 column (50 x 3.0 mm, 2.6 µm, Phenomenex) running at a flow rate of 200 µL/min. Mevalonic acid, farnesyl pyrophosphate, and geranylgeranyl pyrophosphate were separated using a gradient of 5 mM ammonium bicarbonate and 0.05% triethylamine in water (MPA) and 5 mM ammonium bicarbonate and 0.05% triethylamine in 80% acetonitrile (MPB) as follows: starting with 100% MPA, moving to 30% MPA from 0.6 min - 4.0 min, then to 0% MPA from 4.0 min - 5.0 min, 0% MPA was held from 5.0 min - 7.5 min, after 7.5 min the system was returned to 100% MPA and allowed to equilibrate resulting in a total run time of 15 minutes. The following mass transitions (precursor ion to the product ion) were monitored for quantification in multiple reaction monitoring (MRM) mode: mevalonic acid: 147 to 59, farnesyl pyrophosphate: 381 to 79, and geranylgeranyl pyrophosphate: 449 to 79. Data was analyzed and quantified using Analyst Software (AB Sciex).

2.25 Exercise tolerance studies

Adult rats (10 weeks of age) were trained daily on an Exer-3/6 animal treadmill (Columbus Instruments, Columbus, Ohio, USA) over a 7-day period. The treadmill speed was initially set to 10 meters/min with a 10% uphill incline, and thereafter increased by increments of 2 meters/min every 5 min up to a maximum speed of 20 meters/min. Distance to fatigue was determined when the rat was unable to maintain pace with the treadmill belt for > 3 s, despite encouragement to do so by tapping on the treadmill enclosure (electrical stimuli were not used). Following the 7-day training
period, the final testing regimen was as follows: speed of 12.5 meters/min for 1 min, followed by 15 meters/min for 4 mins, 17 meters/min for 4 minutes and 20 meters/min until fatigue. Lowering of the hindquarters and a raised snout, resulting in an altered gait, generally precedes fatigue, which was confirmed by placing the animal on its back and observing a delayed (> 2 s) righting reflex. In the case of a normal righting reflex, the test was repeated daily until fatigue was confirmed. Distance run until fatigue was recorded to the nearest tenth of a meter.

### 2.26 Data Presentation and Statistical Analysis

Values are expressed as means ± SEM, unless otherwise specified. Analyses were performed using Sigma Plot 11.0 (Systat software, San Jose, CA, USA). Where three or more groups were compared, statistical significance (P < 0.05) was determined by one-way ANOVA and Tukey’s *post hoc* test. The Student’s *t*-test was used when two groups were compared.
Chapter 3

Results
Results

3.1 Determining the optimal dose of simvastatin in the newborn rat

We examined the dose-response to simvastatin (0.1, 2.0, 5, 10, and 20 mg/kg/d). The ideal dose was considered to be the lowest amount of simvastatin that would produce the maximum reduction in RhoA/ROCK activity and prevent PHT (decreased PVR and attenuated vascular remodeling) without adversely affecting growth, as determined by body weight at day 14. A dose of 2.0 mg/kg was found to best meet these criteria.

3.2 Lung FPP content and RhoA/ROCK activity were attenuated by simvastatin

Statins inhibit mevalonate synthesis, which reduces isoprenoid intermediate content (55, 125). As shown in Figure 3.1, chronic exposure to hypoxia had no effect on lung FPP content, when compared to normoxia-exposed controls. Treatment with simvastatin led to significantly reduced (p<0.05) lung FPP content in chronic hypoxia compared to vehicle-treated pups (Fig. 3.1). RhoA activity was measured by lung GTP-RhoA content, as a ratio of total (GTP+non-GTP bound) RhoA. Hypoxia-exposed, vehicle-treated pups had significantly (p<0.05) elevated RhoA activity in the lung relative to normoxia controls. Lungs of chronic hypoxia-exposed pups treated with simvastatin had significantly reduced (p<0.05) GTP-RhoA content (Fig. 3.2). Lung ROCK activity was determined by a ratio of pThr850-MYPT1 to pan-MYPT1 content. Hypoxia-exposed, vehicle-treated pups had significantly (p<0.05) higher ROCK activity when compared to normoxia controls. Treatment of chronic hypoxia-exposed pups with
Simvastatin significantly decreased (p<0.05) lung ROCK activity relative to the vehicle-treated animals (Fig. 3.2).

**Figure 3.1:** Pups were exposed to chronic hypoxia (13% O₂) or room air (21% O₂) from postnatal day 1 until day 14. Pups were treated by daily intraperitoneal injections of simvastatin (2 mg/kg) or 20% DMSO in PBS (vehicle control). Simvastatin decreased farnesyl pyrophosphate. *p<0.05, by one-way ANOVA, compared to all other groups. Values represent means ± SEM for n=5-6 samples per group.
Figure 3.2: Pups were exposed to chronic hypoxia (13% O₂) or room air (21% O₂) from postnatal day 1 until day 14. Pups were treated by daily intraperitoneal injections of simvastatin (2 mg/kg) or 20% DMSO in PBS (vehicle control). GTP-RhoA and Total RhoA, pThr850-MYPT1 or pan-MYPT1. Representative immunoblots are shown next to each graph with non-contiguous gel lanes demarcated by black lines. Values represent means ± SEM for n=5-6 samples per group. *p<0.05, by one-way ANOVA compared to all other groups.
3.3 Pulmonary arterial smooth muscle cell ROCK activity was attenuated by simvastatin

20% FBS media significantly increased (p<0.05) pThr850-MYPT1 content in PASMCs. Treatment of PASMC with 1 µM simvastatin added to 20% FBS media reduced P-MYPT1 content comparable to serum starved (1% FBS) cells (Fig. 3.3).

**Figure 3.3:** Pulmonary arterial smooth muscle cells (PASMCs) were treated with DMEM and 20% FBS (positive control), DMEM and 1% FBS (negative control), or DMEM and 20% FBS with 1 µM simvastatin. Ratio of pThr850-MYPT1 and pan-MYPT1. Representative immunoblots are shown below the graph with non-contiguous gel lanes demarcated by black lines (values represent means ± SEM for n=4 PASMC/group). *p<0.05, by one-way ANOVA, compared to 20% FBS with 1 µM simvastatin and 1% FBS groups.
3.4 Simvastatin prevented chronic PHT

Similar to previous findings (75, 254, 268), prolonged exposure to hypoxia led to a significantly increased \( p<0.05 \) PVR index (Fig. 3.4) and Fulton index (Fig. 3.5) in vehicle-treated animals, when compared to normoxia controls. Animals exposed to hypoxia and treated with simvastatin had PVR index (Fig. 3.4) and Fulton index (Fig. 3.5) values comparable to normoxia controls.

**Figure 3.4:** Pups were exposed to chronic hypoxia (13% \( \text{O}_2 \)) or room air (21% \( \text{O}_2 \)) from postnatal day 1 until day 14. Pups were treated by daily intraperitoneal injections of simvastatin (2 mg/kg) or 20% DMSO in PBS (vehicle control). Pulmonary vascular resistance (PVR) index, as estimated by the right-ventricular ejection time (RVET)–to–pulmonary arterial acceleration time (PAAT) ratio. \( *p<0.05 \), by one-way ANOVA compared to all other groups (values represent means ± SEM for \( n=8-12 \) animals/group).
Figure 3.5: Pups were exposed to chronic hypoxia (13% O₂) or room air (21% O₂) from postnatal day 1 until day 14. Pups were treated by daily intraperitoneal injections of simvastatin (2 mg/kg) or 20% DMSO in PBS (vehicle control). Right ventricle (RV)–to–left ventricle + septum (LV+S) dry weight ratios (Fulton index) as a marker of right-ventricular hypertrophy (values represent means ± SEM n=7-9 animals/group). Tiled low-power photomicrographs of hematoxylin and eosin–stained cardiac sections, oriented in the short axis below the atrioventricular valves (right ventricular cavity=RV, scale bar=2000 µm), are shown to demonstrate differences in RV wall thickness between groups. *p<0.001, by one-way ANOVA compared to all other groups.
3.5 Chronic hypoxia-induced pulmonary arterial remodeling was attenuated by simvastatin

Medial wall area is a well-established marker of pulmonary arterial remodeling in PHT (75, 254, 268). As shown previously (75, 254, 268), and in Fig. 3.6 and 3.7, pulmonary arteries of animals chronically exposed to hypoxia had greatly increased medial wall thickness due to increased smooth muscle. Treatment with simvastatin resulted in a significant (p<0.001) decrease in both percentage medial wall area (Fig. 3.6) and proportion of fully muscularized arteries (Fig. 3.7) relative to vehicle-treated controls.
Figure 3.6: Pups were exposed to chronic hypoxia (13% O₂) or room air (21% O₂) from postnatal day 1 until day 14. Pups were treated by daily intraperitoneal injections of simvastatin (2 mg/kg) or 20% DMSO in PBS (vehicle control). Percentage arterial medial wall area (values represent means ± SEM for n=4 animals/group) as a marker of pulmonary arterial remodeling. Representative photomicrographs of elastin staining (dark brown inner and outer elastic laminae delineating the medial vascular wall; scale bar=50 µm) demonstrate medial wall thickening. *p<0.001, by one-way ANOVA compared to all other groups.
Figure 3.7: Pups were exposed to chronic hypoxia (13% O₂) or room air (21% O₂) from postnatal day 1 until day 14. Pups were treated by daily intraperitoneal injections of simvastatin (2 mg/kg) or 20% DMSO in PBS (vehicle control). Degree of muscularization (values represent means ± SEM for n=4 animals/group) as a marker of pulmonary arterial smooth muscle proliferation. Representative photomicrographs of α-smooth muscle actin staining (dark brown staining of artery; scale bar=50 µm) demonstrate proliferation of pulmonary arterial smooth muscle. #p<0.05, by one-way ANOVA compared to normoxia vehicle group for proportion of partially muscularized arteries. †p<0.001, by one-way ANOVA, compared to all other groups for proportions of non-muscularized and fully-muscularized arteries.
3.6 Chronic hypoxia-induced PHT was reversed by rescue treatment with simvastatin

Previous studies have demonstrated that systemic or inhaled treatment with a ROCK inhibitor reversed PHT induced by chronic hypoxia in newborn rats (75, 254). Relative to vehicle-treated animals, sustained rescue treatment with simvastatin significantly reduced (p<0.05) PVR (Fig. 3.8), RVH (Fig. 3.9), percent medial wall area (Fig. 3.10), and arterial muscularization (Fig. 3.11) secondary to chronic hypoxia. These changes were accompanied by significantly (p<0.001) reduced RhoA (Fig. 3.12) and ROCK (Fig. 3.12) activity.

![Graph showing PVR (RVET/PAAT) comparison between Normoxia, Chronic Hypoxia, and d14 Hypoxia Vehicle (Historical Mean). The graph indicates that simvastatin treatment significantly reduced PVR compared to vehicle control.]

**Figure 3.8:** Pups were exposed to chronic hypoxia (13% O₂) or room normoxia (21% O₂) from postnatal day 1 until day 21. Pups were treated by daily intraperitoneal injections of simvastatin (2 mg/kg) or 20% DMSO in PBS (vehicle control) from days 14-21. Pulmonary vascular resistance (PVR) index, as estimated by the right-ventricular ejection time (RVET)–to–pulmonary arterial acceleration time (PAAT) ratio (values represent means ± SEM for n=8-12 animals/group. *p<0.05, by one-way ANOVA compared to all other groups. Dotted line represents mean values of previously published results from postnatal day 14 chronic hypoxia-exposed rats.
Figure 3.9: Pups were exposed to chronic hypoxia (13% O₂) or room normoxia (21% O₂) from postnatal day 1 until day 21. Pups were treated by daily intraperitoneal injections of simvastatin (2 mg/kg) or 20% DMSO in PBS (vehicle control) from days 14-21. Right ventricle (RV)–to–left ventricle + septum (LV+S) dry weight ratios (Fulton index) as a marker of right-ventricular hypertrophy (values represent means ± SEM n=7-9 animals/group). Tiled low-power photomicrographs of hematoxylin and eosin–stained cardiac sections, oriented in the short axis below the atrioventricular valves (right ventricular cavity=RV, scale bar=2000 µm), are shown to demonstrate differences in RV wall thickness between groups. *p<0.05, by one-way ANOVA compared all other groups, #p<0.05, by one-way ANOVA compared to normoxia controls. Dotted line represents mean values of previously published results from postnatal day 14 chronic hypoxia-exposed rats.
Figure 3.10: Pups were exposed to chronic hypoxia (13% O₂) or room normoxia (21% O₂) from postnatal day 1 until day 21. Pups were treated by daily intraperitoneal injections of simvastatin (2 mg/kg) or 20% DMSO in PBS (vehicle control) from days 14-21. Percentage arterial medial wall area (values represent means ± SEM for n=4 animals/group) as a marker of pulmonary arterial remodeling. Representative photomicrographs of elastin staining (dark brown inner and outer elastic laminae delineating the medial vascular wall; scale bar=50 µm) demonstrate medial wall thickening. †p<0.001, by one-way ANOVA compared to all other groups, ‡p<0.001, by one-way ANOVA compared to normoxia-exposed groups. Dotted line represents the mean value from current postnatal d14 chronic hypoxia-exposed rats.
Figure 3.1: Pups were exposed to chronic hypoxia (13% O\textsubscript{2}) or room normoxia (21% O\textsubscript{2}) from postnatal day 1 until day 21. Pups were treated by daily intraperitoneal injections of simvastatin (2 mg/kg) or 20% DMSO in PBS (vehicle control) from days 14-21. Degree of muscularization (values represent means ± SEM for n=4 animals/group) as a marker of pulmonary arterial smooth muscle proliferation. Representative photomicrographs of α-smooth muscle actin staining (dark brown staining of artery; scale bar=50 µm) demonstrate proliferation of pulmonary arterial smooth muscle. Values represent means ± SEM for n=5-6 samples per group. §p<0.05 (non-muscularized), ‖p<0.001 (partially- and fully-muscularized), by one-way ANOVA compared to hypoxia vehicle.
Figure 3.12: Pups were exposed to chronic hypoxia (13% O₂) or room normoxia (21% O₂) from postnatal day 1 until day 21. Pups were treated by daily intraperitoneal injections of simvastatin (2 mg/kg) or 20% DMSO in PBS (vehicle control) from days 14-21. Lung protein content analyzed by Western blot for GTP-RhoA and RhoA. pThr850-MYPT1 or pan-MYPT1. Representative immunoblots are shown next to each graph with non-contiguous gel lanes demarcated by black lines. Values represent means ± SEM for n=5-6 samples per group. *p<0.05, by one-way ANOVA compared to all other groups. #p<0.001, by one-way ANOVA compared to all other groups.

3.7 Rescue treatment with simvastatin improved exercise tolerance in rats.
Exercise capacity in chronic hypoxia, vehicle-treated rats was significantly reduced (p<0.001) compared to air-exposed vehicle- or simvastatin-treated controls. Additionally, female rats ran a significantly (p<0.05) greater distance compared to male rats across all groups and therefore data were stratified by sex. Rescue treatment with simvastatin significantly increased distance run in both females (p<0.001) and males (p<0.05) when compared to hypoxia-exposed, vehicle-treated controls (Fig. 3.13).

**Figure 3.13**: Pups were exposed to chronic hypoxia (13% O₂) or room normoxia (21% O₂) from postnatal day 1 until day 21. Pups were treated by daily intraperitoneal injections of simvastatin (2 mg/kg) or 20% DMSO in PBS (vehicle control) from days 14-21. Maximum exercise capacity measured in meters run (values represent means ± SEM for n=5-8 animals/group). *p<0.05, by one-way ANOVA, compared to hypoxia vehicle group of the same sex. †p<0.001, by one-way ANOVA, compared to hypoxia vehicle group of the same sex.
3.8 Treatment with simvastatin improved weight gain and did not alter systemic blood pressure

We have previously reported significant adverse effects of systemic ROCK inhibition, including worsened growth restriction and greatly decreased systemic blood pressure (75, 268). As previously documented (268), chronic exposure to hypoxia lead to growth restriction (p<0.05) (Fig. 3.14). Preventive simvastatin treatment led to an increased body weight (p<0.05) at the end of the treatment protocol when compared to the vehicle-treated groups (Fig. 3.14). Rescue simvastatin treatment was not significantly different from the vehicle-treated groups (Fig 3.14). Relative to normoxia controls, chronic hypoxia-exposed animals had increased (p<0.05) systolic blood pressure (Fig. 3.14). Acute rescue treatment with simvastatin had no effect on systolic blood pressure.
Figure 3.1: Pups were exposed to chronic hypoxia (13% O₂) or normoxia (21% O₂) from postnatal day 1 until postnatal day 14 or 21. Pups were treated by daily intraperitoneal injections of simvastatin (2 mg/kg) or 20% DMSO in PBS (vehicle control). Final body weight measurements from prevention protocol experiment. Values represent means ± SEM for n=10-12 animals per group. Acute systemic blood pressure cuff readings. Values represent means ± SEM for n=6 animals per group. *p<0.05, by one-way ANOVA compared to all other groups. #p<0.001, by one-way ANOVA compared to normoxia controls. †p<0.05, by one-way ANOVA compared to normoxia controls.
3.9 Preventive treatment with simvastatin did not decrease cholesterol levels in newborn rat serum

Lowering serum cholesterol is the goal of statin therapy in adult humans. As shown in Figure 3.15, neither chronic exposure to hypoxia nor treatment with simvastatin led to any change in total serum cholesterol. To control for the possible binding, uptake, or degradation effects of DMSO on cholesterol (4, 43, 128), normoxia- and hypoxia-exposed animals not injected with vehicle had no alterations to total serum cholesterol compared to all other groups (Fig. 3.15). As shown in Figure 3.15, chronic hypoxia led to significantly increased (p<0.05) serum LDL cholesterol, which was unaffected by treatment with simvastatin.

![Figure 3.15](image_url)

Figure 3.15: Pups were exposed to chronic hypoxia (13% O₂) or normoxia (21% O₂) from postnatal day 1 until day 14. Pups were treated by daily intraperitoneal injections of simvastatin (2 mg/kg) or 20% DMSO in PBS (vehicle control), or naïve to treatment. ELISA analyses for Total Cholesterol and LDL Cholesterol. Values represent means ± SEM for n=6 samples per group. *p<0.05, by one-way ANOVA, compared to hypoxia groups.
3.10 Preventive treatment with simvastatin did not cause adverse effects on the developing brain

Treatment naïve (non-injected) animals exposed to chronic hypoxia had significantly (p<0.05) decreased brain weight compared to naïve normoxia brains (Fig. 3.16). There was a trend towards a significant reduction (p=0.052) of hypoxia vehicle compared to normoxia vehicle brain weights. Treatment with simvastatin had no effects on brain weight in either normoxia or hypoxia-exposed animals (Fig. 3.16). Relative to normoxia-exposed vehicle treated animals, exposure to chronic hypoxia decreased periventricular white matter volume (Fig. 3.17). Preventive treatment with simvastatin had no effect on periventricular myelin density in normoxia-exposed animals, while causing a significant (p<0.05) increase in hypoxia-exposed animals (Fig. 3.17).

![Graph](image)

**Figure 3.16:** Pups were exposed to chronic hypoxia (13% O₂) or normoxia (21% O₂) from postnatal day 1 until day 14. Pups were treated by daily intraperitoneal injections of simvastatin (2 mg/kg) or 20% DMSO in PBS (vehicle control). Brain weight from prevention protocol experiment. Values represent means ± SEM for n=6 samples per group. *p<0.05, by one-way ANOVA compared to normoxia group and normoxia vehicle group.
Figure 3.17: Pups were exposed to chronic hypoxia (13% O₂) or normoxia (21% O₂) from postnatal day 1 until day 14. Pups were treated by daily intraperitoneal injections of simvastatin (2 mg/kg) or 20% DMSO in PBS (vehicle control). Periventricular white matter pixel intensity quantification. Values represent means ± SEM for n=4 samples per group. Scanned representative photomicrographs of Luxol fast blue staining (myelin blue staining; scale bar=2500 µm), oriented to sagittal view, demonstrating neuron myelination between groups. *p<0.05, by one-way ANOVA compared to normoxia controls. #p<0.05, by one-way ANOVA compared to hypoxia vehicle.
3.11 Cleaved ROCK I, but not ROCK I or II content was decreased by preventive treatment with simvastatin

As described previously (75), chronic exposure to hypoxia decreased (p<0.001) cleaved ROCK I (constitutively active form of ROCK) content in the lung when compared to normoxia controls (Fig. 3.18). Treatment with simvastatin decreased cleaved ROCK I in both normoxia- and hypoxia-exposed animals (Fig. 3.18). Hypoxia-exposed, vehicle-treated pups had significantly (p<0.05) lower ROCK I (Fig 3.19), but no change in ROCK II (Fig. 3.19) content in the lung compared to normoxia-exposed vehicle-treated controls. Treatment with simvastatin had no effect on lung content of ROCK I or ROCK II (Fig. 3.19).

**Figure 3.18**: Pups were exposed to chronic hypoxia (13% O₂) or normoxia (21% O₂) from postnatal day 1 until day 14. Pups were treated by daily intraperitoneal injections of simvastatin (2 mg/kg) or 20% DMSO in PBS (vehicle control). Western blot analyses for Cleaved ROCK I and GAPDH. Representative immunoblots are shown next to each graph with non-contiguous gel lanes demarcated by black lines. Values represent means ± SEM for n=5-6 samples per group. *p<0.001, by one-way ANOVA, compared to all other groups. #p<0.05, compared by one-way ANOVA compared to hypoxia vehicle group.
Figure 3.19: Pups were exposed to chronic hypoxia (13% O₂) or normoxia (21% O₂) from postnatal day 1 until day 14. Pups were treated by daily intraperitoneal injections of simvastatin (2 mg/kg) or 20% DMSO in PBS (vehicle control). Western blot analyses for ROCK I, or ROCK II and GAPDH. Representative immunoblots are shown next to each graph with non-contiguous gel lanes demarcated by black lines. Values represent means ± SEM for n=5-6 samples per group. *p<0.05, by one-way ANOVA, compared to the normoxia vehicle group.
3.12 Preventive simvastatin decreased HIF-1α content, and increased lung eNOS content

HIF-1α is known to be up-regulated by hypoxia. Accordingly, neonatal rat pups chronically exposed to hypoxia had significantly (p<0.001) increased HIF-1α protein content in the lung when compared to normoxia controls (Fig. 3.20). Treatment with simvastatin had significantly reduced (p<0.05) HIF-1α content in lungs exposed to chronic hypoxia (Fig. 3.20). eNOS expression has been described to be decreased by ROCK (211). As shown in Fig. 3.21, preventive treatment with simvastatin led to increased eNOS content in both normoxia- (p<0.001) and hypoxia-exposed (p<0.05) animals.

Figure 3.20: Pups were exposed to chronic hypoxia (13% O₂) or normoxia (21% O₂) from postnatal day 1 until day 14. Pups were treated by daily intraperitoneal injections of simvastatin (2 mg/kg) or 20% DMSO in PBS (vehicle control). Western blot analyses for HIF-1α and GAPDH. Representative immunoblots are shown next to each graph with non-contiguous gel lanes demarcated by black lines. Values represent means ± SEM for n=5-6 samples per group. *p<0.001, by one-way ANOVA, compared to normoxia controls. #p<0.05, by one-way ANOVA, compared to hypoxia vehicle group.
Figure 3.21: Pups were exposed to chronic hypoxia (13% O$_2$) or normoxia (21% O$_2$) from postnatal day 1 until day 14. Pups were treated by daily intraperitoneal injections of simvastatin (2 mg/kg) or 20% DMSO in PBS (vehicle control). Western blot analyses for pan-eNOS and GAPDH. Representative immunoblots are shown below the graph with non-contiguous gel lanes demarcated by black lines. Values represent means ± SEM for n=5-6 samples per group. *p<0.001, by one-way ANOVA, compared to all other groups. #p<0.05, by one-way ANOVA, compared to hypoxia vehicle group.
3.13 Preventive treatment with simvastatin did not increase serum markers of muscle or liver toxicity

Statin therapy has been reported in adult humans to cause myopathy (increased CK) and liver injury (increased ALT) (31). As shown in Figure 3.22, neither chronic exposure to hypoxia nor treatment with simvastatin led to any changes in serum ALT or CK. A dose of simvastatin 20 mg/kg/d did not affect serum ALT or CK (Fig. 3.22).

Figure 3.22: Pups were exposed to chronic hypoxia (13% O$_2$) or normoxia (21% O$_2$) from postnatal day 1 until day 14. Pups were treated by daily intraperitoneal injections of simvastatin (2 mg/kg) or 20% DMSO in PBS (vehicle control). ELISA analyses for Alanine Transaminase (ALT) and Creatine Kinase (CK). Values represent means ± SEM for n=6 samples per group.
Chapter 4

Discussion
4.1 Discussion of Results

We have previously established a critical role for the RhoA/ROCK pathway in experimental chronic neonatal PHT (75, 254, 268). In the present study, similar to previous reports in adult rodents (71, 138, 170, 203), we demonstrate that simvastatin both prevents and reverses chronic PHT, presumably via modulation of RhoA activity. The findings from our rescue study are more clinically relevant to human newborn chronic PHT as simvastatin therapy initiated the partial recovery of pathological remodeling, similar to previous ROCK inhibitor studies (75, 254). Reversal was accompanied by improvement to exercise tolerance, suggesting long-term benefits extending into young adulthood. The major novel aspects of this study were the use of simvastatin in neonatal animals, in which the mechanisms of chronic PHT may fundamentally differ from the adult, and for which there are presently no effective therapies. A second novel aspect is the exploration of dose-response, establishing a dose (2 mg/kg), which appeared to effectively balance efficacy and safety. This aspect is critical, as neonates may respond very differently from mature organisms, and therapies designed to ameliorate injury to the lung and pulmonary vasculature could have unanticipated adverse effects on other systems, particularly the developing brain. The goals of treatment for chronic PHT are pulmonary-selective vasodilation and prevention or reversal of pulmonary arterial wall remodeling. This study provides compelling evidence for the therapeutic potential of simvastatin as therapy for chronic neonatal PHT.
We observed that preventively administered simvastatin treatment attenuated the activity of RhoA, normalized PVR, and prevented structural remodeling of the pulmonary arteries. In our rescue protocol, the reduction of RhoA/ROCK activity decreased PVR and partially reversed arterial remodeling. The role of isoprenoid-modulated RhoA activity has not been previously studied in newborn PHT. Both treatment protocols with simvastatin decreased RVH, similar to previous results with direct ROCK inhibition (75). The decrease in RVH could be attributed to the reduction of pressure loading on the right ventricle. However, a recent publication from our lab has shown that the pressure unloading alone is not sufficient to prevent RV dysfunction. Inhaled Fasudil (pulmonary-specific ROCK inhibition) decreased afterload and RVH, but RV RhoA/ROCK signaling remained elevated with concurrent persistent RV dysfunction (75). This suggests that increased pulmonary vasodilation can reduce RVH caused by pressure loading, but does not necessarily improve RV dysfunction. In the present experiments, we did not examine the effects of statin therapy on RhoA or ROCK activity in the RV, nor did we perform experiments (pressure-volume catheter) to examine effects on RV systolic function. The severity of structural markers of chronic PHT (RVH and arterial wall thickening) are stable in the present model from days 14 to 21 of hypoxia exposure. This is important, as it indicates that rescue effects of simvastatin represented reversal of disease rather than prevention of further progression.

Several publications have shown that statins have beneficial cholesterol-independent effects, including isoprenoid inhibition (71, 137, 155, 199, 261). The isoprenoid GGPP contributes to the pathogenesis of PHT by prenylation-mediated intracellular trafficking and membrane localization, and subsequent activation of RhoA
In the present study, we observed an increase in FPP and in HIF-1α content in the lungs of chronic hypoxia-exposed rats. We also observed that simvastatin reduced lung content of FPP, in hypoxia-exposed animals with a trend toward reduction in normoxia controls. Presumably, a reduction of FPP also led to decreased GGPP bioavailability as indicated by reduced RhoA activity; however, we were unable to reliably measure GGPP content in lung tissue. It is known that HIF-1α activity is elevated in hypoxia, which in turn increases transcription of RhoA and/or ROCK (172). Treatment with simvastatin did decrease pulmonary HIF-1α levels in chronic hypoxia-exposed animals, but did not affect lung content of RhoA or of either ROCK isoform (70, 182, 254). Therefore, the effect of statins may be mostly attributed to downregulation of RhoA activity by inhibiting the isoprenoid intermediates, rather than protein content as mediated by HIF-1α. Furthermore, Girgis and colleagues demonstrated that concurrent supplementation of mevalonate with simvastatin treatment negated any beneficial effects in experimental PHT, suggesting a dominant effect for isoprenoid reduction (71).

Vascular remodeling in neonatal animals is a pathognomonic structural finding in chronic PHT (239). Based on previous work, inhibition of the RhoA/ROCK signaling pathway prevented vascular remodeling by inhibiting smooth muscle cell proliferation (268), while blocking the anti-apoptotic effect of ROCK, particularly the ROCK II isoform, prompted the reversal of established remodeling (254). Furthermore, cleaved ROCK I (constitutively active) was found to be downregulated in chronic hypoxia as previously reported (75). The reduction of cleaved ROCK I had reduced apoptotic activity and was observed by the enhanced smooth muscle content in the pulmonary arteries of hypoxia-
exposed lungs. Additionally, simvastatin treatment further reduced cleaved ROCK I content. This suggests that the anti-remodeling effects of statins were primarily related to diminished RhoA activity. VSMC proliferation involves activation of many pro-mitogenic signaling pathways, which disrupt the cell cycle regulation. ROCK is known to be involved in many cellular processes such as mitogenesis. The cyclin-dependent kinase inhibitor p27 is a negative regulator of the cell cycle and VSMC proliferation. Elevated ROCK activity decreases p27 expression, increasing VSMC proliferation (260). Simvastatin inhibition of the RhoA/ROCK signaling pathway may therefore inhibit proliferation of VSMC. ROCK activity also prevents VSMC apoptosis. Previous findings from our lab show attenuated proliferation and increased apoptosis of pulmonary arterial VSMCs (254). Vasodilation of the pulmonary vasculature alone is not effective in reducing PHT-related neonatal mortality. The underlying issue that needs to be addressed is the proliferation of the smooth muscle, which is reduced by inhibiting Rho-dependent ROCK activity (201).

Inhibited NO-cGMP signaling likely plays a major role in the pathogenesis of chronic PHT (113, 162). ROCK is known to inhibit eNOS activity and to disrupt the stability of eNOS mRNA, thus reducing NO bioavailability (60, 103). In the present study, we observed that simvastatin increased eNOS content, which further supports suppression of ROCK activity as the major mechanism for simvastatin effects on chronic neonatal PHT (117). However, the regulation and bioavailability of eNOS remains unclear in either PPHN or chronic PHT (219, 247).

The novel aspect of this study was examination of simvastatin effects on chronic neonatal PHT. Despite promising results in adult experimental chronic PHT (71, 138,
trials employing statins in human adults with chronic PHT have thus far shown an apparent lack of efficacy (192). The implications of these disappointing findings for translation of statins to the neonate remain open, given the potential for maturational differences in response to pharmacological agents that may affect the pulmonary vasculature. For example, selective serotonin reuptake inhibitors (SSRIs) impose a higher risk of PHT in newborns when given to mothers in late pregnancy (33), whereas studies in adults with chronic PHT suggest that SSRIs may improve outcome (107). A small observational study in children with chronic PHT showed improvement in hemodynamic parameters with simvastatin therapy (31). Importantly, the potential utility of statins as a means of targeting RhoA/ROCK activity in the newborn, could extend beyond PHT. We have recently reported a contributory role for up-regulated pulmonary ROCK signaling in experimental BPD-like lung injury (130), a developmental lung disorder characterized by inhibited pulmonary angiogenesis and alveologenesis. Similarly, pulmonary hypoplasia and vascular remodeling in fetal rats with nitrofen-induced congenital diaphragmatic hernia (CDH), in which RhoA may play a pathological role (230), was ameliorated by maternally-administered simvastatin (143). As there are currently no effective preventive therapies for developmental lung disorders such and BPD and CDH, or to prevent or rescue chronic PHT in neonates (93), these findings point toward statins as a potentially useful therapy.

Neonates are particularly susceptible to adverse and off-target effects of pharmacological agents (6). Documented adverse effects of statins in adult humans include hepatic injury and muscle pain (42, 100, 127, 191). In rabbits, high-dose simvastatin (50 mg/kg/d), led to elevations in serum lactate dehydrogenase (LDH), CK,
and ALT (95). In our study, we did not observe any significant increase in serum levels of ALT or CK, even at doses of simvastatin as high as 20 mg/kg. A systematic review and separate meta-analysis suggest that statin therapy does not pose a statistically significant risk for rhabdomyolosis (127) or hepatotoxicity (42). Concern over the prevalence or severity statin side effects may be a holdover from previous toxicity reports involving cerivastatin, which has been withdrawn from the market (220).

Systemic ROCK inhibitors are known to restrict somatic growth and to cause severe systemic hypotension, thus limiting their translational potential in the neonate (75, 268). In the present study, simvastatin did not adversely affect somatic growth in normoxia-exposed animals and actually improved growth in chronic hypoxia-exposed animals. In addition, systemically administered simvastatin did not affect systolic blood pressure. These findings suggest that inhibition of pathological RhoA activity with simvastatin has no effect on somatic growth or basal systemic vascular tone, unlike systemic ROCK inhibition (75, 268). However, we did not examine the systemic effects of statin therapy at the molecular or structural level. We speculate that the lack of systemic side effects could be due to lower basal levels of RhoA and subsequent modulation of ROCK activity. Pathological RhoA activity may only be present in the pulmonary circulation during chronic hypoxia exposure, and is modifiable by statins. This may also be linked to differential expression of RhoA and the ROCK isoforms in the newborn, however there is a limited understanding of the pulmonary or systemic circulation in either adult or newborn physiology (53, 213).

The intended clinical goal of statin therapy is a reduction in circulating cholesterol. In the developing neonate, this may represent an adverse effect, given the
reliance of the developing brain on cholesterol, especially for white matter formation. Interestingly, neither simvastatin, nor the DMSO vehicle altered total serum cholesterol levels or LDL cholesterol in the newborn rat. This finding may be explained by the fact that breastfeeding neonates rely less on endogenous synthesis of cholesterol to maintain serum levels, due to the presence of significant quantities of cholesterol (400-600 mg/dL) in breast milk (64). Lipid soluble statins such as simvastatin can cross the BBB by lactonization, but have not been found to alter brain cholesterol levels (40).

Statins do not accumulate in the brain, being rapidly eliminated via the P-glycoprotein (P-gp) transporter (169). Furthermore, simvastatin metabolism and activation, is very limited in the human brain (252). Brain cholesterol is further protected by a de novo source of cholesterol production, which is required for myelin formation (2, 46, 147, 233). Prematurely-born humans have an increased risk for long-term neurodevelopmental disability due to abnormal axon and dendritic structure in neurons.

In models of newborn neurological injury and development, statins prevented neurological injury through modulation of inflammation to maintain brain morphology, white matter content, and overall brain weight (134, 173, 235). In our present study, we observed potential benefits of simvastatin treatment for white matter content, but did not further explore the outcomes of white matter content on behavioural changes. While there were no reported adverse effects from simvastatin in a small observational study on children with PHT, further study is needed to assess safety and efficacy in the newborn population despite our present reassuring findings (110).
4.2 Limitations and Future Directions

There are a number of limitations to this study. First, our chronic hypoxia model of newborn PHT simulated structural pulmonary vascular and cardiac remodeling as seen in infants with chronic PHT from a variety of causes, including BPD; however, the hypoxic model does not produce inflammation or arrested alveolar development, as observed in human infants with BPD. Therefore, effects of statin treatment should also be studied in a BPD-like model such as secondary to hyperoxia or to systemic bleomycin, where inflammation and arrested lung development are features of injury in addition to chronic PHT (130, 151). Another study on experimental adult PHT has demonstrated the potential of anti-inflammatory benefits with statin therapy (170).

Simvastatin was selected based on its use in multiple clinical and experimental trials, leading to a well-defined dose range and safety profile. While all statins act on the HMG-CoA reductase enzyme, the pharmacological characteristics of each individual statin varies in terms of oral absorption, half-life, hydrophobicity, BBB permeability, metabolism, and whether they are of natural or synthetic origin (19, 200, 214). Future work should test several statin class drugs to determine dose-response for neonatal PHT, as effective doses have been shown to vary greatly in vitro between different statins (5). Lipophilic statins can passively diffuse into systemic cells, while hydrophilic statins have a tendency to remain liver-specific and require carrier proteins for extra-hepatic activity (200). Simvastatin is a lipophilic drug and required DMSO for solubilisation. Other alternatives such as rosuvastatin, are hydrophilic and soluble in PBS, but at the potential cost of variable efficacy in different tissues. However, a study on rosuvastatin (hydrophilic) and atorvastatin (lipophilic) found inhibition of ROCK
activity in extrahepatic cells (183). Use of an alternative statin in future studies, such as rosuvastatin or atorvastatin may be more beneficial to PHT patients, as these statins have a stronger affinity for HMG-CoA reductase and have a greater extent of reduction to LDL cholesterol (19). Presumably, these effects would be accompanied by a greater decrease in mevalonate and therefore greater FPP and GGPP inhibition.

Although PHT was not the target of therapy, pediatric studies employing statins had administered a dose range of 0.1-0.2 mg/kg (67, 110, 188). As an approximation of dose translation by body surface area, 2 mg/kg of simvastatin in rats is roughly equivalent to 0.4 mg/kg in human children (184). However, this does not account for the pharmacokinetics and pharmacodynamics of drug metabolism, which may be greatly accelerated in rats when compared to humans (208). Dose-response studies of the statins in either chronic hypoxia or bleomycin models of PHT, could lead to an optimal selection for safe administration and effectiveness in translation to clinical use.

The use of whole lung tissue in the analyses of biochemical markers in PHT represented an additional limitation. The lung is comprised of many cell types such as smooth muscle, endothelial cells, fibroblasts, both type I and II pneumocytes, and macrophages. We observed an increase to both RhoA and ROCK activity, but we did not characterize all cell types that are modified by chronic hypoxia or simvastatin in the present experiment. Consistent with previous work from our laboratory, chronic hypoxia led to an upregulation of ROCK activity as measured by P-MYPT1 in PASMCs (254) and was reduced with simvastatin treatment. There is a molecular level of cross-talk between multiple cell types with ROCK activity and endothelial dysfunction (166). Statins have been demonstrated to modulate many pathological changes in multiple cell
types including: inhibiting fibroblast proliferation (28), inducing apoptosis in abnormal endothelial cells (231), reducing PASMC migration and proliferation (88). However, the effect of statins on individual lung cell types and candidate pathways are not fully understood, and should be further explored. Future studies could elucidate the mechanisms of statins and direct Rho inhibitors (exoenzyme C3 transferase) through studies evaluating markers of apoptosis, inflammation, and prenylation in vitro.

A technical issue with our methods was encountered when examining the phosphorylated MYPT1 content. The pThr850-MYPT1 band was observed at approximately 80 kDa while the pan-protein was at 135 kDa in both whole lung tissue and PASMCs. Both the phosphorylated and pan-MYPT1 proteins are referenced in the literature as a 135 kDa protein. There is some conflicting information as the antibody against pMYPT-1 is documented in the product datasheet and widely published as an 80 kDa protein. It is possible that the actual molecular weight of the kinase portion of MYPT1 is 115 kDa, and that a 21 kDa subunit contributes to the total weight of 135 kDa. The subunit function is unknown (226) and could be removed once activated, or removed during tissue homogenization for Western blotting (65, 76). The rat isoform of MYPT1 has also been identified as a 110 kDa protein compared to the human or porcine 130 kDa MYPT protein (85). Absence of the leucine zipper (LZ-) portion of MYPT1 has been reported during calcium sensitization, further decreasing the protein sequence and potential molecular weight (262).

There are also limitations with the use of tissue Doppler echocardiography to obtain PVR measurements. It is clinically and experimentally used as a non-invasive surrogate to estimate PVR. The gold-standard for obtaining hemodynamic
measurements is through catheterization. However, a recent publication from our laboratory compared PVR and RV output by echocardiography and catheter measurements in rats. Measurements were estimated by echocardiography, then confirmed by direct catheterization. While catheter measurements were more accurate, echocardiography provides a reliable, non-invasive estimate that could distinguish PHT hemodynamics between experimental groups (51). As echocardiography is an estimate of PVR by the ratio of PAAT and RVET, additional considerations must be made for potential confounders. Heart rate and cardiac output could alter PAAT duration, and therefore PVR, if not controlled.

Evaluation of sex differences should be included with future study in models of PHT. Our current study was not sufficiently powered to stratify by sex. Clinically, it has been reported that there is a significant sex disparity between prevalence and mortality of pediatric PHT, where female patients have a poorer prognosis (114). Females are predominantly affected in adult PHT with ratios as high as 4.3:1 women to men for all forms of PHT (142). The current hypothesis is that estrogen may be injurious to the pulmonary vasculature or the RV. Alternatively, testosterone may be protective of the pulmonary vasculature (179). Regardless, future research could lead to more sex-specific therapy as current treatment does not appear to favour either sex.

The focus on improving treatment of newborn PHT is not limited to the overall survival rate. Follow-up studies have observed that school-age survivors of PHT have reduced physical activity and compromised learning capacity (178, 189, 248). Therefore, long-term outcomes such as neurocognitive performance and exercise tolerance should also be examined. In our model, treadmill testing has demonstrated
that statin treatment improves exercise capacity, secondary to chronic hypoxia. Further examination of potential toxicity and impaired neurological development with statin treatment would be required. We did not observe any major structural brain abnormalities in the infant rat, but this does not necessarily correlate to neurological function. Future examination for effects of statins on behaviour and cognition would be necessary prior to potential translation of this therapy to human newborns (251). Additionally, the impact of statins on hormone production in development is not known, and should be taken into account as cholesterol is a precursor for testosterone and estrogen (188). We did not observe any negative changes in body weight by simvastatin, but did not examine body composition or metabolic changes. Future work could study prolonged statin therapy in the young rat to determine adverse effects with statins, as our study duration of one or two weeks may not have been long enough to induce toxicity.

Future studies would evaluate the hemodynamics of the RV in chronic hypoxia with statin treatment. Our current work demonstrated a reduction to RVH, but we did not determine whether simvastatin improved RV function. Previously, ROCK inhibitors have been shown to improve functional outcomes such as RV stroke volume, RV end systolic and diastolic pressures, and RV output (75). Our study did not incorporate molecular changes in the RV. Previous work had shown that systemically administered ROCK inhibitors modified molecular biomarkers of a failing RV including RhoA, ROCK, and PDE-5 (75). An additional endpoint could be the inclusion of catheter systolic and diastolic blood pressure measurements as a determinant of systemic blood pressure.
Our tail blood pressure cuff was restricted to reading systolic blood pressure, due to limitations in sensitivity imposed by the animal’s small size.

Finally, our present findings focused on the activity of the RhoA small GTPase protein. Many small GTPase proteins also depend on prenylation by isoprenoid intermediates for GDP/GTP exchange, which warrant further investigation. The Ras family is modified by farnesylation. The Rho family of proteins are modified by geranylgeranylation. Of note, Ras, Rac-1, and Cdc42 are best understood and known to be involved in cardiac hypertrophy, actin cytoskeleton remodeling, and smooth muscle cell proliferation (104, 213, 216). Additionally, Rac-1 contributes to ROS generation (140), and oxidative stress may be a potential target in newborn PHT (136). Rac-1 also plays a role in PASMC proliferation via activation of thrombin which can also activate HIF-1α independent of hypoxia (45). In our present experiment, it is likely that simvastatin had reduced prenylation and activity of the other small GTPases, but was directly not accounted for in our findings. Future in vitro studies would compare the Rho inhibitor to other direct small GTPase inhibitors to characterize the range of anti-prenylation effects of statins on experimental PHT.

In summary, simvastatin-mediated inhibition of GGPP prenylation downregulates RhoA, and subsequent ROCK activity. We observed that experimental chronic neonatal PHT is preventable and reversible by simvastatin treatment; leading to reduced PVR, and decreased RV, reduced pulmonary arterial remodeling, and had long-term improvements to exercise capacity. Our findings suggest that simvastatin was well-tolerated, lacked apparent adverse systemic or neurological effects and therefore holds promise as a potentially translatable means of limiting pathological ROCK activity.
References


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>6MWD</td>
<td>6 Minute Walk Distance</td>
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<tr>
<td>ALT</td>
<td>Alanine Transaminase</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>AST</td>
<td>Aspartate Transaminase</td>
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<tr>
<td>BPD</td>
<td>Bronchopulmonary Dysplasia</td>
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<tr>
<td>Ca$^{2+}$</td>
<td>Calcium ion</td>
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<tr>
<td>cGMP</td>
<td>Cyclic Guanosine Monophosphate</td>
</tr>
<tr>
<td>CK</td>
<td>Creatine Kinase</td>
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<tr>
<td>CO</td>
<td>Cardiac Output</td>
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<tr>
<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disease</td>
</tr>
<tr>
<td>CRD</td>
<td>Cysteine-rich Zinc Finger-like Motif Domain</td>
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<tr>
<td>C-terminus</td>
<td>Carboxyl-terminus</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective Tissue Growth Factor</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra-cellular Matrix</td>
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<tr>
<td>ECMO</td>
<td>Extracorporeal Membrane Oxygenation</td>
</tr>
<tr>
<td>ELBW</td>
<td>Extremely Low Birth Weight</td>
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<tr>
<td>eNOS</td>
<td>Endothelial Nitric Oxide</td>
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<tr>
<td>ERK</td>
<td>Extra-cellular Signal-regulated Kinases</td>
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<tr>
<td>ET</td>
<td>Endothelin</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>FPP</td>
<td>Farnesyl Pyrophosphate</td>
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<tr>
<td>FTI</td>
<td>Farnesyl Transferase Inhibitor</td>
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<tr>
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<td>GDP Dissociation Inhibitors</td>
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<tr>
<td>GEF</td>
<td>Guanine Nucleotide Exchange Factors</td>
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PHT  Pulmonary Hypertension
PKG  Protein Kinase G
PND  Postnatal Day
PPHN Persistent Pulmonary Hypertension of the Newborn
PTM  Post-Translational Modifications
PVR  Pulmonary Vascular Resistance
RA   Right Atrium
RBD  Rho-Binding Domain
ROCK Rho-associated Coiled-coiled Kinase
ROS  Reactive Oxygen Species
RV   Right-Ventricular
RVET Right Ventricle Ejection Time
RVF  Right Ventricle Failure
RVH  Right-Ventricular Hypertrophy
SD   Sprague Dawley
Ser  Serine
SGA  Small for Gestational Age
sGC  Soluble Guanylate Cyclase
TGF-β1 Transforming Growth Factor Beta 1
Thr  Threonine
TxB2 Thromboxane
VSMC Vascular Smooth Muscle Cells
WB   Western Blot