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ROLE OF PKCBETA AND OXIDANT SIGNALING IN NEONATAL PULMONARY HYPERTENSION

Joshua R. Sheak

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Joshua R. Sheak

Candidate

Biomedical Sciences Graduate Program: Cell Biology and Physiology

Department

This dissertation is approved, and it is acceptable in quality and form for publication:

Approved by the Dissertation Committee:

Thomas C. Resta, Ph.D. , Chairperson

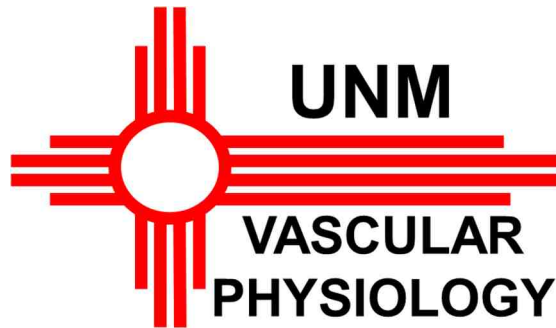
Mary Beth Goens, M.D.

Nancy L. Kanagy, Ph. D.

Nikki L. Jernigan, Ph. D.

Diane S. Lidke, Ph. D.

**ROLE OF PKCBETA AND OXIDANT SIGNALING
IN NEONATAL PULMONARY HYPERTENSION**



by

JOSHUA R. SHEAK

B.S., Biochemistry, University of New Mexico, 2010

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

**Doctor of Philosophy
Biomedical Sciences**

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DEDICATION

To mom and dad

Thank you for inspiring me to work hard for the things I love.

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Thank you, Dr. Tom Resta, for your mentorship, encouragement, and support over the last five years. You have helped me build a foundation for scientific inquiry and hypothesis-driven research that will be with me throughout my career.

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ABSTRACT

Chronic hypoxia (CH)-induced vasoconstriction has been implicated in the pathogenesis of pulmonary hypertension (pHTN) in infants with chronic cardiorespiratory disorders. Although endothelial dysfunction, reduced nitric oxide (NO) bioavailability and oxidative stress contribute to a variety of cardiovascular disorders, their contribution to enhanced vasoconstrictor reactivity in neonatal pHTN is poorly understood. We therefore hypothesized that neonatal CH augments pulmonary vasoconstrictor reactivity by limiting NO-dependent pulmonary vasodilation and by promoting the generation of reactive oxygen species (ROS).

Enzymatic sources of ROS in the vasculature include NADPH oxidase isoforms, xanthine oxidase, and uncoupled endothelial nitric oxide synthase (eNOS). The mitochondria are also a major source of cellular ROS, but surprisingly little is known about the role of mitochondrial-derived ROS (mitoROS) in neonatal pHTN. Based on preliminary studies from our laboratory and evidence that protein kinase C β (PKC β) mediates mitochondrial dysfunction and oxidative stress in neurodegenerative diseases, we further hypothesized that neonatal CH enhances pulmonary vasoconstrictor sensitivity via PKC β -dependent activation of mitoROS generation.

To test these hypotheses, we employed pharmacologic approaches to assess the role of NO, mitoROS and PKC β on basal vascular tone and agonist-induced vasoconstrictor sensitivity in both isolated (*in situ*) lungs and pressurized pulmonary arteries (~150 μ m) from control and CH (2 weeks at 380 mmHg) neonatal rats. CH neonates displayed elevated right ventricular (RV) systolic pressure (*in vivo*) and RV hypertrophy, indicative of pHTN. CH increased both basal pulmonary arterial tone and vasoconstrictor reactivity to the thromboxane analog, U-46619. Interestingly, we observed that endogenous NO limits CH-dependent increases in pulmonary vasoconstriction. Exposure to CH also enhanced NO-dependent vasodilation to arginine vasopressin (AVP), pulmonary expression of NOS III (eNOS), and eNOS phosphorylation at activation residue serine-1177. Additional studies using *in situ* lungs revealed an effect of scavenging ROS or inhibition of PKC β to attenuate CH-dependent increases in basal tone and agonist-induced pulmonary vasoconstrictor sensitivity. Selective inhibitors of PKC β or mitoROS similarly reduced basal tone in arteries from CH rats, while having no effect in control arteries.

We conclude that, in contrast to our hypothesis, enhanced basal tone and agonist-induced vasoconstriction following neonatal CH is limited by increased NO-dependent pulmonary vasodilation resulting from greater eNOS expression and phosphorylation at activation residue serine-1177. Furthermore, both PKC β and mitoROS contribute to enhanced pulmonary vasoconstrictor sensitivity following CH in neonates. These signaling mediators represent new potential therapeutic targets in the treatment of neonatal pHTN.

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CHAPTER 1 – INTRODUCTION

Pulmonary hypertension (pHTN) in newborns results from the failure of the pulmonary circulation to dilate after birth (4, 6). Elevated pulmonary arterial pressure during infancy or childhood contributes significantly to death and disability (5, 14, 27). Current therapies depend on strategies to lower pulmonary arterial pressure, such as inhaled nitric oxide (NO, a potent vasodilator), but have not been demonstrated to improve mortality or length/cost of hospital stay (27, 191, 204, 273). Advances in our understanding of neonatal pHTN are therefore needed to facilitate newer, mechanistically targeted therapies.

Chronic hypoxia (CH) is a common cause of neonatal pHTN and can negatively impact the developing pulmonary circulation (120). CH is a term used to describe chronic reductions in arterial oxygen available to the body (general hypoxia) or to a specific region (tissue hypoxia). Chronic reductions in arterial oxygen levels (hypoxemia) can result from complications occurring during prenatal and antenatal pulmonary development. Reduced fetal oxygen supply resulting from limited maternal oxygen supply (as can occur during exposure to high altitude) or reduced uterine blood flow (found in placental insufficiency) can lead to pHTN (120). Exposure to CH can also occur intrapartum, during labor. Intermittent episodes of interrupted fetal oxygenation can occur during human labor due to uterine contraction, compression of the umbilical cord, head compression, placental abruption, or rupture of fetal vessels (231, 267). Most fetuses tolerate these moments of hypoxia, but some do not and hypoxia-associated disorders such as persistent pHTN of the newborn can result (120). Post-natal exposure to CH usually occurs in newborn babies suffering from parenchymal lung disease such as bronchopulmonary dysplasia (27, 30), meconium aspiration pneumonia

(5, 152), or pulmonary hypoplasia resulting from conditions such as congenital diaphragmatic hernia (249, 260).

Neonatal pHTN resulting from CH exposure is associated with elevated pulmonary vascular resistance (PVR) that is attributed to luminal narrowing of the pulmonary arterial circulation resulting from arterial remodeling and vasoconstriction. Several studies have evaluated factors that influence the degree of pulmonary arterial remodeling that occurs in the setting of neonatal pHTN (19, 33, 142). Therapy for neonates with pHTN aims to reduce and perhaps reverse remodeling of the pulmonary vasculature to facilitate long-term reductions in PVR. Therapies aimed at selective pulmonary vasodilation however are important for the acute management of neonates with pHTN. Enhanced pulmonary vasoconstriction following neonatal CH may result from impaired endothelium-dependent pulmonary vasodilation (19, 87, 89, 129) and increased production of or sensitivity to vasoconstrictor agonists (15, 40, 91, 233). The relative contribution of diminished endothelium-dependent pulmonary vasodilation and enhanced pulmonary vasoconstriction related to increases in basal tone as well as augmented reactivity to endogenous vasoconstrictor agonists has not been fully investigated. The following sections outline the normal development and physiology of the fetal and neonatal pulmonary vasculature and mechanisms contributing to the development of CH-induced neonatal pHTN.

Development of the Pulmonary Vasculature In Utero

Structure of Arteries. Fetal development of the pulmonary vasculature begins near the 5th week of gestation and continues after birth, well into the **postnatal** period (212). The pulmonary circulation is characterized by three structural motifs for arteries – fully muscular, partially muscular, and nonmuscular (263). In the fetal lung, the muscularization of arteries is dependent on vascular external diameter. Based on

microscopic evaluation of the fetal pulmonary vasculature, Hislop and Reid observed, "In all arteries over 200 μm external diameter the wall is muscular. Below 200 μm partially muscular arteries appear and increase until, in the diameter range 100-75 μm , they comprise 68% of the population of arteries. Non-muscular arteries appear between 125 and 100 μm and below 37 μm all arteries have this structure," (127). This size-dependent breakdown of fully to nonmuscularized arteries is present throughout gestation and is similar to that observed in the pulmonary circulation of adults (127, 263).

Although arteries of similar size have comparable muscularization patterns (fully, partially, and nonmuscularized vessels) between the fetal and adult pulmonary circulation, the fetal pulmonary arterial circulation is more muscular and has a larger percent wall thickness (127). Percent wall thickness represents the thickness of the arterial wall as a percentage of the external diameter of the vessel (127). The wall of a given arterial size is twice as muscular in the fetus compared to the adult (127). In the fetus, when the artery contains muscle, wall thickness is inversely proportional to vessel diameter with smaller vessels having the highest percentage wall thickness (127).

Hemodynamic Components of Fetal Pulmonary Circulation. Fetal blood is oxygenated at the placenta and circulated to the fetus (212). A major characteristic of the fetal cardiopulmonary circulation is that more than 90% of cardiac output is diverted away from the lungs to other organs via fetal shunts like the foramen ovale and the ductus arteriosus (153). Shunting of cardiac output away from the lungs *in utero* is accomplished by elevated PVR. *In utero*, fetal PVR is greater when compared to the systemic circulation (3, 187). Elevated PVR is uncharacteristic for the post-natal pulmonary circulation, which is characterized by low pressure and low resistance under normoxic conditions in otherwise healthy adult individuals. Poiseuille's equation

describes elements that impact arterial resistance. Per this equation, we expect that increases in blood viscosity and decreases in luminal radius of the vessel increase PVR.

Increased blood viscosity occurs when hematocrit, or the ratio of the volume of red blood cells to the total volume of blood, increases. *In utero*, circulating red blood cell concentrations gradually increase during the second trimester (weeks 13 – 28), increasing hematocrit values from 30 – 40%. From the second trimester to term (week 40) the red cell content of blood continues to increase to bring fetal hematocrit to an average of around 50% (97). The increase in fetal hematocrit occurring during gestation similarly increases vascular resistance in the pulmonary and systemic circulations. Considering that PVR is so much greater than systemic vascular resistance *in utero*, gestational increase in hematocrit is unlikely to account for enhanced PVR observed in the fetus.

Decreases in radius of the vessel may account for increased fetal PVR. In Poiseuille's equation resistance is inversely proportional to radius to the fourth power, meaning that halving the radius would increase resistance by 16-fold. As mentioned above, arteries in the fetal lung are more muscularized relative to the lungs of adults. This heightened degree of muscularization narrows the lumen of the vessel and contributes to enhanced fetal PVR (212). Small vessels in the fetal lungs have the thickest muscular walls and are believed to contribute to the elevation of PVR *in utero* (127, 212). Early studies evaluating pulmonary vascular structure in the antenatal period demonstrate that these smallest vessels dilate at birth and contribute to an immediate drop in PVR (127). The impact of pulmonary vasodilation occurring at birth suggests that mechanisms of vasoconstriction contribute to enhanced PVR *in utero*.

Mechanisms of Fetal Pulmonary Vasoconstriction.

Increased PVR *in utero* is due to several mechanisms such as mechanical compression of pulmonary vessels by fluid-filled airways in the lungs and vasoconstriction of fetal pulmonary arteries (102, 104).

Fluid-filled airways. Fluid-filled airways are part of gestational lung development with fetal lung fluid formed by the oncotic force generated by chloride secretion of the respiratory epithelium into the lumen of the airway (39, 178). In late gestation fetal lambs, the volume of lung liquid contained in potential airspaces was greater than the volume of air (functional residual capacity) in newborn lambs (131), indicating that fetal lungs are hyperexpanded relative to neonatal lungs. Draining lung liquid increased pulmonary blood flow, whereas tracheal obstruction limited pulmonary blood flow in these fetal lambs (131). These observations suggest that overexpansion of the fluid-filled airway adds extraluminal pressure on the pulmonary vasculature (266) and contributes to increased PVR in the fetus (131).

Hypoxic pulmonary vasoconstriction (HPV). Increase of fetal blood oxygen tension in chronically instrumented lambs either by exposure of pregnant ewes to hyperbaric hyperoxia or *in utero* fetal ventilation with 100% oxygen reduces fetal PVR and increases pulmonary blood flow (24, 26, 188). The pulmonary vasculature is sensitive to changes in blood oxygen tension in near-term fetal lambs, but due to the hypoxic nature of the fetal circulation (57), oxygen-dependent vasodilation is absent (275) and pulmonary arteries demonstrate HPV (77, 78).

HPV describes a physiologic response whereby contraction of pulmonary VSM increases due to both airway and blood-borne hypoxia, with airway hypoxia as the more potent stimulus. Hypoxia exposure has been shown to contract isolated VSM cells from medium and small diameter pulmonary arteries collected from cats, whereas hypoxia-

dependent contraction of VSM cells from cerebral (systemic) arteries was absent (169). In addition to the hypoxia sensitivity of isolated VSM cells, HPV can be demonstrated in isolated, *ex vivo*, perfused lungs (113, 168). Hypoxic sensitivity of isolated VSM cells and lungs indicates that HPV is a local response to hypoxia and not mediated by neurovascular reflexes.

HPV typically occurs in two phases with the rapid, first phase intrinsic to the VSM and the second slower, sustained phase due to the contribution of an endothelium-derived contractile factor (2, 275). The mechanisms that mediate phase 1 of HPV are controversial. One hypothesis proposes that hypoxia decreases the open probability of voltage-gated potassium channels (K_v) present on VSM (282). Hypoxia-dependent reduction of K_v channel open probability can depolarize the VSM membrane (282) and activate voltage-dependent calcium channels (VDCC) leading to entry of extracellular calcium (Ca^{2+}) (179). The resulting increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) leads to Ca^{2+} -dependent pulmonary VSM contraction (1, 2, 179). Hypoxia-dependent inhibition of K_v channels has been linked to decreased production of mitochondrial-derived reactive oxygen species (mitoROS), including superoxide (O_2^-) anion, as oxygen becomes rate-limiting for ROS generation by the mitochondrial electron transport chain (1). Decreases in mitoROS shift the balance of cellular redox state to favor net reduction which further inhibits the K_v channel (282).

An alternative hypothesis for phase 1 of HPV suggests that hypoxia increases mitoROS levels which shifts the cellular redox state to being more oxidized (78, 275). An oxidized cellular state triggers VSM cell contraction through the release of Ca^{2+} from intracellular stores such as the sarcoplasmic reticulum (SR) and subsequent store-operated Ca^{2+} entry (SOCE) (275). Ca^{2+} flux across the plasma membrane secondary to depletion of SR Ca^{2+} stores is termed SOCE. Several studies have shown that phase 1 of HPV is suppressed by depletion of SR Ca^{2+} stores with cyclopiazonic acid, lanthanum

(La^{3+} , a blocker of SOCE), and psalmotoxin (an inhibitor of the store-operated channel, acid sensing ion channel 1) (1, 195, 227).

Mechanisms regulating phase 2 of HPV involve release of an endothelium-derived contractile factor. Removal of the endothelium of pulmonary arteries either eliminates or greatly reduces phase 2 of HPV (2, 275). Vasoconstriction occurring during phase 2 of HPV relies on endothelin-1 (ET-1)-dependent contractile responses in VSM and increases in myofilament Ca^{2+} sensitivity due to signaling involving RhoA and Rho kinase (ROK) (2, 51, 275).

HPV occurring during gestation contributes to elevated PVR and shunting of cardiac output from the lungs to other organs (77, 78). Of interest, conditions of hypoxia *in utero* maintain vasodilation of the ductus arteriosus and the increase in blood oxygen tension occurring with birth has a direct effect to constrict and close the ductus (121). In addition to HPV, other mechanisms of vasoconstriction contribute to enhanced PVR in the fetal pulmonary circulation.

Myogenic response. Unlike the mature pulmonary circulation in adults, the fetal pulmonary vasculature appears to regulate flow through a myogenic response (251, 262). Acute increases in pulmonary arterial pressure *in vivo* accomplished by occlusion of the ductus arteriosus in fetal lambs fitted with an inflatable vascular occluder increased PVR in the presence of NOS inhibition (251). The pressure-dependent increase in pulmonary vasoconstriction suggests myogenic regulation. The myogenic response represents vasoconstriction observed in certain vascular beds that occurs due to an increase in intraluminal pressure. The response is intrinsic to the VSM (69) and although observed in the fetal pulmonary circulation, is better characterized in studies of arteries in the systemic circulation (65, 69, 70, 256).

In studies of the myogenic response in the systemic circulation, increased pressure of an isolated blood vessel elicits VSM depolarization (69). Depolarization opens VDCCs which increases Ca^{2+} influx and leads to subsequent VSM cell contraction (69, 123, 294). Stretch-sensitive, non-selective cation channels such as transient receptor potential channels (TRPs) (64, 123, 192) and epithelial sodium channels (123) have been proposed to mediate depolarization in the myogenic response. Intracellular signaling such as the release of Ca^{2+} from intracellular stores (69, 123) and activation of kinases, ROK (44, 186) and PKC (186), also contribute to myogenic tone.

TRP channels are expressed in the pulmonary circulation of the fetus, however their direct contribution to the fetal pulmonary myogenic response has not been evaluated (40, 102, 108, 203). ROK signaling contributes to the fetal pulmonary vascular myogenic response (262). Signaling of both ROK (202, 262) and PKC (56) are important to pulmonary arterial constriction in the fetus.

Agonist-induced pulmonary vasoconstriction. In the fetal pulmonary circulation, agonist-dependent pulmonary vasoconstriction contributes to fetal PVR (71, 132, 137). Vasoconstrictive agonists such as ET-1, platelet activating factor (PAF), and serotonin (5-HT) have been linked to pulmonary vasoconstriction *in utero* (71, 132, 137). Vasoconstrictive agonists such as ET-1, PAF, and 5-HT promote VSM contraction by binding a g-protein coupled receptor on the VSM membrane (133, 274, 290). When bound to agonist, the receptor's g-protein subunit disassociates and breaks into G_α and $G_{\beta\gamma}$ subunits (17). The subunit G_α activates phospholipase C (PLC) which cleaves membrane phospholipids into diacylglycerol (DAG) and inositol triphosphate (IP_3) (162). DAG has second-messenger properties associated with activation of some downstream kinases such as PKC and is important for 5-HT-induced intracellular signaling (110). IP_3 can bind the IP_3 -receptor on the SR which leads to Ca^{2+} release, increased $[\text{Ca}^{2+}]_i$, and

VSM contraction (133). Vasoconstrictive agonists can also promote G_{α} -dependent activation of Rho A/ROK signaling in VSM (145, 167), a response that can contribute to enhanced myofilament Ca^{2+} sensitivity (17, 145).

In late-gestation fetal lambs, treatment of chronically instrumented near-term fetal lambs with ET-1 infusion increases PVR in a manner sensitive to ET_A (ET-1 receptor linked to VSM contraction) receptor blocker, BQ-123 (136). However in other reports, administration of ET-1 to either chronically instrumented near-term lambs or isolated, perfused lungs from late gestation fetal sheep lead to dose-dependent pulmonary vasodilation (48, 50). These findings suggest that the contribution of ET-1 to regulate fetal pulmonary vascular tone is controversial.

In contrast to the controversial role of ET-1 in the fetal pulmonary circulation, selective inhibition of the PAF receptor with WEB-2170, in near term fetal lambs, facilitated a 68% reduction in fetal PVR, suggesting that PAF contributes to elevated pulmonary vascular tone *in utero* (132). PAF is synthesized from membrane phospholipids by phospholipase A_2 (PLA $_2$) (132) in a variety of cell types, such as platelets, macrophages, endothelial cells, and VSM (29, 134). The levels of PAF and PAF-receptor (PAF-R) in lung tissue are higher in the fetus than in newborn lambs (133, 134). The relatively hypoxic fetal pulmonary circulation may, in part, account for the increase in both PAF and PAF-R (29, 134). In response to hypoxia, transiently cultured pulmonary arterial smooth muscle cells (PASMC) collected from fetal lambs increase protein expression of both PAF-R and PAF (133, 134). Treatment of transiently cultured PASMCs with PAF facilitated IP_3 -dependent increases in intracellular Ca^{2+} that were augmented following exposure to hypoxia (133). Taken together, these results suggest that the hypoxic environment of the fetal pulmonary vasculature may facilitate high PVR through PAF-dependent pulmonary vasoconstriction.

5-HT is another vasoconstrictor agonist that may contribute to enhanced PVR *in utero* (71, 72). 5-HT infusion causes dose-dependent pulmonary vasoconstriction *in vivo* in chronically instrumented late-gestation fetal lambs (71). Interestingly, in near-term fetal lambs, acute infusion of ketanserin, a selective antagonist of a subtype of 5-HT receptors, 5-HT_{2A}, decreases basal PVR (71) suggesting that 5-HT contributes to enhanced PVR *in utero*. Furthermore, selective serotonin reuptake inhibitors (SSRIs), drugs that prolong 5-HT signaling, such as sertraline and fluoxetine also increase PVR in late-gestation fetal sheep (71). SSRI-induced fetal pulmonary vasoconstriction is important because maternal SSRI use in humans has been associated with enhanced newborn PVR in the antenatal period (49). SSRI-dependent increases in antenatal PVR may be due to direct actions on the developing pulmonary circulation considering that SSRIs can cross the placenta (219). Maternal fluoxetine and sertraline exposure in mice leads to premature closure of the ductus arteriosus (130). Fetal closure of the ductus increases PVR in fetal lambs and facilitates persistent elevations of PVR even after delivery (6, 181). Taken together, these studies indicate that 5-HT is an important mediator of enhanced PVR *in utero*.

In summary, enhanced PVR in the fetus is due to several mechanisms: fluid-filled lungs, HPV, myogenic tone, and agonist-induced pulmonary vasoconstriction. These mechanisms are important in maintaining shunting of cardiac output from developing fetal lungs and are vital to the healthy maturation of the pulmonary circulation. High PVR and shunting of blood from the lungs *in utero* is possible due to the role of the placenta in fetal blood oxygenation. After birth, the newborn infant must rely on its lungs for blood oxygenation. This requirement is associated with a massive shift in cardiopulmonary perfusion with cardiac output of the right ventricle no longer shunted from the lungs, but redistributed to them (126). This shift to allow for increased

pulmonary blood flow is associated with profound pulmonary vasodilation and is discussed in following sections.

Adaptation of the Pulmonary Vasculature After Birth

Studies investigating the adaptation that occurs in the pulmonary circulation to allow a newborn to transition from fetal to antenatal life have indicated roles for both reduced arterial muscularity and vasodilation.

Branching Pattern and Structure. Following birth, new arteries and alveoli develop within the acinus (128). Neonatal rats, like humans, undergo the alveolar stage of lung development after birth (61, 260). In rats, the alveolar wall thins, the surface areas of alveoli expand by 20-fold, and capillary surface area expands by 35-fold (260). In the preacinar region, the relative percent wall thickness of arteries gradually decreases throughout infancy. Hislop and Reid compared percent wall thickness in pulmonary arteries from a three-day old child to the mean for children aged 10 months to 10 years and noted, “[In the child aged 3 days], while the arteries over 200 μm external diameter still had a percentage wall thickness in the range of the fetal ages, for those below 200 μm the adult range has been reached. Thinning of the small vessels had been immediate, probably due to dilation. Only after 4 months had the larger vessels lost their fetal wall thickness,” (128). From this study and others, it is clear that initial, rapid reductions in arterial diameter are due to vasodilation and that long-term reductions in percent wall thickness are due to changes in cytoskeletal structure and proliferation (114, 118, 126, 128).

Birth-Related Pulmonary Vasodilation. Pulmonary vasodilation occurring after birth has a rapid onset. In chronically instrumented fetal lambs, pulmonary arterial

pressure decreases and pulmonary blood flow increases within minutes of birth (4, 164). After delivery, the mean pulmonary arterial blood pressure decreases to a value lower than mean arterial blood pressure in the systemic circulation. Greater systemic blood pressure leads to pressure-dependent closure of the foramen ovale (102). The ductus arteriosus begins to close within minutes after birth, but does not fully close for several weeks. Despite the delay in full closure of the ductus, blood flow through the structure stops within 15 hours after birth (190). Reductions of PVR and closure of fetal shunts occurring at birth increase pulmonary blood flow to include total cardiac output in the human infant (102). This is important because at birth the lungs replace the placenta as the organ of gas exchange and blood oxygenation (126).

Mechanisms of Endothelium-Dependent Pulmonary Vasodilation. The anatomy of the blood vessel places the endothelium directly adjacent to the layer of VSM cells and therefore can rapidly influence the tone of VSM cells (95). The endothelium is also lumenally positioned and in direct contact with the blood and can therefore detect birth-related increases in shear stress related to increased pulmonary blood flow (3) and increased oxygen tension (47, 68, 122). Following detection of these birth-related stimuli, the endothelium releases vasoactive mediators such as prostacyclin (164, 165) and NO (4, 46, 100) to facilitate pulmonary vasodilation and decreases in PVR.

Prostacyclin. Endothelial cell prostacyclin production is stimulated by shear stress, changes in blood oxygen tension, and receptor-dependent agonists (164, 235). In response to these stimuli, Ca²⁺-dependent activation of phospholipase A₂ (PLA₂) generates arachidonic acid (AA) through cleavage of membrane phospholipids. Prostacyclin is a product of cyclooxygenase (COX)-dependent metabolism of AA.

Prostacyclin can leave the endothelial cell and bind to the prostacyclin receptor on VSM cells, leading to VSM relaxation through a mechanism reliant on stimulation of adenylyl cyclase and production of cyclic adenosine monophosphate (cAMP) (112). COX expression and the synthesis of prostacyclin are developmentally regulated (42, 164). The increase in blood oxygenation that occurs with the onset of ventilation at birth stimulates prostacyclin synthesis (164). In response to the initiation of ventilation, the decrease in PVR of chronically instrumented newborn lambs occurs in two phases: 1) A rapid decline (< 30 seconds) and; 2) A slower, more gradual decline that occurs during the first 10-20 minutes of ventilation (165). Treatment of these lambs with infusions of indomethacin, an inhibitor of COX, limits only the gradual second phase of ventilation-dependent decrease in PVR (165). Thus, the stimulus of ventilation on prostacyclin release may contribute to postnatal pulmonary vasodilation.

NO. NO derived from the endothelium (100) leads to vasodilation in the perinatal pulmonary circulation (4). NO is produced during the conversion of L-arginine to L-citrulline. The conversion is catalyzed by nitric oxide synthase (NOS) (96, 98). There are three NOS isoforms that contribute to NO formation in the pulmonary circulation of the fetus and neonate, NOS I (neuronal NOS or nNOS), NOS II (inducible NOS or iNOS), and NOS III (endothelial NOS or eNOS).

In whole lung tissue collected from fetal rats, nNOS mRNA and protein expression are increased throughout gestation and then fall rapidly after birth (196). In chronically instrumented fetal lambs, selective nNOS inhibition increases PVR without affecting acetylcholine-induced pulmonary vasodilation (217). Moreover, nNOS protein expression was identified in pulmonary arteries, both with and without the endothelium, collected from late gestation fetal sheep, suggesting that nNOS may be expressed in the VSM or adventitial layer of the pulmonary artery during gestation in sheep (217).

Experiments investigating the contribution of iNOS to PVR in newborn lambs, demonstrated that selective iNOS inhibition mitigates the ventilation- and oxygen-dependent fall in PVR that occurs during early postnatal life (215). However, selective iNOS inhibition in chronically instrumented late gestation fetal lambs only partially inhibited shear stress dependent increases in pulmonary vasodilation (216). Data from these studies is consistent with findings in baboons, where iNOS appears to take on a larger role to impact PVR only after birth (236).

In whole lung tissue collected from fetal rats, both mRNA and protein expression of eNOS are detectable in late stage gestation and near term at their highest levels (196). In a separate study using whole lung tissue from neonatal rats, eNOS mRNA and protein expression were detected at their highest levels within 24 hours after birth with increased eNOS mRNA persisting for 16 days post-partum (150). Additionally, as newborn rats age from one to two weeks eNOS protein expression and NO-dependent regulation of pulmonary vascular tone are increased (53).

Birth-related stimuli such as increases in blood oxygenation (35, 188) and shear stress (96) stimulate eNOS dependent pulmonary vasodilation. In near term lambs, ventilation with 100% O₂ increases eNOS mRNA and protein expression more than ventilation with a gas mixture of N₂/O₂/CO₂ (35). In a study of near-term lambs, raising fetal arterial oxygen tension by exposing pregnant ewes to 100% oxygen at 3 atmospheres of pressure increased the proportion of the right ventricular output distributed to fetal lungs from 8 to 59% (188). Increased pulmonary blood flow occurring at birth would likewise increase shear stress on the endothelium. In isolated pulmonary arterial endothelial cells transiently cultured from late gestation fetal lambs, exposure to shear stress in vitro increased eNOS mRNA and protein expression (35, 276, 279). In chronically instrumented, near term fetal lambs fitted with an inflatable vascular occluder around the ductus arteriosus, compression of the ductus increased pulmonary blood flow

by 300% and decreased PVR (57). These responses were significantly blunted when NOS was inhibited by infusions of nitro-L-arginine (57), an L-arginine analog unavailable for NO production (185). These findings suggest that eNOS contributes to the normal fall in elevated PVR occurring at birth.

Neonatal Pulmonary Hypertension

Newborn pHTN commonly results from the failure of the pulmonary circulation to dilate at birth (6). Elevated PVR impedes blood flow through the pulmonary circulation by remaining greater than systemic vascular resistance (5, 249). This differential leads to right-to-left shunting of unoxygenated blood across the ductus arteriosus and foramen ovale and consequential severe hypoxemia (249). Life-threatening circulatory failure can also result from pHTN (66, 102, 249). Neonatal pHTN resulting from a failure of post-natal pulmonary vasodilation affects 1.9 per 1000 live births in the United States and can complicate the course of up to 10% of all infants admitted to the NICU (246).

Mechanisms of Neonatal Pulmonary Hypertension

There are several elements that mediate increased in PVR observed in the setting of neonatal pHTN and are diagramed in **Figure 1** and discussed below.

Polycythemia. Increased circulating concentration of red blood cells has the potential to increase the viscosity of blood and therefore vascular resistance in a manner described by Poiseuille's equation. Erythropoietin is a hormone that stimulates production of red blood cells in response to either global or tissue hypoxia. Exposure to fetal hypoxia increases erythropoietin concentration (231). Erythropoietin does not cross the placenta and therefore, tissue hypoxia stimulates erythropoietin production in the fetus (283). The liver is the primary site for fetal erythropoietin synthesis and is

Figure

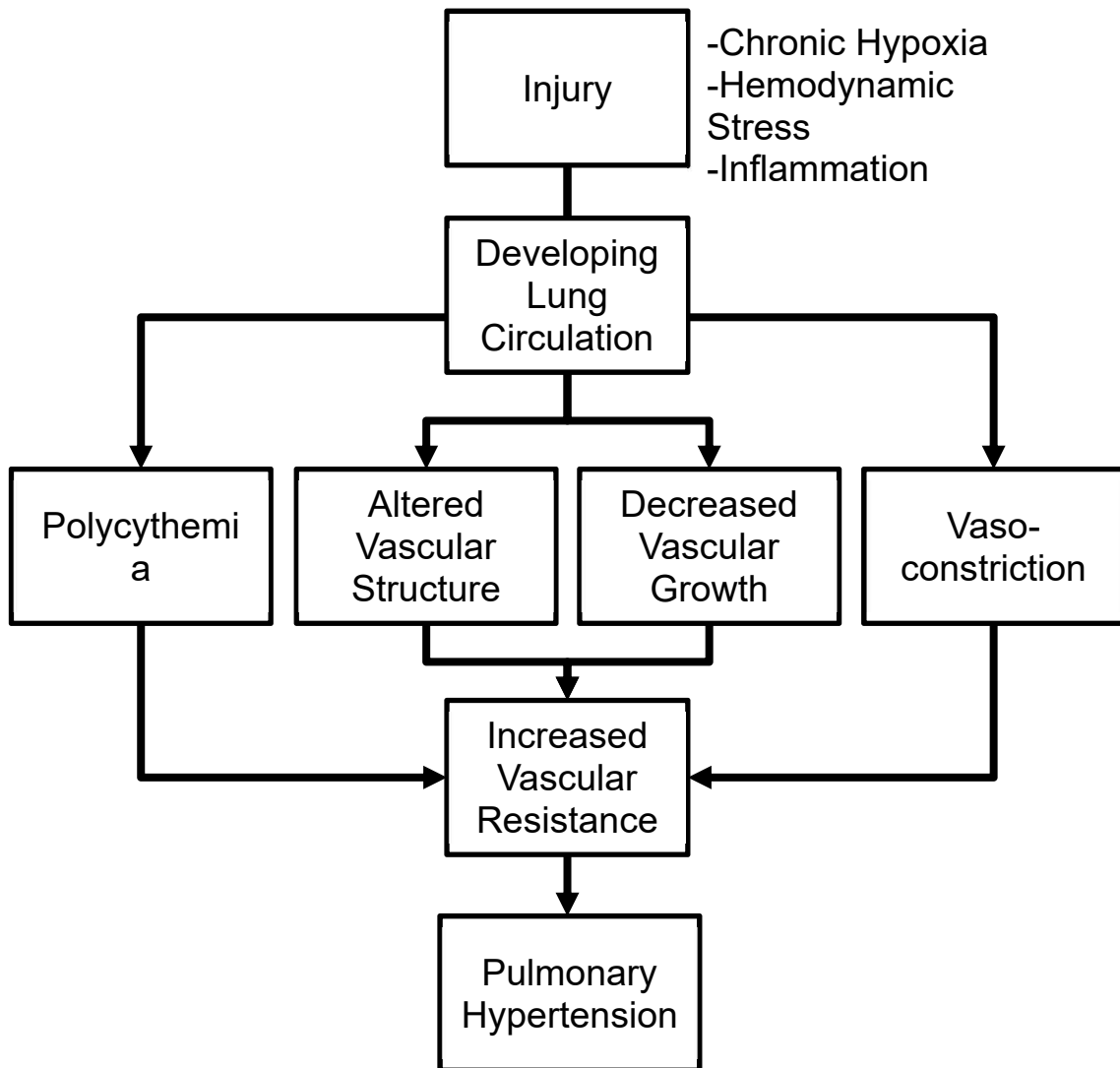


Figure 1. Development of Neonatal Pulmonary Hypertension. Chronic hypoxia (CH) increases pulmonary vascular resistance via polycythemia, altered vascular structure, decreased vascular growth, and vasoconstriction, all of which may contribute to neonatal pHTN.

considered to be the sensor organ for the erythropoietin response to hypoxia (240).

Infants with fetal exposure to CH associated with maternal preeclampsia had elevated erythropoietin concentrations in amniotic fluid, cord blood (231), and in serum (232).

However, erythropoietin returned to near normal levels within 8 hours of delivery (232).

Hypoxia-dependent changes in erythropoietin expression may impact red blood cell concentrations. In animal models of neonatal pHTN involving exposure to CH, hematocrit values in CH exposed neonates are elevated relative to normoxic controls (52, 148). In neonatal rats exposed to 2 weeks of CH, mean hematocrit values for CH exposed rats were approximately 40% whereas mean hematocrit values were 30% in normoxic controls (52, 148). Exposure of adult rats to CH for the same length of time leads to hematocrit values of 61% for CH and 46% for normoxic controls (52). Although hematocrit increases in both newborn and adult rats exposed to CH, the hematocrit of CH exposed neonatal rats equals hematocrit for normoxic adult rats. Modest increases in hematocrit are also observed in newborn piglets (89) and calves (79) with CH-induced pHTN. Due to the small size of the increase in hematocrit, it is unlikely that CH-dependent polycythemia alone is sufficient to explain elevations in PVR observed in neonates with pHTN. In a study involving human infants whether either acute or CH exposure, polycythemia (venous hematocrit >65%) occurred in some patients, but those infants did not require ventilator support or oxygen treatment (232) whereas infants with pHTN do require therapeutic interventions (5, 138, 246).

Altered Vascular Structure. The histology of neonatal pHTN is characterized by thick walled pulmonary arteries (61, 79) that, if untreated, can persist into adulthood (60, 61, 214). The fully muscularized thick arterial wall observed in neonates with pHTN is, in part, due to extension of muscle down the arterial tree into previously partial or non-muscularized arteries (212). The initial thickening of the medial layer of pulmonary

arteries is due to smooth muscle cell hypertrophy and increases in extracellular connective tissue (184). VSM cells in the medial layer of pulmonary arteries from newborn calves and rats show increased cell proliferation and DNA synthesis (183). Extension of smooth muscle down the vascular tree is thought to be due to proliferation of smooth muscle cells as well as differentiation of precursor cells such as pericytes in the non-muscular region of the artery or intermediate cells (fibroblasts) in the partially muscular region to mature VSM cells (184).

Increased circulating levels of ET-1 in human infants with pHTN (228) and in various animal models of neonatal pHTN (20, 143, 144, 247) may promote VSM proliferation and hypertrophy and facilitate arterial remodeling in neonates with pHTN (20, 143, 144). ET-1 signaling also promotes activity of other growth factors like platelet-derived growth factor (141), transforming growth factor- β (139), and nuclear factor of activated T cells isoform c3 (99) that may additionally play a role in vascular remodeling occurring in the setting of neonatal pHTN (21, 25, 33, 117). In a study that compared neonatal fawn-hooded rats (a genetic rat strain that develops pHTN after exposure to mild CH) to Sprague-Dawley rats (a genetic strain that requires exposure to more severe CH to develop pHTN), whole lung ET-1 mRNA expression (preproET-1 mRNA) is elevated 5 days after birth in fawn-hooded rats with no change observed in Sprague-Dawley rats (247). This elevation occurs before pHTN develops, suggesting that ET-1 may play a role in the development of neonatal pHTN (247). Consistent with this possibility, chronic ET_A receptor (ET-1 receptor present on VSM and endothelial cells) blockade prevents and partially reverses arterial remodeling occurring in neonatal rats with CH-induced pHTN (19).

In addition to the role of ET-1 to promote VSM proliferation to vascular remodeling observed in the setting of neonatal pHTN, it may also contribute to reductions in VSM apoptosis. In a study of neonatal rats with pHTN, ET-1 signaling can

limit apoptosis observed in the arterial wall of lungs from neonates with pHTN (140). Additionally, pulmonary arterial expression of Bax, a proapoptotic factor was decreased while that of Bcl-xL, an antiapoptotic factor, was increased in lung sections from neonatal rats with pHTN (140). These results suggests that, in addition to enhanced proliferation, ET-1 may inhibit apoptosis and promote vascular wall thickening.

Decreased Vascular Growth. In human infants with congenital heart disease and subsequent pHTN, the number of peripheral arteries is reduced (184, 213). In models of neonatal pHTN associated with CH such as bronchopulmonary dysplasia (BPD), reduced numbers of small arteries and an abnormal distribution of vessels within the distal lung have been described (60, 61). Neonates who survive neonatal pHTN seem to have a permanent decrease in the number of pulmonary arteries (61). Neonatal fawn-hooded rats show reduced vessel density and alveolar simplification when raised at a high altitude in Denver, CO (60). Housing of neonatal rats in hyperbaric chambers to bring the animals to barometric pressures occurring at sea-level increases vessel number and promotes alveolar septation and development (60).

Reduced signaling of vascular endothelial growth factor (VEGF) may contribute to reduced vascular growth in the setting of neonatal pHTN. VEGF is an important hormone that promotes development and growth of the pulmonary vasculature and airways (201, 261). The addition of a VEGF receptor inhibitor leads to a reduction in artery formation and a failure of alveolar septation in growing rats (61). Human infants and newborn baboons with BPD have reduced lung levels of VEGF and VEGF receptors (32, 170). Neonatal rats with pHTN also have reduced VEGF levels (173, 261) and intratracheal adenovirus-mediated VEGF gene therapy increased survival and promoted development of the lung vasculature and alveoli in these animals (261).

Vasoconstriction. In addition to the role of polycythemia, altered vascular structure, and decreased vascular growth described above, enhanced pulmonary vasoconstriction contributes to increased PVR in neonates with pHTN (88, 180, 250). Indeed, enhanced PVR occurring in animal models of neonatal pHTN can be significantly reduced if not fully alleviated by limiting mechanisms of enhanced pulmonary vasoconstriction (88, 180). In neonatal piglets exposed to CH, elevated PVR is sensitive to exogenous vasodilator treatment with papaverine (88). Additionally, acute inhibition of ROK with fasudil alleviates increased PVR observed in neonates with pHTN (180). In human neonates with pHTN, small precapillary arteries remain undilated after birth with VSM cells containing more contractile elements such as actin and myosin heavy chain isoforms (114, 119). This observation is also true in piglets with CH-induced neonatal pHTN (115). These findings in neonates with pHTN suggests that the VSM of pulmonary arteries is equipped to facilitate more robust VSM contraction and potentially enhanced pulmonary arterial vasoconstriction.

Mechanisms of Enhanced Vasoconstriction in Neonatal Pulmonary Hypertension

Both basal tone and enhanced pulmonary vasoconstrictor agonist sensitivity contribute to enhanced pulmonary vasoconstriction in neonates with pHTN (7, 157, 228, 250). A potential mechanism for enhanced basal tone involves the myogenic response. After birth, the fetal pulmonary myogenic response and subsequent vascular tone is limited by mechanisms of pulmonary vasodilation (53, 250) and, over time, the pulmonary circulation becomes a more passive vascular bed not classically associated with myogenic regulation (44). In newborn lambs fitted with an inflatable vascular occluder around the ductus arteriosus, acute compression of the ductus leads to NO-dependent pulmonary vasodilation (250). In lambs with pHTN however, occluder

inflation increases in PVR and limits pulmonary arterial blood flow in a manner consistent with the myogenic response (250).

Enhanced pulmonary vasoconstriction occurring in neonatal pHTN may also be attributed to increased activity of pulmonary vasoconstrictor agonists. Indeed, in human infants with pHTN, circulating levels of vasoconstrictor agonists such as ET-1 and thromboxane A₂ are increased (7, 8, 157, 228). Additionally, young children with congenital heart disease and pHTN demonstrate an imbalance in the biosynthesis of thromboxane A₂ and prostacyclin (both COX products) with greater urinary excretion of metabolic products of thromboxane A₂ and fewer of prostacyclin (7). The regulation of COX products appears to be important in the development of neonatal pHTN because babies born to mothers who report high NSAID (inhibitors of COX) use in pregnancy are at elevated risk of neonatal pHTN (171).

In animal models of neonatal pHTN, the pulmonary vasoconstrictor agonists 5-HT and thromboxane A₂ contribute to enhanced PVR (72, 90, 93). Acute inhibition of 5-HT signaling alleviates enhanced PVR in lambs with pHTN (72) and administration of an SSRI further increases PVR in these lambs (72) thereby demonstrating that 5-HT enhances basal pulmonary vascular tone, a response that enhances PVR and perhaps contributes to pHTN in neonates. Furthermore, acute inhibition of COX-2 (the enzyme responsible for thromboxane A₂ synthesis from AA) augmented AA-induced pulmonary vasodilation in isolated pulmonary arterial rings from piglets with CH-induced pHTN (90) suggesting that thromboxane A₂ potentiates pulmonary arterial vasoconstriction in neonates with pHTN. Taken together, these results suggest that vasoconstrictor agonists contribute to enhanced vasoconstriction in neonates with pHTN.

There are two potential pathways for enhanced basal tone and pulmonary vasoconstrictor reactivity. The first involves reduced production of vasodilatory compounds that allows fetal pulmonary arterial tone to be uncovered and persist into

antenatal life. The second potential mechanism involves enhanced VSM contractility that may contribute to an augmented myogenic response or augment the action of a vasoconstrictor agonist. In the literature, evidence to support the existence of both possibilities is present and discussed below.

Role of Endothelial Dysfunction. Endothelial production of vasodilatory factors, prostacyclin and NO is limited in the setting of neonatal pHTN (7, 238). In human infants with pHTN, urinary excretion of prostacyclin metabolites is decreased (7). In pulmonary arteries of newborn pigs with CH-induced pHTN, the production of prostacyclin is attenuated (90, 93). In newborn lambs with pHTN, intratracheal administration of prostacyclin decreased PVR and increased pulmonary arterial blood flow (221). Moreover, infusion of milrinone, an inhibitor of phosphodiesterase type 3 (an enzyme that limits prostacyclin-induced vasodilation), decreased baseline PVR and potentiated the therapeutic effect of intratracheal prostacyclin (221). These findings support a role for diminished prostacyclin-dependent pulmonary vasodilation to permit enhanced vasoconstriction in neonates with pHTN.

Limitations on NO-dependent pulmonary vasodilation may additionally contribute to diminished pulmonary vasodilation observed in neonates with pHTN. Indeed, decreased eNOS mRNA and protein expression, reduced production of endothelial-derived NO (EDNO), and impaired NO-dependent pulmonary vasodilation have all been documented in animal models of neonatal pHTN (11, 52, 89, 238). Endogenous inhibitors of eNOS, such as asymmetric dimethylarginine (ADMA) can reduce EDNO production (23, 264). In human infants with pHTN, urinary ADMA levels are elevated relative to healthy control infants (205, 264).

In addition to ADMA-dependent reductions in eNOS-derived NO, other factors that influence eNOS NO production are also altered in neonatal pHTN. Association of

eNOS with heat shock protein 90 (HSP-90) is correlated with increased NO production and decreased superoxide anion (O_2^-) generation (159, 254). Examination of transiently cultured pulmonary arterial endothelial cells from lambs with pHTN noted decreased HSP90-eNOS interaction relative to levels observed in cells from healthy neonates (254). In addition to lower levels of interaction, decreased eNOS-dependent NO production and elevated O_2^- generation was also observed (159, 254). This result suggests that in neonatal pHTN, eNOS association with HSP90 is decreased and eNOS switches from generation of NO to O_2^- , thus limiting NO levels.

To promote VSM relaxation, NO stimulates VSM soluble guanylyl cyclase (sGC)-dependent production of cyclic guanine monophosphate (cGMP) which in turn activates protein kinase G (PKG). PKG causes VSM relaxation in two ways, first by decreasing $[Ca^{2+}]_i$ in the VSM and by decreasing Ca^{2+} -sensitivity of the contractile apparatus (149). In fetal lambs with pHTN, cGMP content and protein expression of sGC is decreased (36, 281). Additionally, increased pulmonary levels of H_2O_2 in lambs with pHTN have been proposed to attenuate both sGC protein expression and activity in pulmonary arterial VSM (281). Increased expression PKG was observed in neonatal lambs with CH-induced pHTN, but activity of PKG was decreased in isolated pulmonary arteries from these lambs (101). These findings suggest that in addition to reduced NO production, the downstream signaling mechanisms that promote NO-induced VSM relaxation are also impaired in neonates with pHTN.

In addition to limitations on factors that promote NO-induced VSM relaxation, there is evidence for augmented levels of enzymes that limit the activity of NO. In studies of lambs with pHTN, protein expression PDE5 is increased (37). Activity of PDE5 in VSM is also increased and acute treatment with PDE5 inhibitors decreased baseline PVR in lambs with pHTN (116) suggesting that PDE5 may potentiate the

limitation on what small amount of NO-dependent pulmonary vasodilation is present in neonates with pHTN.

Decreased production of endogenous NO may also contribute to elevated ET-1 levels observed in neonates with pHTN. NO can limit ET-1 production by inhibition of preproET-1 transcription (151), possibly by decreasing NF- κ B activity, an important transcription factor for ET-1 production (229). ET-1 also regulates NO levels by downregulation of the expression of eNOS and thus may further potentiate ET-1-mediated pulmonary vasoconstriction (277). These studies suggest that reductions in NO not only limit pulmonary vasodilation, but also increase the amount of the contractile factor, ET-1 thereby promoting enhanced vasoconstriction in neonates with pHTN.

Role of Enhanced VSM Contractility. An additional contributor to enhanced pulmonary vasoconstriction in neonatal pHTN is an increase contractility of VSM, independent of changes in vasodilator levels. Potential contributors to enhanced VSM contractility in neonates with pHTN include enhanced Ca^{2+} signaling and increased myofilament Ca^{2+} sensitivity.

In studies of neonatal lambs with pHTN, acute treatment with the VDCC inhibitor nifedipine limits the pulmonary arterial myogenic response (250) suggesting that enhanced Ca^{2+} entry contributes to that response in neonates with pHTN. Nifedipine administration in isolated lungs from piglets exposed to CH leads to profound reductions in pulmonary arterial pressure with little effect in lungs from control piglets (125), suggesting that VDCC are important for elevated pulmonary arterial basal tone. Moreover, isolated pulmonary arterial rings from piglets exposed to CH demonstrate robust reactivity to the Ca^{2+} channel agonist, Bay K 8644, a response that is absent in rings from control neonates (125). Whole cell, voltage-dependent Ca^{2+} currents are also exaggerated in isolated VSM from piglets exposed to CH (125). Taken together, these

studies support a role for VDCC to mediate enhanced pulmonary arterial tone in neonates with pHTN.

One potential explanation for the increased role of VDCC in mediating enhanced VSM contractility can be found in the pathway for HPV. HPV can involve the hypoxia-dependent reduction of the open probability of voltage-gated potassium channels (K_v) present on VSM (282). Reduced K_v channel open probability following exposure to acute hypoxia can depolarize the VSM membrane (282) and activate VDCC leading to entry of extracellular Ca^{2+} (179) and Ca^{2+} -dependent pulmonary VSM contraction (1, 2, 179). K_v channel activity is reduced in neonatal lambs with pHTN in a manner that depends on O_2^- signaling (158). Additionally, O_2^- scavengers restore K_v channel function in isolated arteries from neonatal piglets with CH-induced pHTN (86). Taken together, these findings indicate that neonatal animals with pHTN demonstrate reduced K_v channel activity. This impairment of K_v channels may allow for increased VDCC-induced VSM contractility in neonates with pHTN.

In addition to VDCCs, Ca^{2+} influx through other Ca^{2+} permeable ion channels contributes to increased Ca^{2+} signaling in VSM of neonates with pHTN (203, 223). Enhanced contraction of isolated pulmonary arterial rings from lambs with either pre- or perinatal CH-induced pHTN, is sensitive to inhibitors of TRP channel family members (40, 108, 203). Enhanced protein expression of TRPC4 (203) and TRPC6 (223) may contribute to enhanced SOCE-dependent pulmonary arterial vasoconstriction in neonatal lambs with pHTN (203, 223).

In addition to altered Ca^{2+} -signaling, increased myofilament Ca^{2+} sensitization may contribute to enhanced vasoconstriction observed in neonates with pHTN. Classically, there are two pathways thought to influence myofilament Ca^{2+} sensitization. One pathway involves RhoA/ROK-dependent signaling and the other involves activity of PKC/CPI17. In animal models of neonatal pHTN, levels of activated RhoA are elevated

(105, 180). Increased ROK protein expression and activity is observed in adult and neonatal animals with pHTN, assessed by MYPT1 phosphorylation, a downstream phosphorylation target of ROK (101, 145, 180). These results suggest that increased RhoA/ROK signaling contributes to increased myofilament Ca^{2+} sensitivity due to ROK-dependent MYPT1 phosphorylation. Indeed, ROK contributes to enhanced PVR observed in neonates with pHTN and therapeutic inhibition of ROK normalizes PVR to that of control neonates (180, 250). These results demonstrate that ROK contributes to pHTN in neonates, perhaps through promotion of myofilament Ca^{2+} sensitization.

A separate pathway that promotes myofilament Ca^{2+} sensitization in the setting of pHTN involves PKC-dependent activation of CPI-17 that in turn inhibits activity of myosin light chain phosphatase (242). In studies of isolated lungs from healthy newborn piglets, PKC inhibition with chelerythrine limits ET-1-dependent pulmonary vasoconstrictor activity, but has no effect on baseline pulmonary arterial pressure (31). Moreover, PKC activation with either 1-oleyl-2-acetyl-sn-glycerol (OAG) or phorbol 12,13-dibutyrate (PDBu) leads to dose-dependent pulmonary vasoconstriction in lungs from control piglets (31). Lungs from piglets with CH-induced pHTN showed enhanced baseline pulmonary arterial pressure that was reduced after chelerythrine treatment, suggesting that CH-dependent increases in PVR, in part, depend on PKC signaling (31).

Role of ROS in Vascular Signaling and Pathology

Considering the role of ROK and PKC in mediating enhanced pulmonary vasoconstriction occurring in the setting of neonatal pHTN, it is important to consider how their activity may be stimulated. In models of pHTN in adult animals, both ROK and PKC activity is stimulated by signaling involving reactive oxygen species (ROS) (145, 242). ROS-dependent activation of ROK and PKC signaling may also be occurring in neonates with pHTN. In transiently cultured pulmonary arterial endothelial cells from

neonatal piglets with pHTN, activation of Rac1, an enzymatic subunit of NADPH oxidase (NOX) important for NOX-derived superoxide O_2^- production (55), leads to downstream activation of RhoA/ROK signaling (284).

Several PKC isoforms are activated by oxidant stress (16, 54, 155) and contribute to VSM contraction by increasing $[Ca^{2+}]_i$ (193) and enhancing Ca^{2+} sensitivity of the myofilament contractile apparatus (172, 186, 194). Studies from adult rats with pHTN induced by exposure to intermittent hypoxia, demonstrate augmented agonist-dependent pulmonary vasoconstrictor reactivity through PKC_{β} -mediated VSM Ca^{2+} sensitization (242). PKC also contributes to enhanced pulmonary vasoconstriction and mediates the vasoconstrictor activity of ET-1 in the hypertensive neonatal pulmonary circulation of piglets (31). Taken together, these results suggest that oxidant-dependent activation of Ca^{2+} sensitization pathways involving ROK and PKC contribute to enhanced pulmonary vasoconstriction observed in neonatal pHTN.

Considering the oxidant-dependent stimulation of ROK and PKC dependent Ca^{2+} sensitization, it is interesting to note that in many animal models of neonatal pHTN, enhanced pulmonary levels of ROS are documented from various subcellular sources (9, 111, 144). In newborn rats with pHTN, lung levels of lipid peroxidation products such as 8-isoprostane are elevated and stimulate enhanced pulmonary vascular ET-1 production (144). In newborn lamb models of neonatal pHTN, O_2^- levels in pulmonary vessels are increased (9, 111). In addition to promotion of enhanced VSM contractility through activation of ROK and PKC signaling, increased lung levels of ROS limit the bioavailability of NO due to scavenging and can thus potentiate vasoconstriction (43, 142). Intratracheal treatment of pulmonary hypertensive lambs with recombinant human SOD reduces the high levels of pulmonary ROS observed in these animals, increases eNOS protein expression, and restores NO-dependent pulmonary arterial vasorelaxation (82). This result suggests that lung production of ROS may contribute to enhanced PVR

observed in neonates with pHTN through promotion of enhanced VSM contractility and reductions in NO-dependent pulmonary vasodilation.

Sources of Cellular ROS

A variety of subcellular sources for ROS may contribute to the enhanced level observed in the pulmonary vasculature of neonates with pHTN. Those sources include NOX (43, 92, 111), xanthine oxidase (142), uncoupled eNOS (eNOS-dependent O_2^- production instead of NO) (163), and the mitochondria (9, 234).

The mitochondria are a major source of cellular ROS in VSM, but seldom investigated in the setting of neonatal pHTN (177). MitoROS have been implicated in the development of pulmonary arterial hypertension in adult rats (22). Additionally, lung levels of mitoROS are elevated in neonatal sheep with pHTN (9, 83, 234, 280). Potential stimuli for mitoROS generation in neonates with pHTN includes stretch of VSM cells (278), as might occur in pulmonary arteries of neonates with increased luminal pressures and pHTN (6). Transiently cultured PASMC from lambs with pHTN and healthy controls demonstrated that cyclic stretching increased mitochondrial complex III-dependent production of O_2^- (278). Signaling leading to mitoROS production in the pulmonary circulation has been attributed to uncoupled eNOS (234, 255), reduced SOD2 (the SOD isoform present in the mitochondria) levels (9), and PKC (222). Increased mitoROS levels can lead to diminished NO-dependent pulmonary vasodilation by uncoupling eNOS (234) and promoting the activity of PDE5 (83).

Mitochondrial dysfunction occurring in a number of disease states such as ischemia-reperfusion injury (270), atherosclerosis (210), diabetic cardiomyopathy (244), and multiple sclerosis (252) depends on upstream signaling of PKC_β . PKC_β is an isoform of PKC activated by $[Ca^{2+}]_i$ (268) and cellular redox state (106). Despite the link between PKC_β and mitoROS indicated in a variety of disease states and cell types, the

contribution of PKC β to mitoROS generation in the neonatal pulmonary circulation has not yet been studied.

It is evident that enhanced vasoconstriction plays an important role in mediating pHTN in neonates and reduced NO-dependent vasodilation and enhanced VSM contractility contribute to that enhanced vasoconstriction. However, the relative contribution of both to augmented pulmonary vasoconstriction is unclear. Therefore, the series of experiments described in this dissertation were designed to examine the roles of diminished NO-dependent vasodilation and enhanced VSM contractility as well as the potential contributory signaling mechanisms occurring in neonatal CH-induced pHTN.

Rationale and Specific Aims

Neonatal pHTN affects 1-2 of every 1000 live births in the United States with 20% of cases being fatal. Surviving patients face significant morbidity associated with detriments to neural development and chronic lung disease (5, 246). CH is implicated in the pathogenesis of pHTN (14, 120) and thought to elevate postnatal PVR by increasing basal arterial tone and by augmenting reactivity to endogenous vasoconstrictor agonists (15, 40, 233). Enhanced pulmonary vasoconstriction following neonatal CH may result from a limitation on the production of endothelium-derived relaxing factors or increases in VSM contractility (19, 87, 89, 114, 119, 129). To investigate the relative contribution of each, we first investigated mechanisms of reduced endothelium-dependent pulmonary vasodilation and the contribution of those mechanisms to enhanced vasoconstriction occurring in neonatal rats with pHTN. ROS mediate reduced NO-dependent vasodilation in neonates with pHTN (43, 234) and promote VSM contraction by increasing $[Ca^{2+}]_i$ (51) and enhancing myofilament Ca^{2+} sensitivity (51, 145, 156). Therefore, we next sought to evaluate the role of ROS in enhanced pulmonary vasoconstriction occurring in neonates with CH-induced pHTN. Finally, PKC_{β} contributes to augmented pulmonary arterial vasoconstriction in adult rodents with pHTN (242) and leads to mitoROS production in a variety of disorders (210, 244, 252, 270). Understanding that mitoROS contribute to neonatal pHTN (9, 10, 83), we set out to investigate the role of PKC_{β} in stimulating mitoROS production in neonatal PASMC and the contribution of this signaling pathway to enhanced pulmonary vasoconstriction in neonates with CH-induced pHTN.

Specific Aim 1: Determine the contribution of endothelial dysfunction to enhanced basal tone and agonist-induced vasoconstriction in neonatal pHTN.

Hypothesis: Neonatal CH increases basal tone and pulmonary vasoconstrictor sensitivity by attenuating NO-dependent pulmonary vasodilation.

1. Assess effects of neonatal CH on regulation of basal tone and agonist-induced vasoconstriction by endogenous NO.
2. Evaluate the effect of CH on NO-dependent pulmonary vasodilation in neonatal rats.
3. Assess the effect of neonatal CH on expression of pulmonary NOS.

Approach

To test our hypothesis, we used an isolated (*in situ*) perfused lung preparation to evaluate the contribution of endogenous NO to basal and agonist-induced pulmonary vasoconstriction in normoxic and CH neonatal (2 week old) rats. Additional experiments examined vasodilatory responsiveness to the receptor-mediated, NO-dependent pulmonary vasodilator arginine vasopressin (AVP), and assessed pulmonary eNOS expression and phosphorylation status by western blotting. Our findings demonstrate a novel effect of neonatal CH to augment NO-dependent vasodilation and to increase pulmonary eNOS expression and phosphorylation at activation residue Ser-1177, responses that lessen enhanced basal tone and vasoconstrictor sensitivity to the thromboxane analog U-46619.

Specific Aim 2: Establish the contribution of ROS to enhanced vasoconstriction in CH-induced neonatal pHTN.

Hypothesis: MitoROS contribute to enhanced pulmonary arterial vasoconstriction in neonates with CH-induced pHTN.

1. Determine the contribution of ROS to enhanced basal tone and vasoconstrictor reactivity following CH in isolated (*in situ*) lungs.
2. Evaluate the role of mitoROS in enhanced pulmonary arterial vasoconstriction following CH in isolated pulmonary arteries.

Approach

To evaluate the hypothesis, we used scavengers of cytosolic ROS in an isolated (*in situ*) lung preparation to investigate the role of pulmonary ROS to mediate enhanced basal tone and agonist-induced vasoconstriction observed in neonates exposed to CH. In addition to *in situ* lung experiments, this study included protocols featuring isolated, pressurized small pulmonary arteries (100-200 μm) from control and CH neonates to evaluate the contribution of mitoROS to arterial tone. We observed that ROS contribute to enhanced basal tone and agonist-induced vasoconstriction in lungs from neonatal rats with pHTN. We additionally found that mitoROS contribute to enhanced tone in isolated pulmonary arteries from CH-exposed neonates. Our findings demonstrate that mitoROS contribute to enhanced pulmonary arterial tone in neonates with CH-induced pHTN.

Specific Aim 3: Define the contribution of PKC β -signaling to enhanced vascular reactivity following CH exposure in neonates.

Hypothesis: PKC β -dependent mitoROS generation in pulmonary vascular smooth muscle contributes to enhanced pulmonary arterial tone following neonatal CH.

1. Determine the contribution of PKC β to CH-dependent increases in vasoconstriction in both isolated (*in situ*) lungs and isolated pulmonary arteries.
2. Determine the effect of PKC β stimulation on mitoROS generation in neonatal pulmonary VSM.
3. Assess the effect of neonatal CH on PKC β expression.

Approach

To address our hypothesis, we used both isolated (*in situ*) lungs and pulmonary arteries to study the contribution of PKC β to enhanced pulmonary vasoconstriction observed in neonates following CH exposure. Additional studies examined PKC β -dependent mitoROS generation in transiently cultured PASMCs from control neonates and assessed PKC β protein expression using western blotting. Our findings suggest that neonatal CH leads to PKC β -dependent increases in pulmonary arterial tone and increased agonist-induced vasoconstriction without increasing PKC β protein expression. Additionally, PKC β promotes mitoROS production in pulmonary VSM of neonatal rats, a response that may contribute to PKC β -dependent enhancement of pulmonary vasoconstriction following CH exposure.

CHAPTER 2 – METHODS

General Methods

All protocols used in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of New Mexico Health Sciences Center.

Experimental Groups and Hypoxic Exposure Protocol

Timed-pregnant Sprague-Dawley (Harlan Industries) rats were allowed to acclimate for 1 wk before giving birth at ambient normobaric pressure (~630 mmHg in Albuquerque, NM). Litters selected for exposure to CH were housed in a hypobaric hypoxic chamber with barometric pressure maintained at 380 ± 5 mmHg for 12 days [beginning on postnatal day 2 of life]. Age-matched normoxic controls were housed in similar sham conditions at ambient barometric pressure. Neonates were housed with their birthing dam. The chamber was opened two times per wk to provide animals with food, water, and clean bedding. Animals were housed on a 12:12 hour light-dark cycle.

Assessment of Right Ventricular Systolic Pressure (RVSP), Right Ventricular Weight, and Hematocrit

RVSP and RV hypertrophy were evaluated as indices of pHTN (33, 248). Rats from each group were anesthetized with ketamine (40 mg/kg) and xylazine (6 mg/kg) (180). An upper transverse laparotomy was performed, and RVSP was assessed using a closed-chest transdiaphragmatic approach with a 25-gauge needle attached to a pressure transducer (Hugo Sachs Elektronik-Harvard Apparatus). Entry into the right ventricle (RV) was confirmed by monitoring the pressure waveform. RVSP and heart rate were obtained using AT-CODAS data-acquisition software (Dataq Instruments). Fulton's index, expressed as the percent ratio of RV to left ventricle and interventricular

septum (LV+S) weight was used to assess the degree of CH-induced RV hypertrophy. The polycythemic response to CH was determined by measuring hematocrit (Hct) from blood samples taken by direct cardiac puncture at the time of lung isolation.

Isolated (in situ) Perfused Rat Lung Preparation

To evaluate the effect of neonatal CH on baseline as well as agonist-dependent changes in PVR, neonatal rats were anesthetized with pentobarbital sodium (200 mg/kg, i.p.). The animal was placed on a heating block, the trachea was cannulated and lungs were ventilated with a positive-pressure mouse ventilator (Hugo Sachs Elektronik-Harvard Apparatus) at a tidal volume of 5 ml/kg body wt. and a rate of 100 breaths/min with a warmed and humidified gas mixture (21% O₂/6% CO₂/balance N₂). End-expiratory pressure was maintained at 3 cm H₂O. After removal of the frontal ribcage, heparin (5 U, 50 µl) was injected into the RV and the pulmonary artery was cannulated with custom fitted PE-90 tubing (Becton-Dickinson). The preparation was immediately perfused with a peristaltic pump (Ismatec) at a rate of 0.1 ml/min with a physiological saline solution (PSS) containing in mM: 129.8 NaCl, 5.4 KCl, 0.83 MgSO₄, 19 NaHCO₃, 1.8 CaCl₂, and 5.5 glucose with 4% (wt/vol) bovine serum albumin (BSA) (all from Sigma). Following cannulation of the pulmonary artery, the left ventricle was cannulated with custom fitted PE-160 tubing. Perfusion rate (Q) was gradually increased to a maximum of 15 ml/min·kg body wt. Perfusion was non-recirculating until the perfusate exiting the lung was nearly free of blood. Recirculation was then initiated and maintained for the duration of the experiment. Total recirculating volume was ~3.5 ml. Following a 20 min equilibration period, an effluent sample of PSS was collected for determination of pH, Pco₂, and Po₂ using a handheld iSTAT 1 blood gas analyzer (Abbott) and iSTAT test cartridges (G3+, Abbott). Lungs were kept in zone 3 conditions by maintaining venous pressure at 3 mmHg and airway pressure at 1 mmHg (53).

Pulmonary arterial (P_a) and venous (P_v) pressures were recorded continuously. Pressures were measured using a P-75 pressure transducer (Hugo Sachs Elektronik-Harvard Apparatus) connected to the pulmonary arterial and venous lines. Data were recorded and processed using data acquisition software and hardware (AT-CODAS, Dataq Instruments). Total PVR was calculated as $(P_a - P_v)/Q$.

Assessment of Segmental Vascular Resistance in Isolated (in situ) Rat Lungs

Effects of neonatal CH on basal and agonist-induced changes in arterial and venous resistance were determined using a double occlusion method as previously reported (80, 226). Briefly, lungs were isolated as described above and inflow and outflow lines were simultaneously occluded, allowing arterial and venous pressures to rapidly equilibrate to an approximation of microvascular capillary pressure (P_c). Pulmonary arterial resistance was calculated using the formula, $(P_a - P_c)/Q$, and pulmonary venous resistance was calculated using, $(P_c - P_v)/Q$.

Western Blot in Whole Lung Homogenates and Isolated Pulmonary Arteries

Western blots were performed as previously reported (52). Whole lungs collected from neonates on post-natal day 14 (P14) were snap-frozen in liquid nitrogen and stored at -80°C . On the day of experimentation, frozen lungs were fragmented with a mortar and pestle pre-cooled in liquid nitrogen.

In experiments requiring investigation of arterial protein content, pulmonary arteries were collected from lungs of P14 neonatal pups and snap frozen in liquid nitrogen and stored at -80°C until assayed.

Frozen lung tissue or pulmonary arteries were added to a Dounce homogenizer containing ice-cold homogenization buffer [10 mM Tris·HCl (pH 7.4), 255 mM sucrose, 2 mM EDTA, 12 μM leupeptin, 4 μM pepstatin A, 1 μM aprotinin, 1% v/v phosphatase

inhibitor cocktail 3 (cantharidin, (-)-p-bromolevamisole oxalate, and calyculin A) from Sigma]. Tissue homogenates were sonicated on ice with an ultrasonic homogenizer (Cole Parmer). Homogenates were then spun at 1500 x g for 10 min in a desktop centrifuge (Beckman) at 4°C. Sample protein concentrations were assessed using a NanoDrop (Thermo Fisher Scientific). Samples (15-45 µg/lane) were resolved by SDS-PAGE using 7.5% acrylamide gels along with molecular-weight standards (Bio-Rad). The separated proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad) and blocked for 1 hour at room temperature with 5% nonfat milk (Carnation) and 0.05% Tween-20 (Bio-Rad) in a Tris-buffered saline solution (TBS) containing 10 mM Tris·HCl and 50 mM NaCl (pH 7.5). Blots were then incubated overnight at 4°C with slight agitation in primary antibody. For each protein of interest, various primary antibodies were used and are indicated in specific methods for each aim of this study.

Blots were then probed with a horseradish peroxidase-conjugated goat secondary antibody raised against the animal of origin for the primary antibody (1:3000; Bio-Rad) in TTBS plus 5% nonfat milk to achieve immunochemical labeling. Chemiluminescence labeling was performed according to manufacturer instructions (ECL substrate, Thermo Scientific). Protein bands were detected using chemiluminescent-sensitive film and band intensity was quantified by densitometric analysis using ImageJ (NIH). To control for protein loading, all membranes were treated with Coomassie Brilliant Blue (Bio-Rad) to stain total protein. All bands were normalized to total protein content in the membrane as assessed by coomassie staining (208).

Calculations and Statistics

Data are expressed as mean ± standard error of the mean (SEM). Values of “n” indicate numbers of animals per experimental group. A t-test, one-way analysis of variance (ANOVA), or two-way ANOVA was used to make comparisons between groups

when appropriate. If differences were detected using ANOVA, individual groups were further evaluated using a Student-Newman-Keuls post-hoc comparison. Percentage data were arcsine transformed before statistical comparison. *P* values of <0.05 were considered significant.

Protocols for Specific Aims

Protocols for Specific Aim 1

Determine the contribution of endothelial dysfunction to enhanced basal tone and agonist-induced vasoconstriction in neonatal pHTN.

Protocol Series 1.1: Assess effects of neonatal CH on regulation of basal tone and agonist-induced vasoconstriction by endogenous NO.

To assess the role of endogenous NO to baseline PVR, lungs from each group were perfused with PSS containing either saline vehicle or the NOS inhibitor, N^ω-nitro-L-arginine (L-NNA; 300 μM). We have previously demonstrated that this dose of L-NNA effectively inhibits NO-dependent pulmonary vasodilation in isolated, perfused lungs from adult rats (230). After a 20 min equilibration period, P_c was assessed by double occlusion technique. The contribution of active tone to baseline PVR was assessed by administration of the NO donor, 1,3-propanediamine, N-{4-[1-(3-aminopropyl)-2-hydroxy-2-nitro-sohydrazino]butyl} (spermine NONOate; 100 μM) to maximally dilate the pulmonary vasculature. A second double occlusion was performed after stabilization of the response to spermine NONOate. Basal tone is expressed as the change in resistance to spermine NONOate.

The effect of endogenous NO to limit vasoconstrictor reactivity was determined by performing cumulative concentration-response curves to the thromboxane analog 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin F_{2α} (U-46619) in the presence and

absence of L-NNA. Double occlusions were performed to assess P_c under baseline conditions and following development of a stable pressor response to each concentration of U-46619.

Protocol Series 1.2: Evaluate the effect of CH on NO-dependent pulmonary vasodilation in neonatal rats.

Endothelium-Dependent Vasodilatory Responses. Receptor-mediated vasodilation to the endothelium-derived NO (EDNO)-dependent vasodilator, AVP (226), was measured in lungs from control and CH neonatal rats. After a 20 min equilibration period, lungs were precontracted with the thromboxane analog, U-46619 to achieve a stable arterial pressor response of ~ 10 mmHg. AVP (25 nM) was then added to the perfusate to induce vasodilation. Parallel protocols were performed in the presence of L-NNA to verify the contribution of endogenous NO to this response. Vasodilatory responses are expressed as a percent reversal of U-46619-mediated constriction.

Responses to Exogenous NO. Considering that EDNO-dependent responses following CH may be influenced by altered NO bioavailability or VSM sensitivity to NO, we assessed concentration-response curves to the NO donor, spermine NONOate, in lungs from each group of rats. After the equilibration period, lungs were precontracted with U-46619 to ~ 10 mmHg above baseline P_a . Once the constriction stabilized, a cumulative concentration-response curve to spermine NONOate was performed. Considering that lungs from neonates exposed to CH display greater basal pulmonary arterial constriction than control lungs, we repeated these experiments in lungs precontracted with U-46619 to achieve similar overall P_a between groups. All experiments were conducted in the presence of L-NNA to eliminate the contribution of

endogenous NO to these responses. Segmental vascular resistances were evaluated by double occlusion technique at baseline, after stabilization of the U-46619 constriction, and following stabilization of the response to each concentration of NONOate.

Protocol Series 1.3: Assess the effect of neonatal CH on expression of pulmonary NOS.

The effect of CH on the expression of NOS isoforms: NOS I (neuronal or nNOS), NOS II (inducible or iNOS), NOS III (endothelial or eNOS), as well as the post-translational phosphorylation of eNOS at serine-1177, a modification associated with increased eNOS-dependent NO production (75), were also investigated. Western blots were performed using whole lung homogenates from both control and CH neonates as outlined above in the general western blot protocol. Samples (45 µg protein/lane) were resolved by SDS-PAGE with 7.5% acrylamide along with molecular-weight standards (Bio-Rad). Antibodies used to probe for NOS isoforms included mouse monoclonal antibodies against nNOS (1:500; BD Biosciences-Transduction Labs), iNOS (1:500; BD Biosciences-Transduction Labs), eNOS (1:1000; BD Biosciences-Transduction Labs), and phospho(Ser-1177)-eNOS (1:1000; BD Biosciences-Transduction Labs). Protein expression was normalized to total protein to account for any variance in protein loading.

Protocols for Specific Aim 2

Establish the contribution of ROS to enhanced vasoconstriction in CH-induced neonatal pHTN.

Protocol Series 2.1: Determine the contribution of ROS to enhanced basal tone and vasoconstrictor reactivity following CH in isolated (*in situ*) lungs.

To evaluate the role of ROS in enhanced vasoconstriction following CH, lung studies similar to those in Protocol Series 1.1 were repeated in the presence of the

superoxide dismutase (SOD) mimetic, 1-oxy-2,2,6,6-tetramethyl-4-hydroxypiperidine (218) (TEMPOL; 1 mM). All lungs were pretreated with L-NNA (300 μ M) to avoid complications of endogenous NO on observed studies. Lungs from P14 neonates in both groups were allowed to equilibrate for 20 minutes, after which a baseline double occlusion was performed. The contribution of vascular tone to baseline PVR was assessed via administration of spermine NONOate. A double occlusion was performed after stabilization of the response to NONOate. Basal tone is expressed as the change in resistance to spermine NONOate.

To determine the effect of ROS on vasoconstrictor sensitivity of the pulmonary vasculature in control and CH pups, a cumulative dose-response curve to U-46619 (50, 100, 150, and 200 nM) was performed in the presence and absence of TEMPOL. A double occlusion was performed after a stabilized pressor response at each concentration.

Protocol Series 2.2: Evaluate the role of mitoROS in enhanced pulmonary arterial vasoconstriction following CH in isolated pulmonary arteries.

We assessed the role of ROS to mediate enhanced pulmonary arterial vasoconstrictor reactivity in pressurized small pulmonary arteries isolated from each group of animals (P14) as previously described (44, 197, 242). Pulmonary arteries [\sim 150-200 μ m inner diameter (i.d.)] from the left lung were dissected from surrounding parenchyma and cannulated on glass microcannulae secured in a vessel perfusion chamber (Living Systems Instrumentation). Cannulated, pressurized vessels were superfused with recirculating, heated (37°C) PSS bubbled with a 6% CO₂/10% O₂/balance N₂ gas mixture for a 20 min equilibration period. Vessel i.d. was simultaneously measured using bright-field videomicroscopy. Following a 20 min equilibration period, L-NNA (300 μ M) was added to the superfusate. In the presence of

L-NNA, the vessels developed vasomotor tone. Experiments were allowed to progress for a minimum of 20 min or until the vasoconstrictor response stabilized. Following stabilization of basal tone, vessels were superfused with non-recirculating Ca²⁺-free PSS that contained (in mM) 129.8 NaCl, 5.4 KCl, 0.83 MgSO₄, 19 NaHCO₃, 5.5 glucose, and 3 ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) for 30 min. Following a 30 min Ca²⁺-free PSS washout, recirculation was initiated and ionomycin [3 μM; Ca²⁺ ionophore (198, 243)] was added to maximally dilate the vessel. Basal tone was expressed as % of maximally dilated, Ca²⁺-free diameter using the formula: [(Ca²⁺-free i.d. – Constricted i.d.)/Ca²⁺-free i.d.]*100.

In isolated vessel studies conducted in the presence of ROS inhibitors, the inhibitor was present throughout the experiment – during the vessel dissection, cannulation, equilibration, and L-NNA exposure. Basal tone studies were performed in the presence and absence of the mitochondrial specific SOD mimetic, MitoTEMPO (74) (200 μM), MitoQ (67, 109, 241) (1 μM; restores electron flux through the electron transport chain to lower oxidative stress), or vehicle.

Protocols for Specific Aim 3

Define the contribution of PKC_β-signaling to enhanced vascular reactivity following CH exposure in neonates.

Protocol Series 3.1: Determine the contribution of PKC_β to CH-dependent increases in vasoconstriction.

Isolated (in situ) lung studies

To evaluate the role of PKC_β to mediate enhanced vasoconstriction following CH, lung studies similar to those in Protocol Series 2.1 were repeated in the presence of

either the general PKC inhibitor, Ro 31-8220 (242) (10 μM) or the selective PKC β inhibitor LY-333,531 (84, 146, 242) (10 nM). As in Protocol Series 2.1, all lungs were pretreated with L-NNA (300 μM). Following a 20 minute equilibration period, a baseline double occlusion was performed in all *in situ* lung preparations. The contribution of vascular tone to baseline PVR was assessed through administration of spermine NONOate. A double occlusion was performed after stabilization of the response to NONOate. Basal tone is expressed as the change in resistance to spermine NONOate.

To determine the contribution of PKC and PKC β to vasoconstrictor sensitivity of the pulmonary vasculature in neonates from both groups, a cumulative dose-response curve to U-46619 (50, 100, 150, and 200 nM) was performed in the presence and absence of either Ro 31-8220 (10 μM) or LY-333,531 (10 nM). A double occlusion was performed after a stabilized pressor response at each concentration.

Isolated pulmonary arteries

Parallel to Protocol Series 2.2, we assessed effects of PKC β inhibition with LY-333,531 on basal tone development in isolated, pressurized pulmonary arteries (~150-200 μm i.d.) collected from both control and CH neonates. Similar to Protocol Series 2.2, cannulated arteries were equilibrated in bubbled PSS for 20 min. Following a 20 min equilibration period, L-NNA (300 μM) was added to the superfusate. In the presence of L-NNA, the vessels developed vasomotor tone. Experiments were allowed to progress for a minimum of 20 min or until the vasoconstrictor response stabilized. Following stabilization of tone, vessels were superfused with non-recirculating Ca $^{2+}$ -free PSS for 30 min. Following a 30 min Ca $^{2+}$ -free PSS washout, recirculation was initiated and ionomycin (3 μM) was added to maximally dilate the vessel. Basal tone was expressed as % of maximally dilated, Ca $^{2+}$ -free diameter using the formula: $[(\text{Ca}^{2+}\text{-free i.d.} - \text{Constricted i.d.})/\text{Ca}^{2+}\text{-free i.d.}] * 100$.

To evaluate the contribution of PKC β to arterial tone development, isolated pulmonary arteries from both control and CH neonates were evaluated in the presence and absence of LY-333,531 (10 nM). When present, LY-333,531 was maintained throughout the experimental duration – during the vessel dissection, cannulation, equilibration, and L-NNA exposure.

Protocol Series 3.2: Determine the effect of PKC β stimulation on mitoROS generation in neonatal pulmonary VSM.

PKC-dependent increases in levels of mitochondrial ROS (mitoROS) were assessed in transiently cultured, primary PASMCs as previously described (208). Briefly, neonatal rats (P14) from control neonates were anesthetized with pentobarbital sodium (200 mg/kg i.p.) and the heart and lungs were exposed via midline thoracotomy. The left and right lungs were removed and placed in sterile 20 mM Ca $^{2+}$ Hank's buffered saline solution (HBSS) plus 1% (v/v) penicillin/streptomycin (pen/strep). Intrapulmonary arteries (~2nd-5th order) were dissected from surrounding lung parenchyma. To isolate PASMCs, isolated pulmonary arteries from both lungs were enzymatically digested in 20 mM Ca $^{2+}$ HBSS containing papain (9.5 U/ml), type-1 collagenase (1,750 U/ml), and dithiothreitol (1 mM) at 37°C for 15 min. Following enzymatic digestion, collected pulmonary arteries were added to a 70 μ m cell strainer and rinsed with 10 mL Ca $^{2+}$ -free HBSS. Pulmonary arteries were then added to a fresh Eppendorf tube containing Ca $^{2+}$ -free HBSS and dispersed with a Pasteur pipette. The cell suspension was then placed on gelatin-coated 25 mm glass coverslips and cultured in Ham's F-12 media supplemented with 5% fetal bovine serum (FBS) and 1% pen/strep for 48 hrs at 37°C.

Cultured PASMCs from control neonates were serum starved for 24 hours in Ham's F-12 media plus 1% FBS and 1% pen/strep prior to experimentation. Cells were then treated with PMA (10 μ M) to stimulate PKC-dependent mitoROS production.

Mitochondrial-derived O_2^- was detected with the mitochondrial specific O_2^- indicator MitoSOX (147) (5 μ M). Depending on the experimental protocol, cells were pre-treated with scavengers of cellular ROS, 1-oxy-2,2,6,6-tetramethyl-4-hydroxypiperidine (218) (TEMPO; 1 mM) and 4,5-Dihydroxy-1,3-benzenedisulfonic acid disodium salt (145) (Tiron, 10 mM). A selective scavenger of mitochondrial O_2^- was also used (2-(2,2,6,6-tetramethylpiperidin-1-oxy-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (mitoTEMPO, 200 μ M). ROS scavenger pre-treatment occurred for 20 minutes at 37°C and remained throughout the duration of MitoSOX treatment. Following 20 min pre-treatment, PSMCs were then exposed to MitoSOX for 30 min before being fixed with 4% paraformaldehyde (PFA). The coverslips containing MitoSOX labeled PSMCs were then secured to a glass slide using FluoroGel (Electron Microscopy Sciences) and imaged using confocal microscopy.

Fluorescence images were acquired with a x63 80% glycerol immersion objective on a confocal microscope (Leica, TCS SP5, 1.3 numerical aperture). MitoSOX was excited with a DPSS 561 laser and the emission processed through an Acousto-Optical Beam Splitter (AOBS, Leica) crystal set to an emission bandwidth of 570 nm – 620 nm. Images were obtained without an optical zoom (x-y pixel dimensions of 0.49 μ m x 0.49 μ m), all images were obtained using a 512 x 512 frame, and frame averaging of 2 scans. Scan speed was set to 400 Hz. Laser power was 60% for the DPSS 561 laser. Z-stacks were obtained at z-steps of 0.8 μ m. Detector gain and offset setting were held constant for all acquired z-stacks at a gain setting of 985 and an offset of -12 for the photomultiplier tube set to collect bandwidth of 570 nm – 620 nm.

Quantification of fluorescence was performed using SlideBook 6 (3i: Intelligent Imaging Innovations). MitoSOX fluorescence intensity is represented as an average fluorescence intensity using an inclusive threshold of 10,000 to 65,535 (maximum for 16 bit images). Each z-stack was thresholded to select for positive-stained areas with

fluorescence intensity values above background (cells not treated with MitoSOX). Data are reported as mean \pm SEM and n represents the number of individual animals that yielded cells for experimentation. Each individual n is an average of 2 technical replicates of cells from the same animal. To explore PKC β -dependent mitoROS production, we conducted studies parallel to Protocol Series 2.2, in the presence and absence of LY-333,531.

Protocol Series 3.3: Assess the effect of neonatal CH on PKC β expression.

Western blots were performed to evaluate protein expression of PKC β splice variants (PKC β I and PKC β II) protein expression in isolated pulmonary arteries from each group of rats (209, 269). The following primary antibodies were used: α -PKC β I (1:200, Santa Cruz) and α -PKC β II (1:200, Santa Cruz). Protein expression was normalized to total protein, as determined by staining with Coomassie, to account for variance in protein loading.

CHAPTER 3 – RESULTS

General

Evidence for pHTN and Right Ventricular Hypertrophy in Neonatal CH Rats

CH neonates developed higher RVSP compared to normoxic control rats (Figure 2A, B). Heart rate was not significantly different between groups (**Table 1**). Consistent with elevated RVSP, the ratio of RV to LV+S weight was increased by CH exposure, demonstrating RV hypertrophy indicative of pHTN (**Figure 2C**). Similar results were obtained for RV weight normalized to either body weight or total heart weight (**Table 1**). CH also increased LV+S weight normalized to body mass, which may reflect a lower body weight of CH neonates compared to age-matched controls (**Table 1**). Hematocrit was greater in rats exposed to CH compared to controls, reflecting the polycythemic response to CH (**Table 1**).

Saline gases in isolated (in situ) lung experiments

Effluent collected from both control and CH rats and analyzed by iStat demonstrated that pH, P_{CO_2} , and P_{O_2} did not differ between groups (**Table 2**).

Specific Aim 1

Determine the contribution of endothelial dysfunction to enhanced basal tone and agonist-induced vasoconstriction in neonatal pHTN.

Hypothesis:

We hypothesized that neonatal CH enhances basal tone and pulmonary vasoconstrictor sensitivity by limiting NO-dependent pulmonary vasodilation.

Figure 2

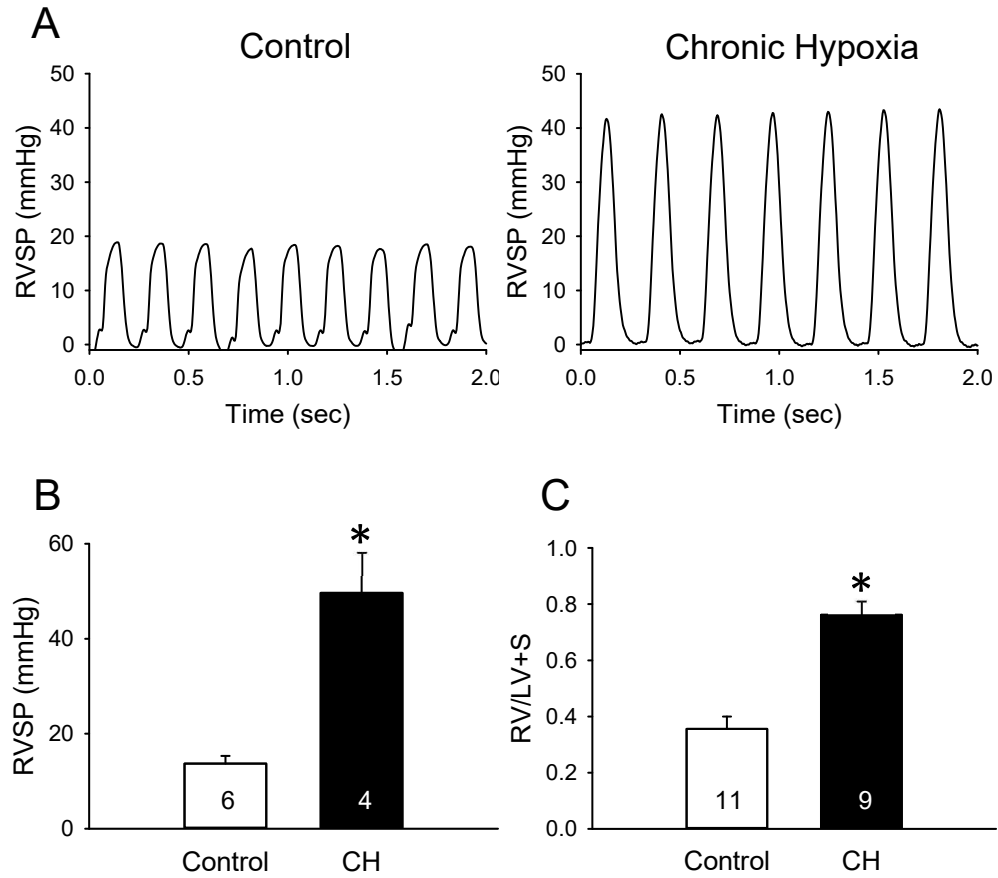


Figure 2. Chronic hypoxia (CH) increases peak right ventricular systolic pressure (RVSP) and induces RV hypertrophy in neonatal rats. (A) Sample pressure traces and (B) Summary data for peak RVSP in control and CH neonates. (C) Ratio of right ventricular (RV) mass to left ventricular plus septal (LV+S) mass for each group. Values are means \pm SEM; n = 4-11/group (indicated in bars); *p<0.05 vs. control, analyzed by unpaired t-test.

Table 1

	Control	CH
Final BW (g)	26.2 ± 0.5 (17)	20.0 ± 0.8 (9)*
RV/BW (mg/g)	1.12 ± 0.05 (11)	4.40 ± 0.39 (9)*
LV+S/BW (mg/g)	3.15 ± 0.07 (11)	5.85 ± 0.25 (9)*
RV/Total (mg/mg)	0.26 ± 0.01 (11)	0.43 ± 0.02 (9)*
Hematocrit (%)	31 ± 1 (11)	37 ± 1 (9)*
Heart Rate (beats/min)	255 ± 11 (6)	301 ± 26 (4)

Table 1. Body weight, heart weights, hematocrit, and heart rate from control and CH neonatal rats. Values are means ± SEM; n = 4-11 animals/group (indicated in parentheses). *p<0.05 vs. control; analyzed by unpaired t-test. RV, right ventricle; BW, body weight; LV+S, left ventricle plus the interventricular septum; Total, total heart weight.

Table 2

	Control	CH
pH	7.47 ± 0.04 (11)	7.49 ± 0.03 (22)
pCO ₂ (mmHg)	18.93 ± 1.03 (11)	18.03 ± 0.78 (22)
pO ₂ (mmHg)	106.64 ± 2.14 (11)	105 ± 2.84 (22)

Table 2. Saline gas values from recirculating perfusate of lungs from control and CH neonates. Perfusate samples were taken following a 20 min equilibration period. Values are mean ± SEM with n in parenthesis; n = 11-22/group; analyzed by t-test, no significant differences between groups.

Endogenous NO Limits CH-Dependent Increases in Basal Pulmonary Vascular Resistance

In situ lungs from neonates exposed to CH displayed elevated total and arterial baseline vascular resistance compared to normoxic controls (**Figure 3A, B**). In contrast, venous resistance was unaltered by CH exposure (**Figure 3C**). L-NNA increased total, arterial and venous baseline PVR in lungs from CH neonates, while having no effect in control pups (**Figure 3**).

The contribution of basal tone to CH-dependent increases in PVR was assessed by evaluating changes in total and segmental resistances to the NO donor, spermine NONOate, in lungs from each group. CH increased basal tone (**Figure 4A**) in the absence of NOS inhibition. This effect of CH appeared to result from arterial constriction since changes in arterial resistance to spermine NONOate tended to be greater in lungs from CH rats compared to controls (**Figure 4B**), although this response did not reach statistical significance. NOS inhibition increased basal pulmonary vascular tone in CH rats (**Figure 4A**) by further augmenting arterial constriction (**Figure 4B**), while having no significant effect in control lungs. In contrast, venous tone was minimal and unaltered by either CH exposure or treatment with L-NNA (**Figure 4C**).

Endogenous NO Attenuates U-46619-Induced Pulmonary Vasoconstriction

In addition to effects of CH to increase basal tone (**Figure 4**), total, arterial and venous constrictor responses to U-46619 were greater in lungs from CH compared to normoxic neonates in the absence of NOS inhibition (**Figure 5**). L-NNA markedly augmented vasoconstrictor reactivity to U-46619 in lungs from CH rats, while having a more modest effect in control lungs. Enhanced vasoconstrictor sensitivity to U-46619 following CH was primarily a function of greater arterial constriction (**Figure 5B**), as venous responses to U-46619 were minimal (**Figure 5C**).

Figure 3

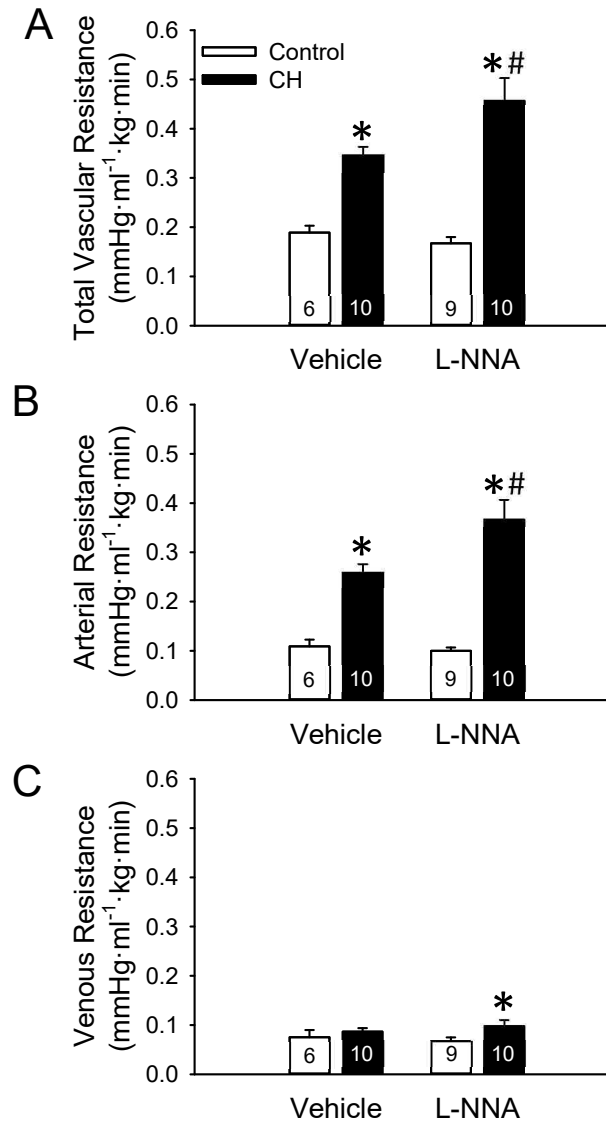


Figure 3. Endogenous NO limits CH-dependent increases in baseline pulmonary vascular resistance. (A) total, (B) arterial, and (C) venous baseline vascular resistances in lungs (in situ) from control and CH neonatal rats in the presence or absence of the NOS inhibitor L-NNA (300 μM). Values are means \pm SEM; n = 6-10/group (indicated in bars); * $p < 0.05$ vs. control, # $p < 0.05$ vs. vehicle, analyzed by two-way ANOVA followed by Student-Newman-Keuls post-hoc comparison.

Figure 4

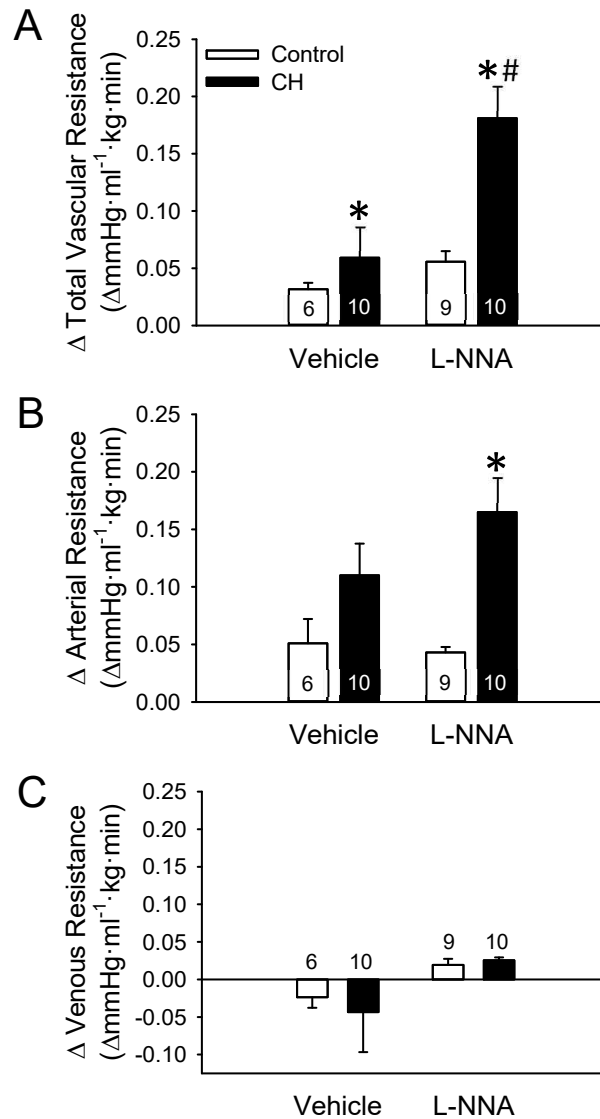


Figure 4. Endogenous NO limits CH-dependent increases in basal pulmonary arterial tone.

The contribution of basal tone to (A) total, (B) arterial and (C) venous resistance is expressed as the change in resistance to spermine NONOate (100 μM) in lungs (in situ) from control and CH neonates. Experiments were conducted in the presence or absence of L-NNA (300 μM). Values are means \pm SEM; n = 6-10/group (indicated in or above bars); *p<0.05 vs. control, #p<0.05 vs. vehicle, analyzed by two-way ANOVA followed by Student-Newman-Keuls post-hoc comparison.

Figure 5

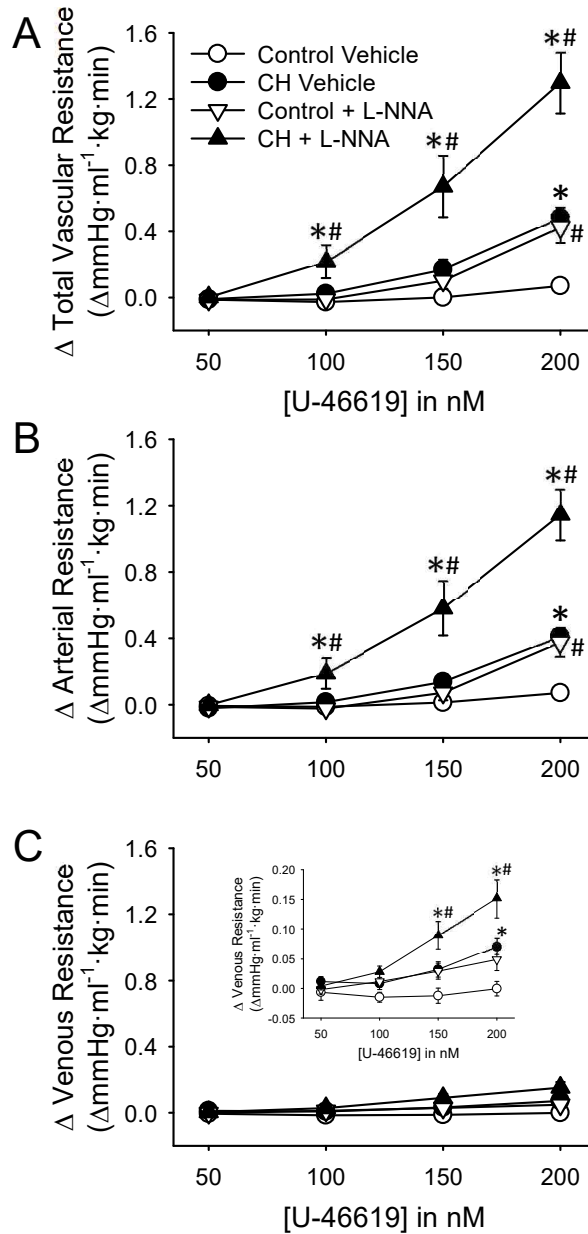


Figure 5. Endogenous NO limits CH-dependent increases in vasoconstrictor sensitivity to U-46619. Changes in (A) total, (B) arterial, and (C) venous resistance to U-46619 in lungs (in situ) from control and CH neonates. Experiments were conducted in the presence or absence of L-NNA (300 μ M). Values are means \pm SEM; n = 8 for Control Vehicle, n = 10 for CH Vehicle, n = 9 for Control L-NNA, n = 9 for CH L-NNA; *p<0.05 vs. control, #p<0.05 vs. vehicle; analyzed by two way ANOVA at each concentration of U-46619 followed by Student-Newman-Keuls post-hoc comparison.

CH Augments EDNO-Dependent Pulmonary Vasodilation

Lungs from control and CH neonates were precontracted with a concentration of U-46619 sufficient to achieve similar pressor responses between groups (**Table 3**) and then treated with AVP to dilate the pulmonary circulation. Vasodilatory responses to AVP were greatly augmented following exposure to CH (**Figure 6A**). L-NNA nearly abolished AVP-induced pulmonary vasodilation in lungs from each group demonstrating that this response depends on NOS-derived NO.

CH Does Not Alter Vasodilatory Responsiveness to Exogenous NO

In contrast to effects of CH to augment EDNO-dependent vasoreactivity (**Figure 6A**), vasodilatory responses to exogenous NO (spermine NONOate) in L-NNA pre-treated lungs were not different between lungs from control and CH neonatal rats (**Figure 6B**). Similar responses to spermine NONOate were observed whether U-46619 was added to achieve similar changes in PVR or total PVR between groups (**Table 4**).

CH Increases levels of Pulmonary eNOS as well as Serine-1177-Phosphorylated eNOS

Both total eNOS and the ratio of Ser-1177 phosphorylated eNOS to total eNOS were greater in lung samples from CH neonates compared to controls (**Figure 7**) as determined by western blotting. In contrast, CH had no significant effect on pulmonary nNOS or iNOS levels (**Figure 8**).

Table 3

	Control Vehicle	Control L-NNA	CH Vehicle	CH L-NNA
Baseline R	0.18 ± 0.01 (14)	0.22 ± 0.02 (6)	0.50 ± 0.03* (6)	0.70 ± 0.04*# (5)
Constricted R	0.75 ± 0.06 (14)	0.98 ± 0.13 (6)	1.35 ± 0.1* (6)	1.53 ± 0.16* (5)
ΔR to U-46619	0.57 ± 0.05 (14)	0.76 ± 0.13 (6)	0.84 ± 0.1 (6)	0.83 ± 0.16 (5)
ΔR to AVP	-0.17 ± 0.03 (14)	-0.05 ± 0.02 (6)#	-0.76 ± 0.08* (6)	-0.08 ± 0.01*# (5)

Table 3. Pulmonary vascular resistance values for lungs from control and CH neonates treated with AVP in the presence or absence of LNNA. When indicated, 300 μM L-NNA present throughout experiment. Units of resistance (R) are mmHg · ml⁻¹ · kg · min. Values are means ± SEM with n in parenthesis; n = 5-14/group; *p<0.05 vs. control, #p<0.05 vs. vehicle; analyzed by two way ANOVA, individual groups were further evaluated using a Student-Newman-Keuls post-hoc comparison.

Figure 6

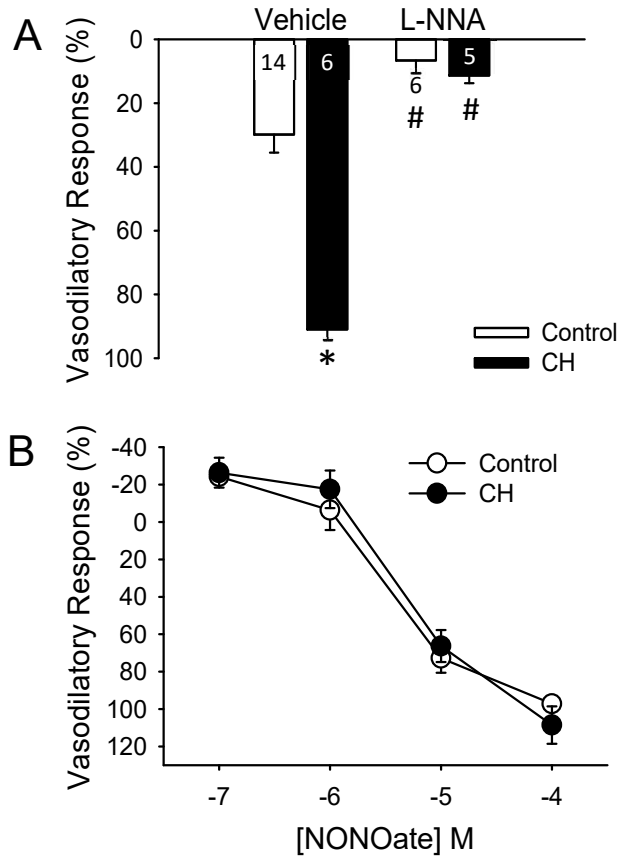


Figure 6. CH augments EDNO-dependent vasodilation to arginine vasopressin (AVP) without altering smooth muscle sensitivity to NO.

A. Total vasodilatory responses (percent reversal of U-46619-induced constriction) to AVP (25 nM) in lungs (in situ) from control and CH neonates. Experiments were conducted in the presence or absence of L-NNA (300 μ M). Values are means \pm SEM; n = 5-14/group (indicated in or below bars); *p<0.05 vs. control, #p<0.05 vs. vehicle, analyzed by two way ANOVA followed by Student-Newman-Keuls post-hoc comparison. **B.** Total vasodilatory responses (percent reversal of U-46619-induced constriction) to spermine NONOate. Experiments were conducted in the presence of L-NNA to limit the contribution of endogenous NO production. Values are means \pm SEM; n = 5 for Control, n = 5 for CH; analyzed by two way ANOVA.

Table 4

	U-46619 added to achieve similar change in PVR between groups		U-46619 added to achieve similar total PVR between groups	
	Control	CH	Control	CH
Baseline R	0.20 ± 0.03 (5)	0.49 ± 0.04* (5)	0.2 ± 0.01 (4)	0.49 ± 0.05* (4)
Constricted R	0.87 ± 0.07 (5)	1.26 ± 0.09* (5)	1.13 ± 0.93 (4)	1.33 ± 0.08 (4)
ΔR to U-46619	0.67 ± 0.06 (5)	0.77 ± 0.10 (5)	0.93 ± 0.08 (4)	0.84 ± 0.09 (4)
ΔR to 10 ⁻⁷ M NONOate	0.16 ± 0.04 (5)	0.22 ± 0.09 (5)	0.25 ± 0.03 (4)	0.26 ± 0.01 (4)
ΔR to 10 ⁻⁶ M NONOate	0.04 ± 0.11 (5)	0.16 ± 0.09 (5)	0.22 ± 0.07 (4)	0.21 ± 0.10 (4)
ΔR to 10 ⁻⁵ M NONOate	-0.53 ± 0.03 (5)	-0.53 ± 0.11 (5)	-0.55 ± 0.06 (4)	-0.63 ± 0.06 (4)
ΔR to 10 ⁻⁴ M NONOate	-0.65 ± 0.05 (5)	-0.86 ± 0.14 (5)	-0.88 ± 0.07 (4)	0.98 ± 0.08 (4)

Table 4. Pulmonary vascular resistance values for lungs from control and CH neonates treated with NONOate. 300 μM L-NNA present throughout experiment. Left side of table shows data from lungs with [U-46619] sufficient to raise baseline pulmonary arterial pressure by 10 mmHg in both groups. Right side of table shows data from lungs with [U-46619] to equalize baseline pulmonary arterial pressure between groups. Units of resistance (R) are mmHg · ml⁻¹ · kg · min. Values are means ± SEM with n in parenthesis; n = 5-14/group; *p<0.05 vs. control; analyzed by unpaired t-test.

Figure 7

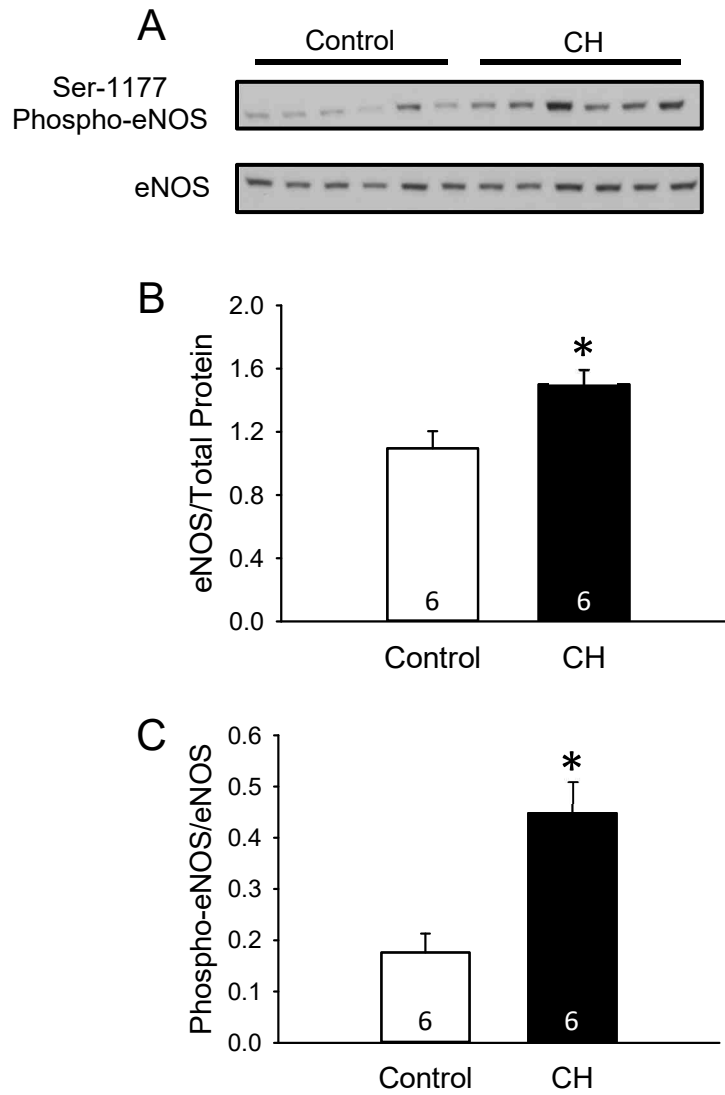


Figure 7. CH increases levels of eNOS and phosphorylated eNOS (Ser-1177) in neonatal rat lungs. (A) Western blots for eNOS and phospho-eNOS, and (B, C) summary data for total eNOS and the ratio of phosphorylated eNOS to total eNOS in lungs from control and CH neonatal rats. Values are means \pm SEM; $n = 6$ /group (indicated in bars); * $p < 0.05$ vs. control, analyzed by unpaired t-test.

Figure 8

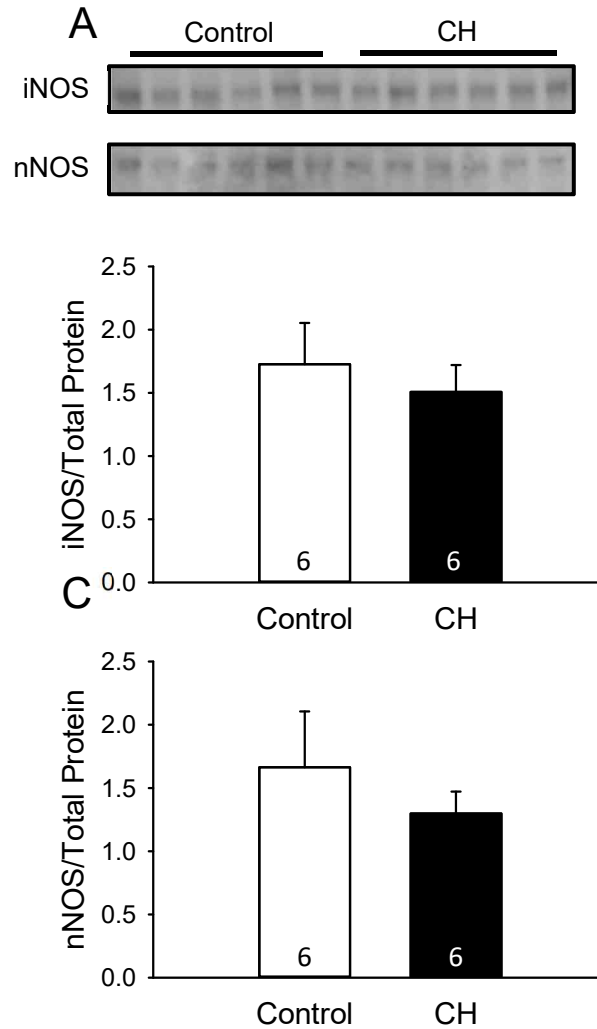


Figure 8. CH does not alter iNOS or nNOS protein expression in neonatal lungs. (A) Western blots and (B, C) summary data for total iNOS and nNOS in lungs from neonatal control and CH rats. n = 6/group (indicated in bars), analyzed by unpaired t-test.

Specific Aim 2

Establish the contribution of ROS to enhanced vasoconstriction in CH-induced neonatal pHTN.

Hypothesis:

We hypothesized that mitoROS contribute to enhanced basal and agonist-induced pulmonary arterial constriction in neonates with CH-induced pHTN.

ROS Facilitate Augmented Basal Pulmonary Arterial Tone Following CH

To eliminate the contribution of endogenous NO on observed pulmonary vascular tone, this study and all subsequent isolated (*in situ*) lung studies were done in the continued presence of L-NNA. CH-dependent increases in baseline PVR were fully relieved by TEMPOL (**Figure 9A**). Furthermore, TEMPOL normalized arterial resistance in lungs from both control and CH groups while neither CH nor TEMPOL altered venous resistance (**Figure 9B, C**). This result suggested that the CH-induced elevation in baseline PVR was due entirely to the effect of CH to increase arterial resistance.

To evaluate the contribution of basal tone to CH-dependent elevations in PVR, we assessed vasodilatory responses to the NO donor, spermine NONOate. Exposure of neonatal rats to CH lead to an elevation in basal tone, due to greater pulmonary arterial tone (**Figure 10A, B**). Pre-treatment with the SOD mimetic, TEMPOL prevented CH-dependent increases in basal tone and pulmonary vascular tone was normalized between control and CH neonates (**Figure 10A**). This finding suggested that augmented PVR observed following neonatal CH exposure was completely due to mechanisms of increased vasoconstriction.

Figure 9

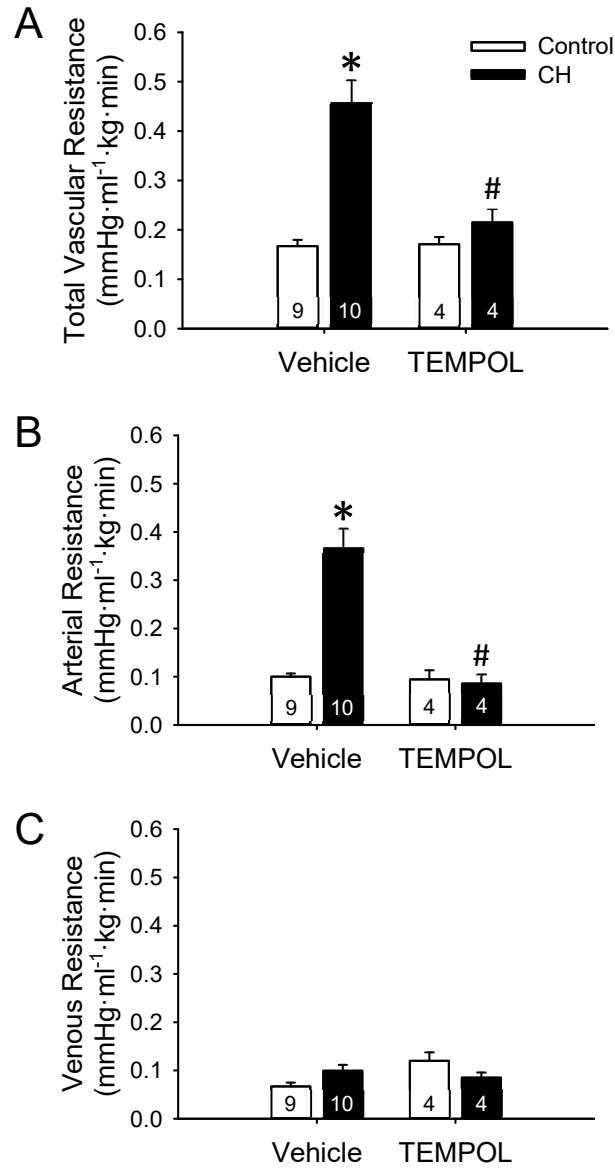


Figure 9. CH increases baseline pulmonary vascular resistance through ROS signaling. (A) total, (B) arterial, and (C) venous baseline vascular resistances in lungs (*in situ*) from control and CH neonatal rats in the presence or absence of the SOD mimetic TEMPOL (1 mM). Experiments were conducted in the presence of L-NNA (300 μ M). Values are means \pm SEM; n = 4-10/group (indicated in bars); *p<0.05 vs. control, #p<0.05 vs. vehicle, analyzed by two-way ANOVA followed by Student-Newman-Keuls post-hoc comparison.

Figure 10

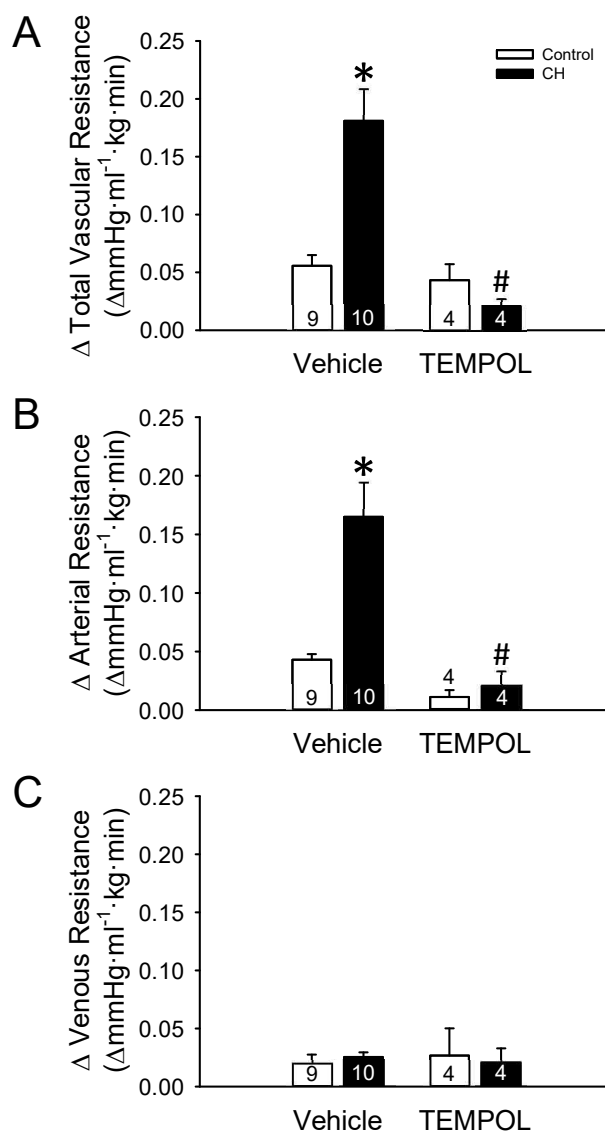


Figure 10. ROS contribute to augmented basal tone in lungs from neonatal rats exposed to CH. The contribution of basal tone to (A) total, (B) arterial and (C) venous resistance is expressed as the change in resistance to spermine NONOate (100 μ M) in lungs (in situ) from control and CH neonates. Experiments were conducted in the presence of L-NNA (300 μ M) with or without the ROS scavenger, TEMPOL (1 mM). Values are means \pm SEM; n = 4-10/group (indicated in or above bars); *p<0.05 vs. control, #p<0.05 vs. vehicle, analyzed by two way ANOVA with a Student-Newman-Keuls post-hoc test.

ROS Mediate Enhanced Vasoconstrictor Sensitivity to U-46619 Following CH

Exposure

U-46619 produced concentration-dependent increases in total PVR in lungs from both control and CH neonates (**Figure 11A**). This effect of U-46619 was largely a result of arterial constriction (Figure 11B), as venous responses were relatively small in lungs from both groups of rats (Figure 11C).

Vasoconstrictor reactivity to U-46619 was significantly augmented by CH (Figure 11A) Neonatal pHTN induced by exposure to CH elevates vasoreactivity to U46619 (Figure 11A). This response to CH was primarily a function of increased arterial reactivity to U-46619 (Figure 11B), although venoconstriction to U-46619 was also augmented by CH exposure (Figure 11C). TEMPOL attenuated total, arterial and venous responses to U-46619 in lungs from CH rats (Figure 11A, B, C). Although TEMPOL also tended to reduce vascular reactivity to U46619 in control lungs, this effect did not achieve statistical significance (Figure 11). These findings suggest that O_2^- contributes to elevated pulmonary vasoconstrictor sensitivity to U46619 following neonatal CH.

MitoROS Contribute to Enhanced Pulmonary Arterial Tone Following CH

Exposure to CH lead to greater pulmonary arterial tone in isolated, pressurized pulmonary arteries collected from control and CH neonates (Figure 12, 13, and 14). Pretreatment with either the mitochondrial selective O_2^- scavenger MitoTEMPO or the inhibitor of mitoROS production, MitoQ, eliminated the CH-dependent increase in arterial tone. There was no significant effect of either MitoTEMPO or MitoQ to alter tone in vessels from control neonates (Figures 13 and 14). Ca^{2+} -free inner and outer diameters for pulmonary arteries included in this study are listed in Tables 5 and 6. Taken together, these results support a major contribution of mitoROS to mediate enhanced pulmonary arterial tone in neonatal rats exposed to CH.

Figure 11

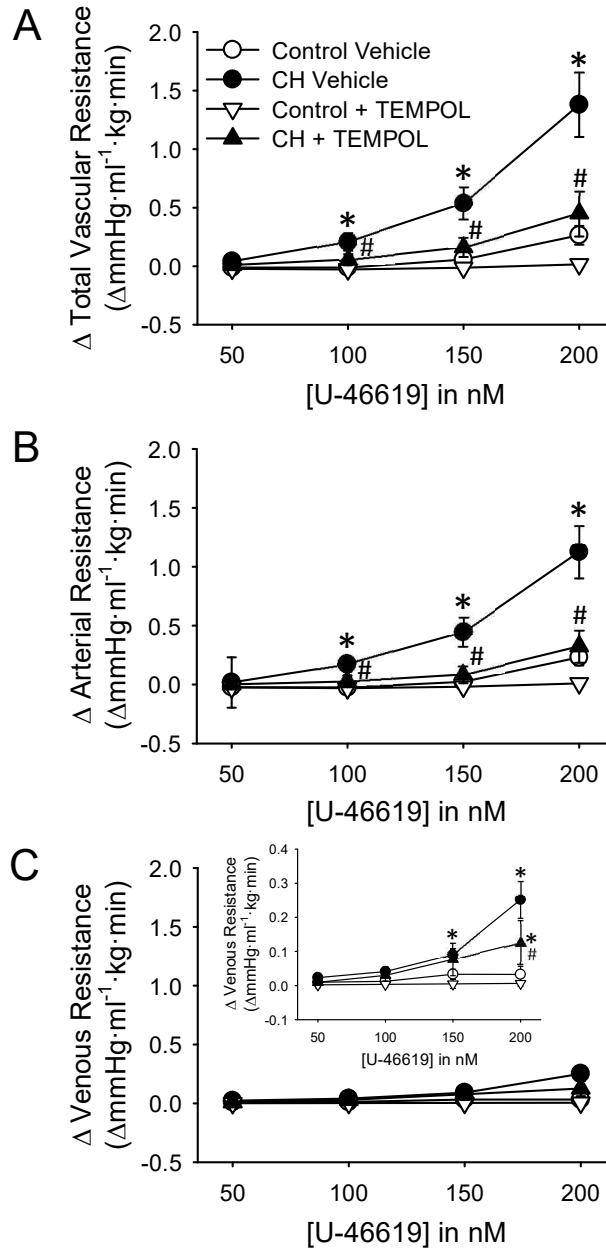


Figure 11. ROS facilitate enhanced vasoconstrictor sensitivity in lungs from CH neonates. Changes in (A) total, (B) arterial, and (C) venous resistance to U-46619 in lungs from control and CH neonates. Experiments were conducted in the continued presence of L-NNA (300 μM) with or without the ROS scavenger, TEMPOL (1 mM). Values are means ± SEM; n = 6 for Control Vehicle, n = 5 for CH Vehicle, n = 6 for Control TEMPOL, n = 4 for CH TEMPOL; *p < 0.05 vs. control, #p < 0.05 vs. vehicle; analyzed by two way ANOVA at each [U-46619] with a Student-Newman-Keuls post-hoc comparison.

Figure 12

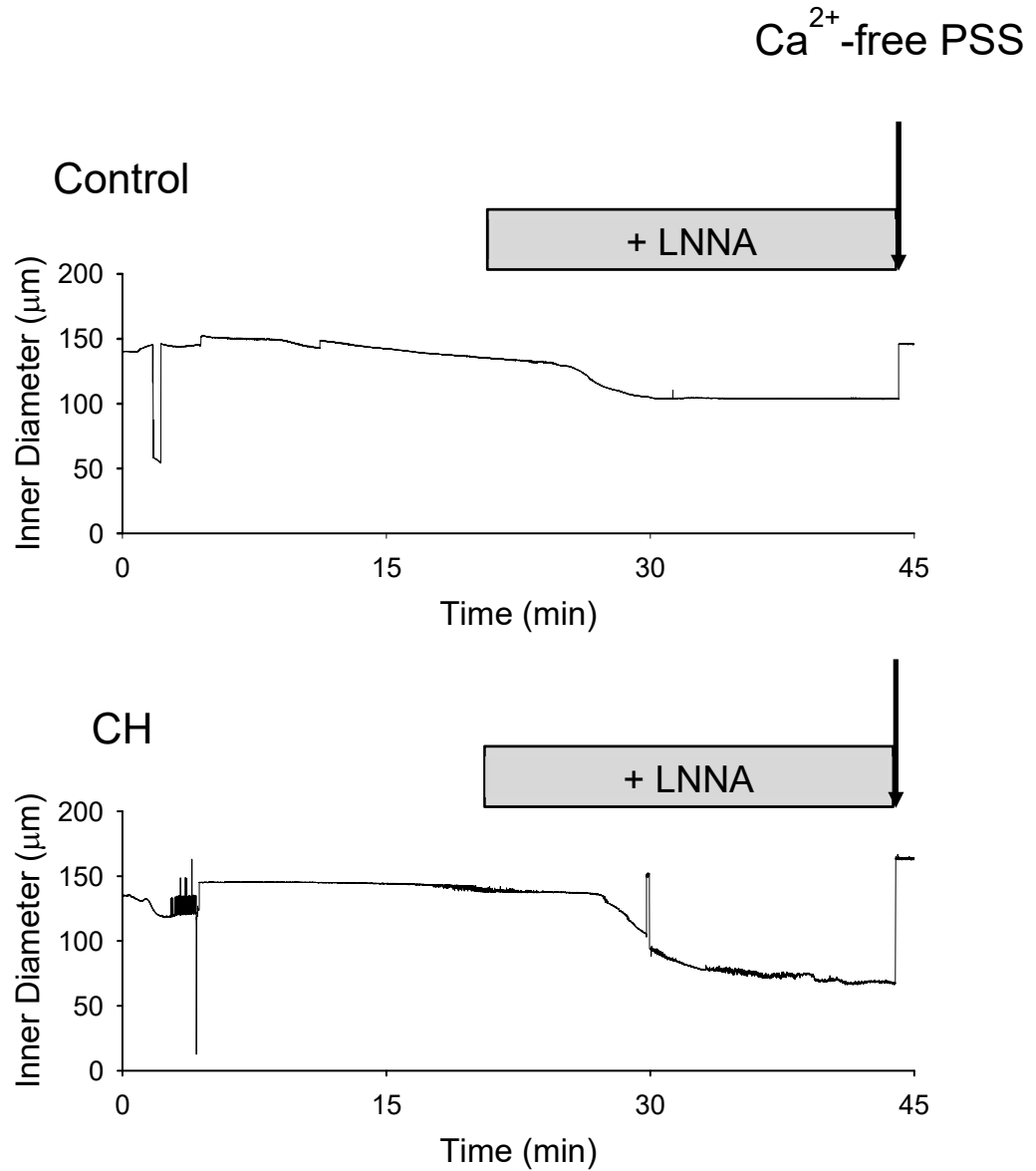


Figure 12. CH increases basal tone development in isolated, small pulmonary arteries from neonatal rats. Representative pressure traces from control and CH groups showing arterial tone development in the presence of LNNA (300 µM) following 20 min equilibration period. Arrow indicates pausing of data acquisition to allow for 30 min Ca^{2+} -free PSS washout followed by 30 min in Ca^{2+} -free PSS + ionomycin (3 µM).

Figure 13

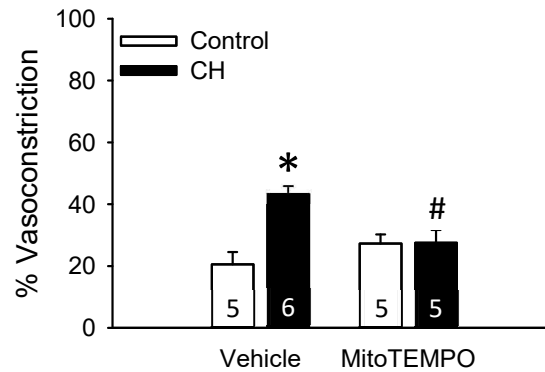


Figure 13. MitoROS contribute to increased basal tone isolated pulmonary arteries from CH neonates.

Vasoconstriction is % maximally dilated (Ca^{2+} -free) diameter, calculated using the formula: $[(\text{Ca}^{2+}$ -free i.d. - Constricted i.d.)/ Ca^{2+} -free i.d.]. Experiments were conducted in the continued presence of MitoTEMPO, (200 μM). The vessel was superfused with L-NNA (300 μM) following 20 min equilibration time. Values are means \pm SEM; n = 5-6/group (indicated in bars); * $p < 0.05$ vs. control, # $p < 0.05$ vs. vehicle, analyzed by two way ANOVA followed by Student-Newman-Keuls post-hoc test.

Figure 14

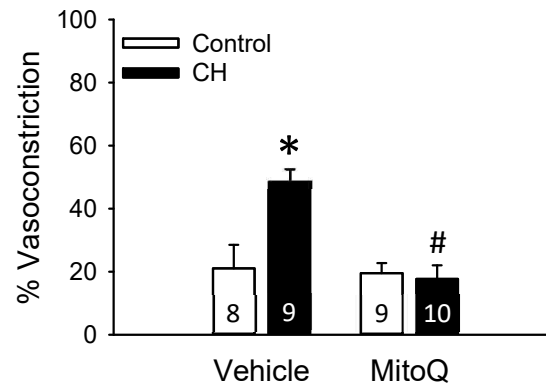


Figure 14. MitoROS contribute to increased basal tone in isolated pulmonary arteries from CH neonates.

Vasoconstriction is % maximally dilated (Ca^{2+} -free) Diameter, calculated using the formula: $[(\text{Ca}^{2+}$ -free i.d. - Constricted i.d.)/ Ca^{2+} -free i.d.]. Experiments were conducted in the continued presence of MitoQ, (1 μM , restores electron flux through the electron transport chain to lower oxidative stress). The vessel was superfused with L-NNA (300 μM) following 20 min equilibration time. Values are means \pm SEM; n = 8-10/group (indicated in bars); * $p < 0.05$ vs. control, # $p < 0.05$ vs. vehicle, analyzed by two way ANOVA followed by Student-Newman-Keuls post-hoc test.

Table 5

	Ca²⁺-Free Inner Diameter (μm)	Ca²⁺ Free Outer Diameter (μm)
Control Vehicle	191 ± 17 (5)	229 ± 15 (5)
CH Vehicle	180 ± 14 (6)	226 ± 15 (6)
Control MitoTEMPO	209 ± 12 (5)	262 ± 14 (5)
CH MitoTEMPO	181 ± 13 (5)	219 ± 8 (5)

Table 5. Ca²⁺-free diameters for isolated pulmonary arteries from both control and CH neonates ± MitoTEMPO. Values are mean ± SEM with n in parenthesis; n = 5-6/group; analyzed by two way ANOVA followed by Student-Newman-Keuls post-hoc test, no significant differences between groups.

Table 6

	Ca²⁺-Free Inner Diameter (μm)	Ca²⁺ Free Outer Diameter (μm)
Control Vehicle	183 ± 10 (8)	222 ± 9 (8)
CH Vehicle	167 ± 6 (9)	191 ± 4* (9)
Control MitoQ	180 ± 12 (9)	217 ± 12 (9)
CH MitoQ	162 ± 6 (10)	199 ± 9 (10)

Table 6. Ca²⁺-free diameters for isolated pulmonary arteries from both control and CH neonates ± MitoQ. Values are mean ± SEM with n in parenthesis; n = 8-10/group; *p<0.05 vs. control; analyzed by two way ANOVA followed by Student-Newman-Keuls post-hoc test.

Specific Aim 3

Define the contribution of PKC β -signaling to enhanced vascular reactivity following CH exposure in neonates.

Hypothesis:

PKC β -dependent mitoROS generation in pulmonary vascular smooth muscle contributes to enhanced pulmonary arterial tone following neonatal CH.

PKC β Contributes to Enhanced Pulmonary Arterial Vasoconstriction in Lungs from Neonates Exposed to CH

Pretreatment with PKC inhibitor Ro 31-8220 did not significantly reduce baseline PVR in lungs from CH neonates (Figure 15A), however PKC β inhibition did significantly lower augmented baseline PVR (Figure 17A). CH-dependent increases in arterial resistance were significantly limited by either PKC or PKC β inhibition (Figure 15B and 17B), suggesting that PKC and PKC β signaling contribute to increased pulmonary arterial resistance following CH exposure. Neither CH nor PKC inhibitors altered venous resistance (Figure 15C and 17C) demonstrating that changes in arterial resistance following CH exposure are responsible for elevated baseline PVR in neonates exposed to CH.

The general PKC inhibitor, Ro 31-8220, attenuated CH-dependent increases in basal pulmonary arterial tone (Figure 16) as well as U-46619-induced vasoconstriction (Figure 19). Ro 31-8220 limited CH-dependent increases in both basal and U-46619-dependent changes in arterial tone (Figures 16 and 19). Pretreatment with LY-333,531 significantly reduced pulmonary arterial tone and agonist-induced vasoconstriction to U-46619 in lungs from CH neonates (Figure 18 and 20). Neither PKC nor PKC β inhibition altered basal tone or pulmonary vasoreactivity to U-46619 in lungs from control neonates (Figure 16, 18, 19, and 20). Collectively, these findings suggest that PKC β

Figure 15

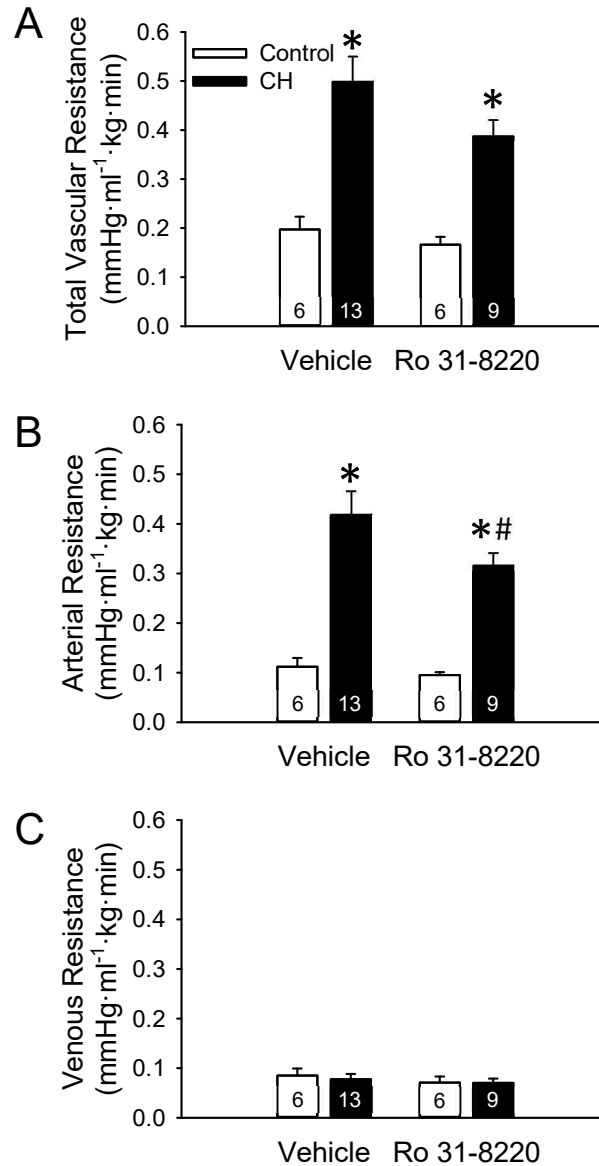


Figure 15. CH increases baseline pulmonary arterial resistance through PKC signaling. (A) total, (B) arterial, and (C) venous baseline vascular resistances in lungs (*in situ*) from control and CH neonatal rats in the presence or absence of the PKC inhibitor Ro 31-8220 (10 μ M). Experiments were conducted in the presence of L-NNA (300 μ M). Values are means \pm SEM; n = 6-13/group (indicated in bars); *p<0.05 vs. control, #p<0.05 vs. vehicle, analyzed by two-way ANOVA followed by Student-Newman-Keuls post-hoc comparison.

Figure 16

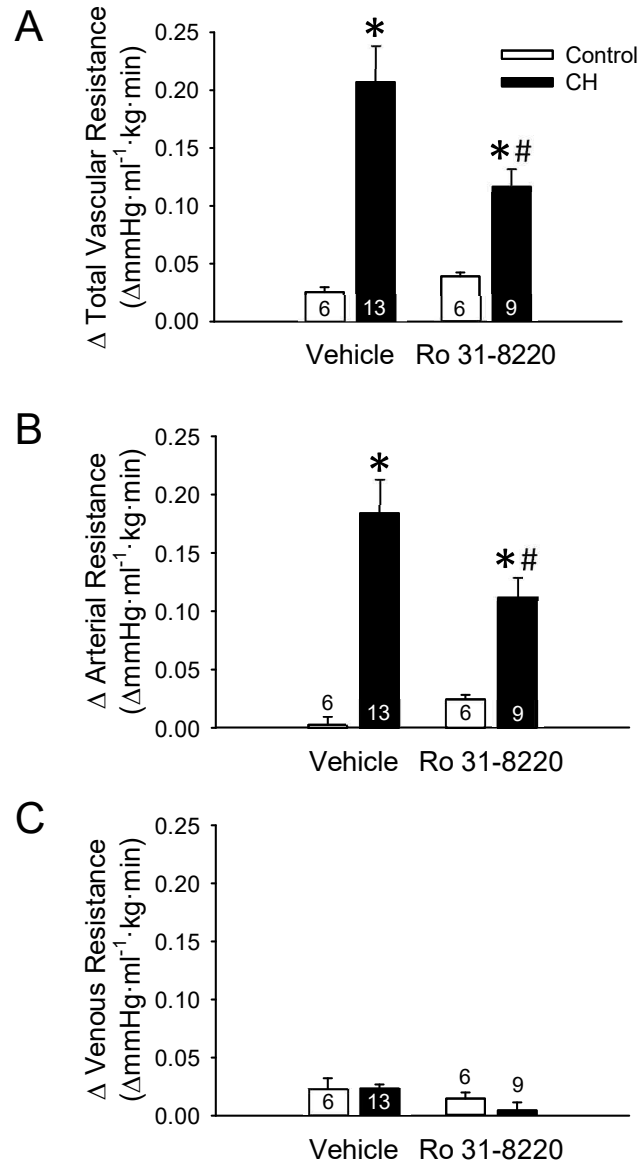


Figure 16. PKC contributes to augmented basal tone in lungs from neonatal rats exposed to CH. The contribution of basal tone to (A) total, (B) arterial and (C) venous resistance is expressed as the change in resistance to spermine NONOate (100 μ M) in lungs (in situ) from control and CH neonates. Experiments were conducted continued presence of L-NNA (300 μ M) with or without the PKC inhibitor, Ro 31-8220 (10 μ M). Values are means \pm SEM; n = 6-13/group (indicated in or above bars); *p<0.05 vs. control, #p<0.05 vs. vehicle, analyzed by two way ANOVA with a Student-Newman-Keuls post-hoc test.

Figure 17

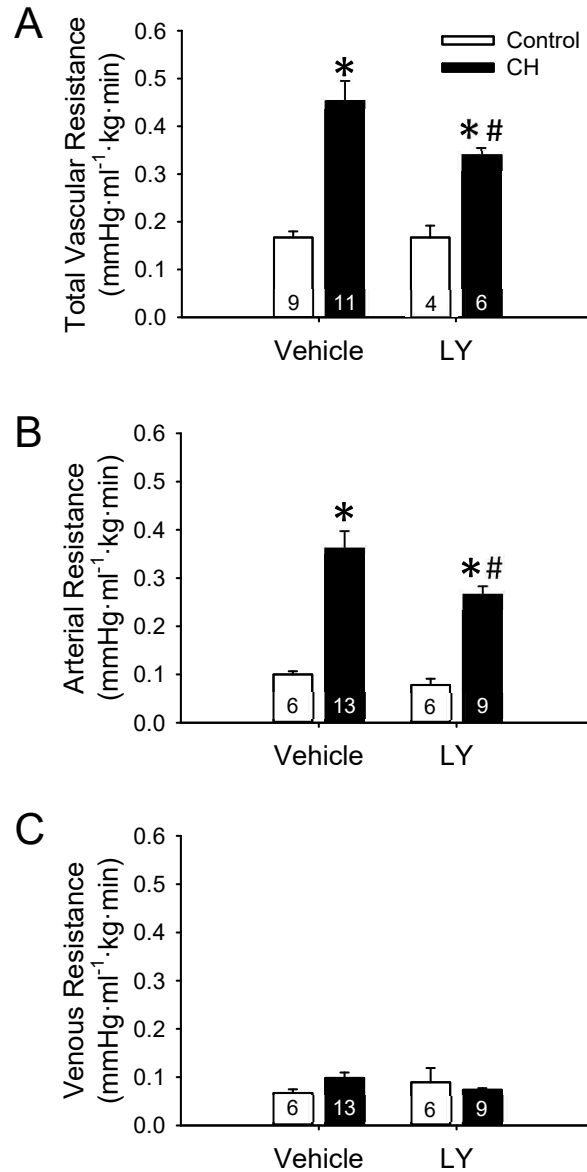


Figure 17. CH increases baseline pulmonary vascular resistance through PKC β signaling. (A) total, (B) arterial, and (C) venous baseline vascular resistances in lungs (*in situ*) from control and CH neonatal rats in the presence or absence of the PKC β inhibitor LY-333,531 (10 nM). Experiments were conducted in the presence of L-NNA (300 μ M). Values are means \pm SEM; n = 6-13/group (indicated in bars); *p < 0.05 vs. control, #p < 0.05 vs. vehicle, analyzed by two-way ANOVA followed by Student-Newman-Keuls post-hoc comparison.

Figure 18

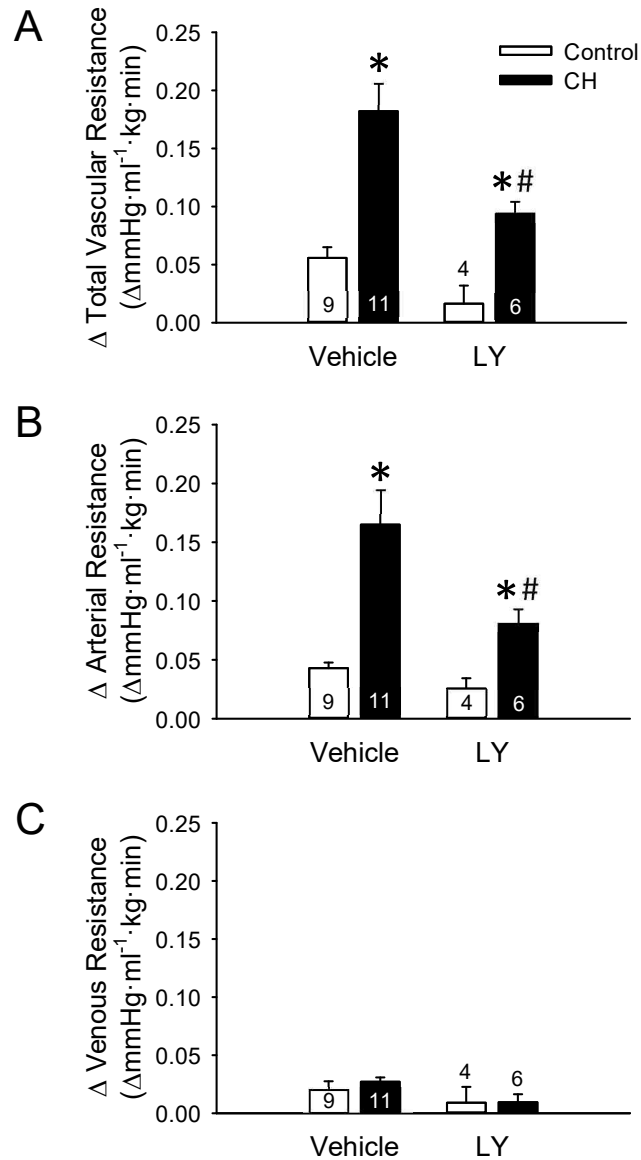


Figure 18. PKC_β inhibition limits CH-dependent increases in basal tone in lungs from neonatal rats. The contribution of basal tone to (A) total, (B) arterial and (C) venous resistance is expressed as the change in resistance to spermine NONOate (100 μM) in lungs (in situ) from control and CH neonates. Experiments were conducted continued presence of L-NNA (300 μM) with or without the PKC_β inhibitor, LY-333,531 (10 nM). Values are means ± SEM; n = 4-11/group (indicated in or above bars); *p<0.05 vs. control, #p<0.05 vs. vehicle, analyzed by two way ANOVA with a Student-Newman-Keuls post-hoc test.

Figure 19

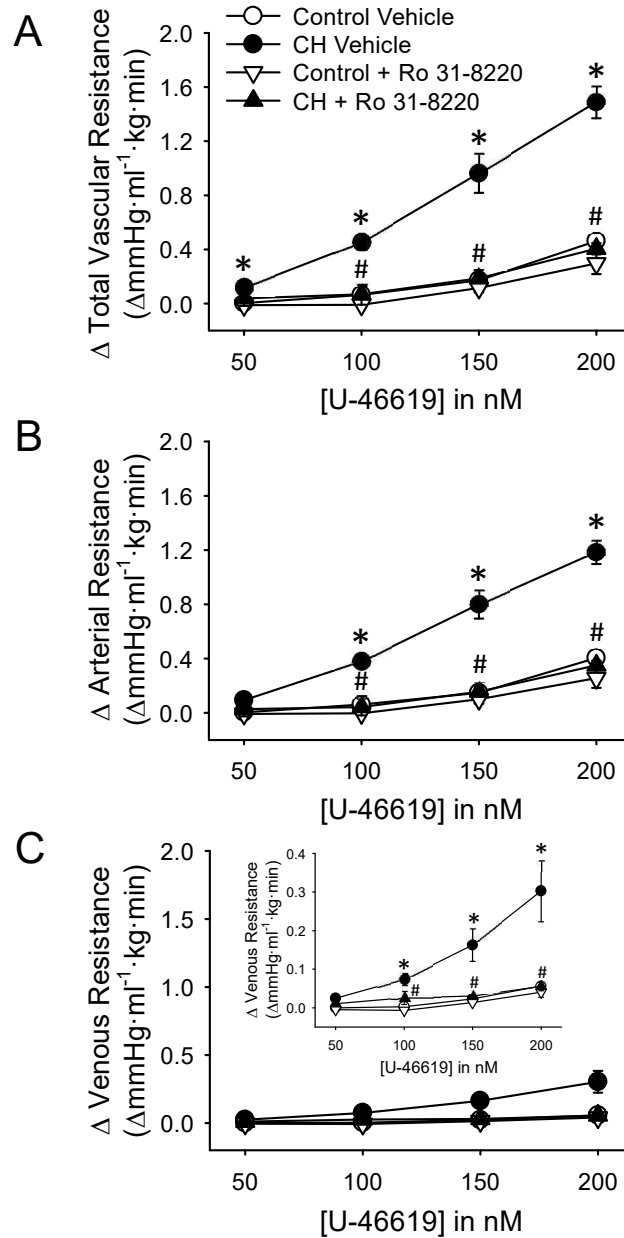


Figure 19. PKC facilitates enhanced vasoconstrictor sensitivity in lungs from CH neonates. Changes in (A) total, (B) arterial, and (C) venous resistance to U-46619 in lungs from control and CH neonates. Experiments were conducted in the continued presence of L-NNA (300 μ M) with or without the PKC inhibitor, Ro 31-8220 (10 μ M). Values are means \pm SEM; n = 5 for Control Vehicle, n = 6 for CH Vehicle, n = 5 for Control Ro 31-8220, n = 4 for CH Ro 31-8220; *p<0.05 vs. control, #p<0.05 vs. vehicle, analyzed by two way ANOVA at each [U-46619] with a Student-Newman-Keuls post-hoc test.

Figure 20

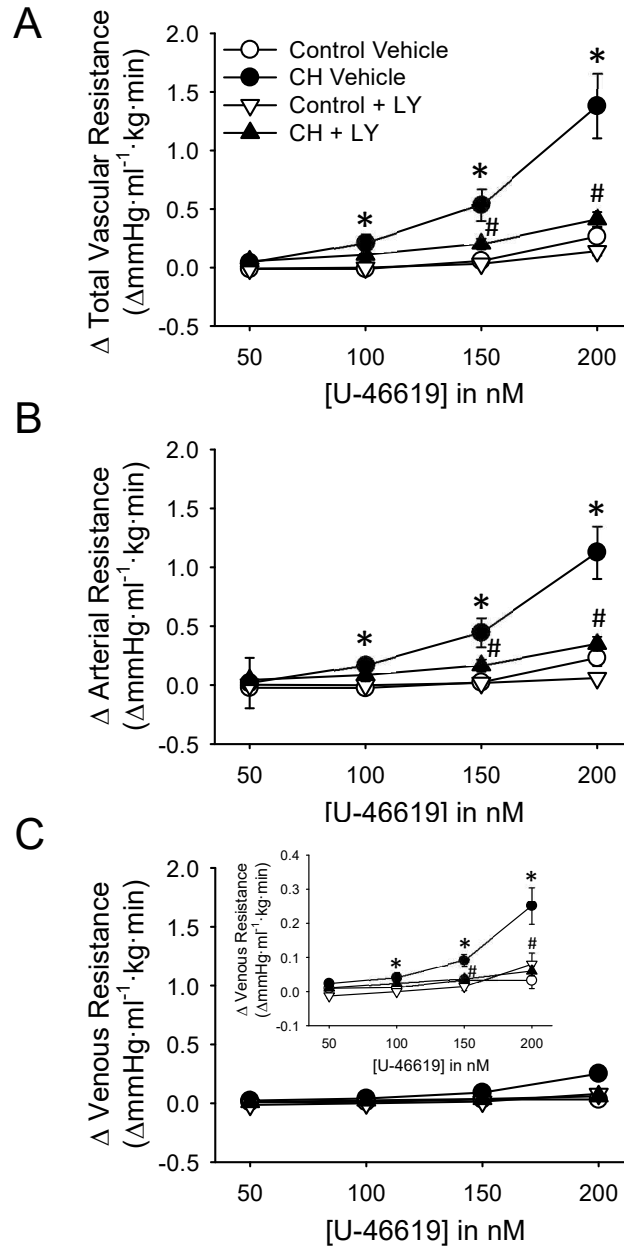


Figure 20. PKC β facilitates enhanced vasoconstrictor sensitivity in lungs from CH neonates. Changes in (A) total, (B) arterial, and (C) venous resistance to U-46619 in lungs from control and CH neonates. Experiments were conducted in the continued presence of L-NNA (300 μ M) with or without the PKC β inhibitor, LY-333,531 (10 nM). Values are means \pm SEM; n = 6 for Control Vehicle, n = 5 for CH Vehicle, n = 5 for Control LY-333,531, n = 5 for CH LY-333,531; *p<0.05 vs. control, #p<0.05 vs. vehicle, analyzed by two way ANOVA at each [U-46619] with a Student-Newman-Keuls post-hoc test.

contributes to enhanced pulmonary arterial tone and pulmonary arterial vasoconstrictor sensitivity to U-46619 in neonates with CH-induced pHTN.

PKC β Mediates CH-induced Basal Tone in Isolated Pulmonary Arteries

Consistent with effects of LY-333,531 to reduce CH-dependent increases in basal tone in the *in situ* lung preparation, PKC β inhibition reduced basal tone development in isolated, pressurized pulmonary arteries from CH rats (Figure 21). We also observed no effect of PKC β inhibition on basal tone in arteries from normoxic control neonates (Figure 21). Inner and outer Ca²⁺-free diameters of vessels included in this study are listed in Table 7.

PMA Stimulates mitoROS Production in a PKC β -Dependent Manner in Isolated PASMCs from Control Neonates

In transiently cultured PASMCs collected from pulmonary arteries of control neonatal rats, we evaluated PKC β -dependent mitoROS production using the PKC activator, PMA to simulate an increase in mitochondrial O₂⁻ production assessed by fluorescence of the mitochondrial selective O₂⁻ indicator, MitoSOX. Upon stimulation with PMA, we observed that MitoSOX fluorescence increased in a manner sensitive to either PKC β inhibition with LY-333,531 or O₂⁻ scavenging via either TEMPOL or tirion (Figure 22). MitoTEMPO similarly prevented PMA-mediated increases in mitoROS generation (Figure 22). These inhibitors were without effect on MitoSOX fluorescence in the absence of PMA treatment (Figure 22).

PKC β Protein Expression in lungs from neonatal control and CH rats

Western blot analysis revealed protein expression of both PKC β I or PKC β II isoforms in lungs from each group of rats (Figure 23). Although levels of each isoform

Figure 21

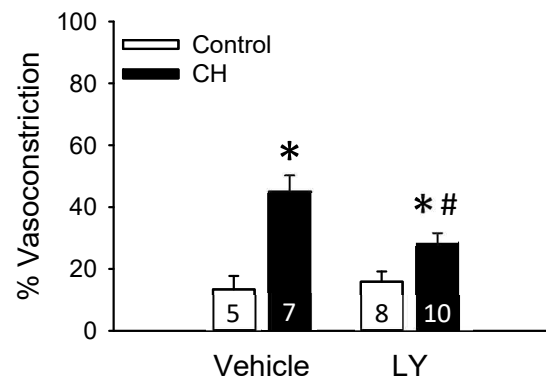


Figure 21. PKC β contributes to increased basal tone in isolated pulmonary arteries from CH neonates.

Vasoconstriction is % maximally dilated (Ca²⁺-free) diameter, calculated using the formula: [(Ca²⁺-free i.d. – Constricted i.d.)/Ca²⁺-free i.d.]. Experiments were conducted in the continued presence of PKC β inhibitor, LY-333,531 (10 nM). The vessel was superfused with L-NNA (300 μ M) following 20 min equilibration time. Values are means \pm SEM; n = 5-10/ group (indicated in bars); *p<0.05 vs. control, #p<0.05 vs. vehicle, analyzed by two way ANOVA followed by Student-Newman-Keuls post-hoc test.

Table 7

	Ca²⁺-Free Inner Diameter (μm)	Ca²⁺ Free Outer Diameter (μm)
Control Vehicle	191 ± 14 (5)	233 ± 12 (5)
CH Vehicle	183 ± 10 (7)	225 ± 12 (7)
Control LY	185 ± 8 (8)	214 ± 12 (8)
CH LY	152 ± 7*# (10)	187 ± 8*# (10)

Table 7. Ca²⁺-free diameters for isolated pulmonary arteries from both control and CH neonates ± LY-333,531. Values are mean ± SEM with n in parenthesis; n = 5-10/group; *p<0.05 vs. control, #p<0.05 vs. vehicle; analyzed by two way ANOVA followed by Student-Newman-Keuls post-hoc test.

Figure 22

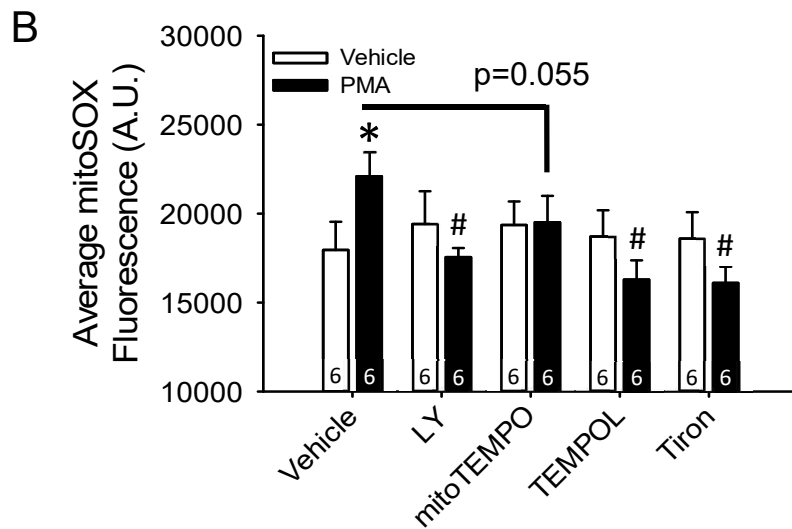
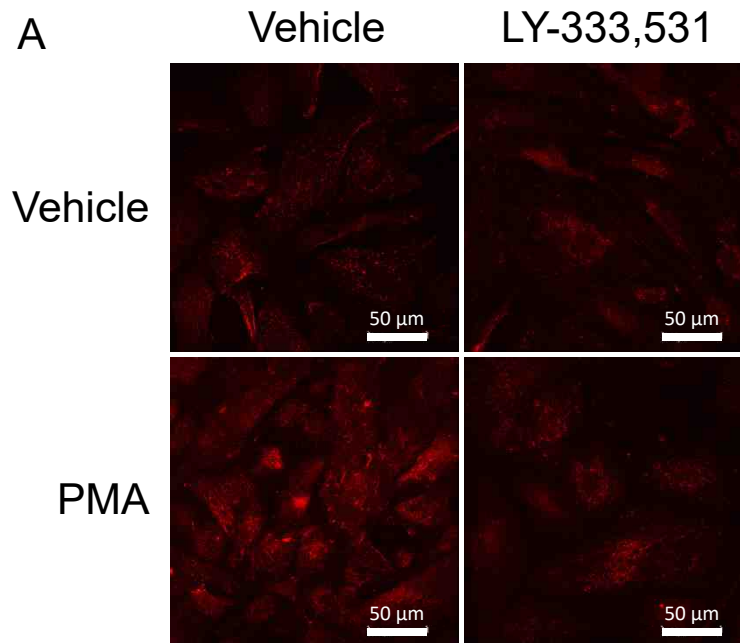
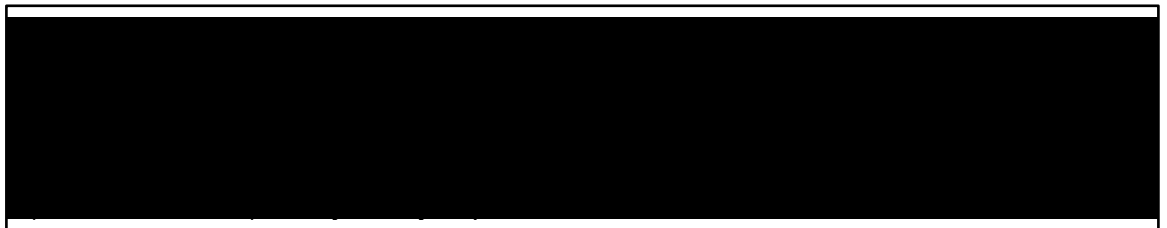
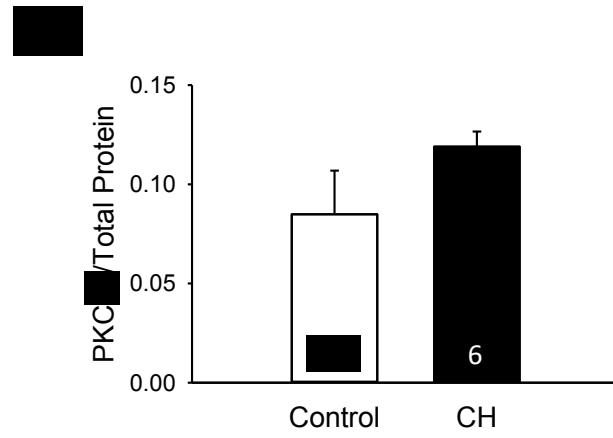
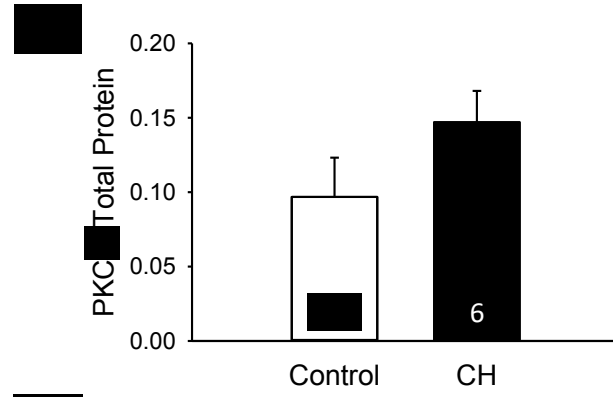
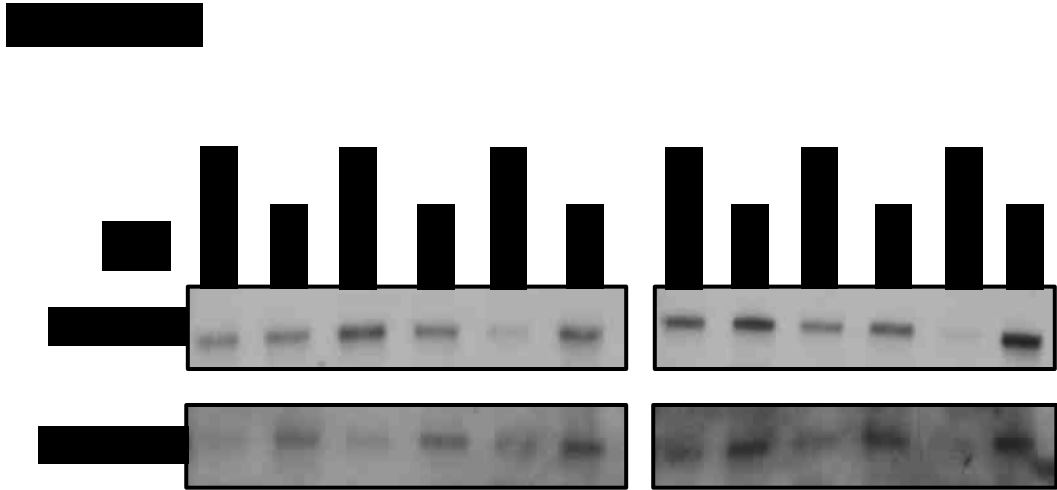


Figure 22. PKC β signaling leads to mitoROS production in PASMCS from control neonates. (A) Representative photomicrographs of mitoSOX fluorescence from transiently cultured PASMCS collected from pulmonary arteries of control neonates. (B) Summary data for 6 biological replicates for each treatment condition. Values are means \pm SEM; n = 6/group (indicated in bars), analyzed by two way ANOVA followed by Student-Newman-Keuls post-hoc test.



tended to be greater in lungs from CH compared to control neonates, this effect did not achieve statistical significance.

CHAPTER 4 - DISCUSSION

Specific Aim 1: Determine the contribution of endothelial dysfunction to enhanced basal tone and agonist-induced vasoconstriction in neonatal pHTN.

Vasoconstrictor mechanisms play an important role in the development of CH-induced neonatal pHTN (15, 40, 91, 129). However, whether vasoconstriction in this setting is a consequence of diminished endothelium-dependent vasodilatory capacity, enhanced production of a contractile factor, or an inherent increase in VSM contractility is poorly understood. Considering that endothelial dysfunction is implicated in the development of neonatal pHTN (89, 129), the present aim *examined the hypothesis that neonatal CH enhances basal tone and pulmonary vasoconstrictor sensitivity by limiting NO-dependent pulmonary vasodilation*. The major findings from this aim are that 1) neonatal CH enhances both basal pulmonary arterial tone and agonist-induced vasoconstrictor sensitivity; 2) endogenous NO limits these responses to CH, while having little to no effect in lungs from normoxic control animals; 3) CH augments receptor-mediated, EDNO-dependent pulmonary arterial dilation, but is without effect on vasoreactivity to exogenous NO; and 4) CH increases pulmonary expression of eNOS as well as eNOS phosphorylation at Ser-1177, a post-translational modification associated with enhanced NO generation (75). Collectively, these data demonstrate an effect of CH to enhance both basal pulmonary arterial tone and receptor-mediated vasoconstrictor sensitivity despite a compensatory increase in eNOS expression and activity.

Exposure to CH leads to neonatal pHTN in a variety of animals models (40, 87, 148) by elevating PVR through increased basal arterial tone and augmented reactivity to endogenous vasoconstrictor agonists (15, 40, 233). Direct evidence supporting an

important contribution of vasoconstriction to this response includes findings that ROK inhibition (180) or inhaled NO (81) acutely reverse PVR in neonatal CH rats. Furthermore, pulmonary vasoconstrictor sensitivity to serotonin is enhanced in newborn lambs housed at high altitude (3801 meters) relative to low altitude (335 meters) lambs in a manner sensitive to ROK inhibition (40). Following exposure to chronic normobaric hypoxia in neonatal piglets, endothelium-derived thromboxane products lead to pulmonary arterial vasoconstriction that is absent in normoxic controls (91). In a neonatal lamb model of pHTN that involves banding of the ductus arteriosus acute increases in pulmonary blood flow lead to vasoconstriction in a manner consistent with a myogenic response (250). Although enhanced pulmonary vasoconstriction in the setting of neonatal pHTN may be due to enhanced production of and sensitivity to endogenous vasoconstrictor agonists, there is also evidence for diminished production of endothelium-derived relaxing factors as well (87, 238). However, the relative contributions of diminished endothelium-dependent vasodilation and increased VSM contractile responsiveness to the vasoconstrictor component of neonatal pHTN is not well defined.

One potential mechanism by which neonatal pHTN may occur is through limitation of the normal NO-dependent fall in PVR. During gestation, the fetal pulmonary circulation is marked by high resistance to pulmonary blood flow that is maintained by hypoxic pulmonary vasoconstriction (246). At birth, the increase in O₂ tension stimulates pulmonary arterial eNOS-dependent vasodilation that relieves fetal pulmonary arterial tone (4, 102). However, even after delivery, the pulmonary circulation retains residual arterial tone that can be uncovered by eNOS inhibition. For example, in studies using isolated, perfused lungs from neonatal rats, acute administration of L-NNA increased baseline PVR by three fold, with the majority of the response in the pulmonary arterial circulation (53). Furthermore, occlusion of the ductus arteriosus in neonatal lambs

following NOS inhibition leads to an increase in PVR in a manner consistent with a myogenic response (251). NO-dependent pulmonary vasodilation is an important mediator of the transition from high resistance fetal to low resistance antenatal pulmonary circulation. Therefore, enhanced vasoconstriction may occur in neonatal pHTN through reduction in NO-dependent vasodilatory capacity.

As mentioned above, neonatal pHTN is often associated with reduced NO-dependent vasodilatory responsiveness, which has been attributed to both endothelial dysfunction and impaired VSM sensitivity to NO. In neonatal lambs with pHTN, pulmonary eNOS levels are reduced (238) and expression of PDE5 in the pulmonary circulation is elevated (37). Increased protein expression of PDE5 can limit the vasodilatory effect of NO in the setting of neonatal pHTN due to disruption of the signaling cascade of NO (37). In animal models of CH-induced neonatal pHTN, NO and eNOS levels are also diminished (52, 87, 129). Given evidence for endothelial dysfunction in the setting of neonatal pHTN, the purpose of the present study was to address whether neonatal CH elevates PVR through a similar mechanism related to diminished NO-dependent pulmonary vasodilation.

Our present findings demonstrate that, in contrast to the evidence in the literature of diminished NO-dependent pulmonary vasodilation, CH exposure enhanced EDNO-dependent vasodilation in the pulmonary circulation of newborn rats. In this aim, we observed increased basal tone and vasoconstrictor sensitivity to U-46619 in lungs from neonatal CH rats that was greatly enhanced following inhibition of NOS, supporting an effect of endogenous NO to limit CH-dependent vasoconstriction. In addition, the majority of the CH-dependent increases in PVR resulted from arterial constriction as determined by double occlusion technique. These results suggest that neonatal CH preferentially impacts arterial function, perhaps by increasing arterial smooth muscle

contractility due to enhanced Ca^{2+} -dependent mechanisms (250) and/or by enhancing Ca^{2+} sensitivity via ROK-dependent signaling (180, 262).

Consistent with effects of NOS inhibition to markedly augment basal tone and agonist-induced arterial constriction following neonatal CH, we found that CH also augments endogenous NO-dependent pulmonary vasodilation to AVP while having no effect on reactivity to the NO donor, spermine NONOate. These findings suggest that CH augments EDNO-dependent pulmonary vasodilation without altering responsiveness to exogenous NO, and are consistent with previous observations in adult rats from our group and others that CH selectively increases pulmonary arterial eNOS expression and enhances NO-dependent pulmonary vasodilation (62, 224, 226, 237).

Our study further suggests a potential mechanism by which CH elevates endogenous NO production through CH-dependent increases in protein expression of eNOS as well as levels of phosphorylated eNOS at Ser-1177, while having no detectable effect on levels of nNOS or iNOS. The lack of CH-dependent changes in iNOS protein expression is interesting considering that iNOS is a hypoxia-inducible gene (200) and increased in lungs from adult rats exposed to CH (63, 225). However, in isolated, pressurized pulmonary arteries from neonatal piglets exposed to either CH or normoxia, selective iNOS inhibition with aminoguanidine had no effect on baseline arterial diameter in arteries from either group suggesting that iNOS does not play a role in regulation of baseline tone in the neonatal pulmonary circulation (87).

The mechanism by which CH increases pulmonary eNOS expression and Ser-1177 phosphorylation is not clear, but may result from increased arterial shear stress associated with CH exposure (253). Ser-1177 phosphorylation of eNOS occurs mainly due to activity of the serine/threonine kinase, Akt (75). Akt-dependent Ser-1177 phosphorylation of eNOS is increased by shear stress on endothelial cells *in vitro* (220) and shear stress increases eNOS mRNA and protein expression in fetal lambs (35).

Our findings of enhanced endothelium-dependent pulmonary vasodilation and associated increases in eNOS protein expression/Ser-1177 phosphorylation differ from previous reports of diminished NO-dependent pulmonary vasodilation in neonatal pHTN (37, 87). Some of this difference could be attributable to study design as well as species difference. Whereas the present study employed a CH model of pHTN involving chronic exposure to hypobaric hypoxia, others have studied effects chronic exposure to normobaric hypoxia (19, 148) or occlusion of the ductus arteriosus (9, 181). Other reports describe pulmonary endothelial dysfunction in response to chronic hypobaric hypoxia in alternate species, including piglets (87) or lambs (40), suggesting a possible species difference in the neonatal response to hypobaric CH.

Effects of neonatal CH to augment basal and agonist-induced vasoconstriction following NOS blockade may be explained by an increase in intrinsic VSM contractility or possibly greater sensitivity to endogenous contractile factors. In neonatal piglets exposed to CH, endothelium-derived thromboxane products contribute to pulmonary vasoconstriction (91). Additionally, in neonatal lambs with pHTN, Ca^{2+} influx through voltage-gated Ca^{2+} channels facilitates a myogenic response (250). Furthermore, enhanced Ca^{2+} entry via a store-operated mechanism contributes to vasoconstriction in adult mice with CH-induced pHTN (166, 195, 271). In addition to Ca^{2+} -dependent mechanisms of enhancing pulmonary VSM contractility, ROK-induced myofilament Ca^{2+} sensitization has been implicated in both the fetal pulmonary myogenic response (262) and in mediating enhanced vasoconstrictor reactivity in neonatal pHTN (40, 180, 287). Similar effects of ROK-dependent Ca^{2+} sensitization contribute to enhanced VSM contractility and pressure-dependent tone in lungs from adult CH rats (44, 145).

The literature also points to a potential role for ROS in enhanced pulmonary vasoconstrictor sensitivity in neonatal pHTN. Levels of ROS resulting from NADPH oxidase (73), xanthine oxidase (142), uncoupled eNOS (159), and mitochondria (9) are

elevated in models of neonatal pHTN. ROS-signaling has additionally been linked to increases in ROK-dependent Ca^{2+} sensitization (145). Although not directly tested, ROS-induced ROK-dependent Ca^{2+} sensitization may play a role in mediating enhanced VSM contractility and subsequent augmentation of pulmonary arterial vasoconstrictor sensitivity in the setting of neonatal pHTN.

In conclusion, this aim demonstrates a previously undescribed effect of neonatal CH to augment both basal pulmonary arterial tone and receptor-mediated vasoconstrictor sensitivity despite a compensatory increase in NO-mediated pulmonary vasodilation, eNOS expression and activity. These results advance our understanding of protective mechanisms that may limit the severity of neonatal pHTN, and are consistent with an effect of CH to increase basal VSM tone and contractile responsiveness rather than to impair endothelium-dependent vasodilation.

Specific Aim 2: Establish the role of ROS in enhanced vasoconstriction in the setting of pHTN in neonates exposed to CH.

Studies in Aim 1 argue against a role for impaired EDNO-dependent pulmonary vasodilation in mediating augmented vasoconstrictor sensitivity following CH in neonatal rats. Considering that lung levels of cellular and mitochondrial-derived ROS are elevated in neonates with pHTN (10, 43, 142) and that ROS contribute to enhanced arterial vasoconstriction in the hypertensive neonatal pulmonary circulation (43, 145, 197), the present aim *examined the hypothesis that mitoROS contribute to enhanced pulmonary arterial vasoconstriction in neonates with CH-induced pHTN*. The major findings from this aim are that 1) CH-dependent increases in basal and agonist-induced vasoconstrictor reactivity rely on ROS and 2) mitochondrial O_2^- augments pulmonary arterial tone following neonatal CH exposure. Taken together, these data demonstrate

an effect of CH to enhance both basal pulmonary arterial tone and receptor-mediated vasoconstrictor sensitivity in a mitoROS dependent manner. Additionally, the normalization of baseline total vascular and arterial resistance between lungs from control and CH neonates after TEMPOL pretreatment suggests that CH-dependent increases in PVR are due entirely to mechanisms of pulmonary arterial vasoconstriction, independent of remodeling effects.

Our data suggest that a specific ROS species, O_2^- , is responsible for mediating enhanced pulmonary vasoconstriction in neonates with pHTN. In this aim, we used both TEMPOL and MitoTEMPO to scavenge pulmonary ROS. Both TEMPOL and MitoTEMPO are SOD mimetics meaning that they convert O_2^- to H_2O_2 (74, 161). Lowering lung levels of O_2^- correlating with reductions in elevated pulmonary vasoconstrictor sensitivity in neonates with pHTN has been demonstrated in the literature (43, 73, 82, 142), however those studies focused on the restoration of NO-dependent pulmonary vasodilation. Elevated vascular levels of O_2^- can scavenge and therefore limit the bioavailability of NO (43, 82). In this aim, all experiments were done in the presence of L-NNA to inhibit endogenous NO production. We observed that pretreatment of lungs with TEMPOL abolishes CH-dependent increases in pulmonary arterial tone. This finding is consistent with an interpretation that O_2^- potentiates pulmonary vasoconstriction at the level of the VSM, perhaps by increasing contractility. Indeed, in adult rodents with CH-induced pHTN, increased levels of pulmonary arterial O_2^- activates RhoA/ROK-dependent myofilament Ca^{2+} sensitization and promotes pulmonary arterial pressure-dependent tone and enhanced vasoconstrictor sensitivity (44, 145, 197). This signaling mechanism may contribute to enhanced VSM contractility in neonates with CH-induced pHTN. We cannot however eliminate the possibility that the observed effect of TEMPOL and MitoTEMPO (both SOD mimetics which dismutate O_2^- to H_2O_2) to reduce vasoconstrictor reactivity could be due to the pulmonary vasodilatory

effect of H₂O₂ (45, 163, 174). Additionally, TEMPOL has been shown to promote vasodilation through direct activation of K⁺-channels in the membranes of vascular smooth muscle cells (288, 289). However, the finding that MitoQ, a molecule that is not an SOD mimetic, but instead an inhibitor of mitochondrial O₂⁻ production, similarly abolishes CH-dependent enhancement of pulmonary arterial tone argues against this possibility.

There are several sources of ROS that may contribute to enhanced lung levels of ROS observed in the setting of neonatal pHTN. ROS generation by NOX, xanthine oxidase, and uncoupled eNOS contribute to ROS production in the pulmonary circulation of neonates with pHTN (92, 142, 254). The mitochondria are also a potent source of cellular O₂⁻ in the pulmonary vasculature (12). In the setting of neonatal pHTN, there are several potential mechanisms whereby lung mitoROS levels might be enhanced. In transiently cultured PASMCs from newborn lambs, cyclic stretch stimulates mitochondrial O₂⁻ production (278). This finding suggests that stretch on the VSM, as might be expected in neonates with elevated pulmonary arterial pressure, stimulates mitochondrial O₂⁻ production. Moreover, recent studies in lambs with pHTN, demonstrate that basal mitochondrial-derived O₂⁻ levels are increased in endothelial cells from pulmonary hypertensive lambs due to lower SOD2 (the SOD isoform present in mitochondria that scavenges O₂⁻) protein expression and activity relative to cells from healthy controls (9). Mitochondrial-derived O₂⁻ limits NO bioavailability and therefore potentiates pulmonary vasoconstrictor sensitivity (9, 10). Restoration of SOD2 expression in pulmonary arterial endothelial cells from lambs with pHTN using adenoviral transfection not only limits mitochondrial O₂⁻ levels, but also improves NO-dependent pulmonary vasodilation and increases eNOS mRNA and protein expression to levels observed in cells from control lambs (9). These findings from the literature suggest that increased basal mitochondrial O₂⁻ generation in neonates with pHTN

potentiates pulmonary vasoconstriction not only by limiting NO-dependent pulmonary vasodilation through scavenging NO, but also by reducing mechanisms for NO production. Findings from Aim 1 of this dissertation however suggest that neonatal CH exposure potentiates NO-dependent pulmonary dilation in animals with pHTN. Furthermore, findings from Aim 2 indicate that mitoROS are important mediators of augmented vasoconstriction, perhaps through a direct effect to promote enhanced VSM contractility.

Findings from this aim suggest that mitochondrial-derived O_2^- contributes to enhanced pulmonary arterial tone following neonatal CH exposure. Our studies in isolated pulmonary arteries from both control and CH neonates used MitoTEMPO and MitoQ, both mitochondrial-targeted strategies for reducing levels of mitochondrial-derived O_2^- . In the presence of either compound, CH-dependent increases in pulmonary arterial tone were eliminated suggesting a pivotal role of mitochondrial O_2^- following CH exposure to augment arterial tone in the pulmonary circulation. Furthermore, the effect of both MitoTEMPO and MitoQ to remove enhanced arterial tone in the presence of L-NNA suggests that mitochondrial O_2^- enhance tone by promoting mechanisms of vasoconstriction such as increased production of an endothelium-derived contractile factor and/or by enhancing VSM contractility, not by reducing NO-dependent pulmonary arterial vasodilation. Although we identify a potential subcellular source of O_2^- production, we do not examine the cell-type responsible for mitochondrial O_2^- production. Future studies in endothelium denuded (removed), isolated pulmonary arteries are important to determine whether enhanced pulmonary arterial tone observed in CH exposed neonates is a consequence of mitochondrial O_2^- production in the endothelium or in VSM of the medial layer/fibroblasts in the adventitia.

Furthermore, findings related to mitochondrial-derived O_2^- relied on studies in isolated, endothelium-intact, pulmonary arteries which also presents some limitations.

The lung is a multicellular tissue with a variety of cell types such as endothelial cells (46) and adventitial fibroblasts (189) that may contribute to regulation of pulmonary vascular tone in the setting of pHTN. There are also factors such as NO, AVP, ET-1, and 5-HT, released from the lung and other endocrine organs that can alter the level of tone within the pulmonary circulation *in vivo*. Additionally, our studies focus on only a segment of one branch in the arterial tree, making it difficult to extend our findings to the entire pulmonary circulation. However, our finding that TEMPOL eliminates CH-dependent increases in pulmonary vascular resistance in the isolated (*in situ*) lung, where the entire pulmonary circulation is present, by limiting pulmonary arterial tone bolsters a role for our findings related to mitochondrial O_2^- in the intact pulmonary circulation.

MitoROS generation in the endothelium may lead to increased production of an endothelium-derived contractile factor such as ET-1 (144). Enhanced mitochondrial O_2^- levels in endothelial cells from lambs with pHTN limit eNOS mRNA and protein expression and limit vasodilation to endogenous NO (9). NO can limit ET-1 production and secretion by pulmonary arterial endothelial cells through downregulating expression of preproET-1 mRNA (151). In the setting of neonatal pHTN, reduced pulmonary vascular NO may contribute to elevations in ET-1 levels observed in neonates with pHTN (144, 228). Findings from Aim 1 of this dissertation however, make this interpretation unlikely in our model due to the observed effect of CH to increase NO-dependent pulmonary vasodilation in neonates with pHTN. The literature also supports a role for O_2^- to lead to lipid peroxidation-dependent increases in ET-1 lung levels in neonates with pHTN (144). It is possible that in our model, mitochondrial O_2^- may increase ET-1 production through other means, such as promotion of lipid peroxidation.

In addition to the possible role of mitoROS to promote activity of ET-1, mitochondrial O_2^- is also important for 5-HT-induced pulmonary arterial vasoconstriction as well. In intrapulmonary arteries from adult rats, mitochondrial O_2^- is important for 5-

HT-induced vasoconstriction (34, 103). In isolated PASMCs from these animals, 5-HT treatment leads to mitochondrial O_2^- production that is sensitive to 5-HT receptor antagonists (103). 5-HT binds 5-HT_{2A} receptors on rat PASMCs and stimulates influx of extracellular Ca^{2+} which then leads increased mitochondrial Ca^{2+} uptake, mitochondrial depolarization, and subsequent mitochondrial O_2^- generation from complex I (103). Moreover, mitoROS (9, 83), 5-HT-induced pulmonary vasoconstriction (40, 49, 72), VSM $[Ca^{2+}]_i$ (124), and vascular tone (180, 250, 251) are known to be increased in the setting of neonatal pHTN, suggesting that 5-HT-induced mitochondrial O_2^- may be an important mediator of neonatal pHTN.

Mitochondrial O_2^- may increase VSM contractility in pulmonary arteries of neonates with CH-induced pHTN by altering Ca^{2+} signaling and/or by increasing myofilament Ca^{2+} sensitivity. In addition to their energy-generating capacity, mitochondria also participate in regulation of $[Ca^{2+}]_i$ (176). Studies of transiently cultured PASMCs from adult mice have demonstrated that mitoROS contribute to acute-hypoxia dependent increases in $[Ca^{2+}]_i$ and subsequent VSM contraction (272). It is also possible that in addition to a potential role to alter $[Ca^{2+}]_i$ signaling, mitoROS may contribute to enhanced myofilament Ca^{2+} -sensitization. As mentioned earlier, cellular ROS contribute to RhoA/ROK-dependent Ca^{2+} sensitization in adult rodents with CH-induced pHTN (145). ROK has been shown to activate actin polymerization (18). Actin polymerization is thought to promote myofilament Ca^{2+} sensitization by enhancing transmission force to the extracellular matrix (258, 293) and by increasing the assembly of contractile units between actin and myosin (38). In cultured mouse and human fibroblasts, treatment with rotenone, an electron transport chain complex 1 inhibitor, reduces mitoROS production and limits cellular attachment and membrane spreading (292), processes driven by actin polymerization (291). These findings suggest that mitoROS promote actin polymerization, a mechanism that increases myofilament Ca^{2+}

sensitization.

Several intracellular signaling pathways can promote mitoROS generation. In primary cultures of mouse microvascular endothelial cells, extracellular O_2^- generated by NOX can stimulate increased $[Ca^{2+}]_i$ which in turn results in mitochondrial O_2^- production (94). In disease models such as cardiac ischemic preconditioning and angiotensin II-induced systemic hypertension, mitoROS production in cardiac myocytes (175) and bovine aortic endothelial cells (76) is a downstream event in several signaling pathways such those impacted by Akt, MAP kinases, PKA, and PKC (76, 175). In disease states such as diabetes, ischemia-reperfusion injury, multiple sclerosis, obesity, and osteoporosis the β isoform of PKC contributes to mitoROS production and cellular oxidant stress that may facilitate disease pathogenesis (16, 182, 244, 270). The effect of mitochondrial O_2^- to mediate enhanced pulmonary arterial vasoconstriction may be a downstream consequence of kinase signaling, perhaps PKC_β considering its role to mediate cellular dysfunction in several disease states.

In conclusion, this aim demonstrates that augmented pulmonary arterial tone and pulmonary vasoconstrictor sensitivity rely on mitochondrial O_2^- signaling. Considering the link between PKC_β , mitoROS, and cellular dysfunction, studies in Aim 3 were designed to pursue the contribution of PKC_β to enhanced pulmonary vasoconstriction occurring in neonates with CH-induced pHTN.

Specific Aim 3: Define the contribution of PKC_β -signaling to enhanced vascular reactivity following CH exposure in neonates.

Findings from Aim 2 of this dissertation demonstrate that mitochondrial O_2^- contributes to enhanced pulmonary arterial vasoconstriction in neonates with CH-induced pHTN. The upstream signaling mechanism for mitochondrial O_2^- generation

however was still unknown. Considering previous evidence linking PKC β signaling with mitoROS production in several disease states, the present aim *examined the hypothesis that PKC β -dependent mitoROS generation in pulmonary vascular smooth muscle contributes to enhanced pulmonary arterial tone following neonatal CH*. The major findings from this aim are that 1) PKC β signaling leads to increases in mitoROS production in transiently cultured PASMCs from control neonates; 2) PKC β signaling contributes to enhanced pulmonary arterial tone and vasoconstrictor sensitivity following CH; and 3) PKC β I and PKC β II are both expressed in pulmonary arteries from both control and CH neonates, but they were not significantly altered by CH. Taken together, these data demonstrate that PKC β signaling leads to mitoROS generation in PASMCs from neonatal rats and that PKC β and mitoROS are important mediators of enhanced pulmonary arterial vasoconstriction occurring in the setting of neonatal pHTN.

PKC is an important regulatory kinase in the pulmonary vasculature where it contributes to VSM contractility and proliferation (28, 41). In the setting of neonatal pHTN, PKC signaling is an important mediator of enhanced basal pulmonary vascular tone (31). Additionally, studies in isolated pulmonary arteries from adult rats with intermittent hypoxia-induced pHTN demonstrate that enhanced vasoconstrictor sensitivity of ET-1 is sensitive to myrPKC (a myristoylated PKC α / β pseudosubstrate inhibitor) and LY-333,531 (a selective PKC β inhibitor), but not rottlerin (a PKC δ inhibitor) (242). These results demonstrate that enhanced pulmonary arterial vasoconstrictor reactivity occurring in the setting of pHTN rely on PKC isoform signaling, likely PKC β .

PKC β is a classical PKC isoform which is regulated by both changes in [Ca $^{2+}$] $_i$ and diacylglycerol (268). Findings from aim 3 of this dissertation demonstrate that both splice variants of PKC β , PKC β I and PKC β II, are expressed pulmonary arteries of both control and CH neonates. As protein expression of PKC β splice variants was unaltered by CH, mechanisms other than changes in protein content account for the PKC β -

dependency of tone development and agonist-induced pulmonary vasoconstriction observed only in neonates exposed to CH. Subcellular localization of PKC isoforms is an important regulator of their activity (193) with increased cell membrane association correlated with enhanced activity of PKC isoforms (193, 211, 265). In adult rats with intermittent hypoxia-induced pHTN, PKC β protein expression was unaltered by intermittent hypoxia exposure, but the basal cell membrane association of PKC β is increased as is ET-1-dependent translocation away from the plasma membrane in pulmonary arteries from animals exposed to intermittent hypoxia compared to normoxic controls (242). This finding suggests that although neonatal CH does not increase protein expression of PKC β , it may alter the subcellular localization of PKC β to be more membrane associated and therefore more active.

Considering the role of Ca $^{2+}$ in promoting activation of classical PKC isoforms such as PKC β , it is also possible that following CH exposure in neonatal rats, increased PASMCMC [Ca $^{2+}$] $_i$ activates PKC β -dependent pulmonary arterial vasoconstriction. In PASMCMCs collected from both control and CH-exposed piglets, basal Ca $^{2+}$ is elevated in cells from piglets exposed to CH (124). Additionally, exposure of PASMCMCs from either control or CH piglets to 3 days of hypoxia *in vitro* increased [Ca $^{2+}$] $_i$ (124). Finally, stimulation of cultured PASMCMCs with U-46619 was associated with greater changes in [Ca $^{2+}$] $_i$ in cells from CH exposed piglets (124). In transiently cultured PASMCMCs from both healthy control lambs and those with pHTN, Ca $^{2+}$ release from intracellular stores as well as SOCE was enhanced in cells from neonates with pHTN (223). However, this group did not report whether there was a difference in basal Ca $^{2+}$ between PASMCMCs collected from control lambs and those with pHTN (223). Findings in pulmonary VSM from neonatal piglets and lambs suggest that intracellular Ca $^{2+}$ levels may be elevated in neonates with pHTN which may then contribute to PKC β activation.

In addition to [Ca $^{2+}$] $_i$ contributing to PKC β activation in pulmonary VSM of

neonatal rats exposed to CH, PKC β is also regulated by cellular redox state. Oxidative stress associated with certain diseases such as multiple sclerosis, obesity, and osteoporosis is thought to activate PKC β signaling (16, 182, 252, 295). The enzymatic structure of PKC β contains an auto-inhibitory zone with cysteine thiols vulnerable to reversible oxidation to form a sulfenylated (-SOH) side group (106). Protein sulfenylation of the auto-inhibitory zone is thought to produce an increase in the catalytic activity as well as potentiate agonist-dependent activation of PKC β (107, 155).

An important downstream consequence of PKC β activation and signaling is mitoROS generation (16, 252). In this aim, we observe that PKC β signaling promotes mitoROS generation in isolated PSMCs from neonatal rats. PKC β -induced mitoROS generation occurring in disorders such as aging, multiple sclerosis, obesity, and osteoporosis involves PKC β -dependent translocation of the redox enzyme p66shc to the mitochondria (16, 182, 252, 295). Normally the factor p66shc resides in the cytoplasm, but under conditions of oxidative stress, such as that observed in neonatal pHTN, PKC β -dependent phosphorylation of p66shc stimulates its translocation to the mitochondrial inner membrane. Once there, p66shc oxidizes cytochrome c and then reduces molecular oxygen into O $_2^-$ (207, 252).

An additional potential mechanism whereby PKC β may promote the generation of mitoROS involves the mitoK $_{ATP}$ channel. The mitoK $_{ATP}$ channel is expressed on the mitochondria and appears to be distinct from K $_{ATP}$ channels located in the plasma membrane in terms of its regulation and composition (135). In cardiomyocytes collected from adult rabbits, mitoK $_{ATP}$ channel opening was associated with increased mitoROS generation (199). Opening of the mitoK $_{ATP}$ channel permits K $^+$ influx into the mitochondrial matrix and matrix alkalization as incoming K $^+$ ions replace H $^+$ pumped out by the respiratory chain (59, 85). Matrix alkalization uncouples the electron transport chain and leads to mitoROS production (59, 85). The opening of mitoK $_{ATP}$ channels is

promoted by PKC signaling. In isolated mitochondria collected from homogenates of adult rat hearts, inhibition of PKC prevented PMA-induced mitoK_{ATP} channel opening. Although this group went further and demonstrated that PKC_ε contributed to mitoK_{ATP} opening in isolated cardiac mitochondria (58), they did not test a role for PKC_β. Findings from this aim evaluating PMA-dependent increases in mitochondrial O₂⁻ production assessed using mitoSOX fluorescence in transient cultures of neonatal PASMCs, demonstrate that PKC_β signals to induce mitoROS generation. There may be a difference in which PKC isoform modifies mitoK_{ATP} channel kinetics depending on a given cell type with VSM and cardiac muscle regulated in different ways.

We observe in this aim that PKC_β is upstream of mitochondrial O₂⁻ production and findings from Aim 2 of this dissertation indicate that mitochondrial O₂⁻ are important mediators of elevated pulmonary arterial tone following neonatal CH exposure. Therefore, it is possible that PKC_β-dependent mitochondrial O₂⁻ contributes to enhanced pulmonary arterial tone in neonates with CH-induced pHTN. Of interest, whereas inhibition of mitochondrial O₂⁻ signaling fully alleviated elevated tone in pulmonary arteries from CH neonates, PKC_β inhibition was only partially effective. This finding is also true in results from the isolated (*in situ*) lung preparation where TEMPOL eliminated CH-dependent enhancement of both basal tone and agonist-induced vasoconstrictor sensitivity, whereas PKC_β inhibition only partially relieved those responses. These findings together suggest that perhaps PKC_β is not fully inhibited by the concentration of LY-333,531 used in these studies or that there are other mechanisms for mitochondrial O₂⁻ production. Additionally, it is possible that there may be sources of ROS in the whole lung that are not mitochondrial. In the discussion section for Aim 2, it was suggested that mechanisms such as increased [Ca²⁺]_i (103) and/or elevated cytoplasmic or extracellular levels of O₂⁻ (94) may stimulate mitoROS generation, independent of PKC_β signaling.

PKC β may also facilitate enhanced pulmonary vasoconstriction independent of downstream mitoROS signaling. Classical PKC signaling leads to the inhibition of myosin light-chain phosphatase via phosphorylation by the peptide CPI-17. CPI-17-dependent phosphorylation of the myosin light chain leads to greater Ca²⁺-sensitization and subsequent increases in vasoconstrictor sensitivity (154). Considering that PKC β contributes to enhanced pulmonary arterial tone and pulmonary vasoconstrictor sensitivity in neonatal rats with CH-induced pHTN, CPI-17-dependent increases in myofilament Ca²⁺-sensitivity may be involved in this response.

However, in adult rats with pHTN induced by exposure to intermittent hypoxia, PKC β contributed to enhanced ET-1 vasoconstrictor sensitivity and Ca²⁺-sensitization without altering levels of phosphorylated myosin light-chain (242). Additionally, activation of PKC in uterine arteries of pregnant sheep increased arterial tone without altering levels of phosphorylated myosin light-chain (286). These findings suggest PKC signaling, even involving PKC β , may use pathways independent of CPI-17 to promote Ca²⁺ sensitization and enhanced pulmonary vasoconstriction. As mentioned above, actin polymerization may also contribute to enhanced vasoconstrictor reactivity independent of changes in [Ca²⁺]_i. CH exposure of pregnant sheep increased PKC-dependent uterine artery constriction in a manner dependent on actin polymerization (285). This finding suggested that PKC-induced actin polymerization is important for uterine artery constriction following CH exposure during pregnancy. We observe that antenatal CH exposure of neonatal rats increases pulmonary arterial tone and vasoconstrictor sensitivity in a PKC β -dependent manner, perhaps dependent on actin polymerization.

Actin polymerization can also be promoted by mitochondrial-derived O₂⁻ (291, 292). Findings from Aims 2 and 3, demonstrate that PKC β signaling in transiently cultured PASMCs from neonatal rats increases mitochondrial O₂⁻ generation and that

PKC β and mitoROS contribute to enhanced pulmonary arterial tone following CH exposure. It is possible that PKC β -dependent mitoROS generation in PASMCs of neonatal rats contributes to mitoROS-dependent actin polymerization and subsequent promotion of myofilament Ca $^{2+}$ sensitivity.

In conclusion, this aim demonstrates that augmented pulmonary arterial tone and pulmonary vasoconstrictor sensitivity rely on PKC β and that PKC β signaling in neonatal PASMCs is upstream of mitochondrial O $_2^-$ generation in PASMCs from control neonates. We know from aim 2 that mitoROS are important mediators of enhanced pulmonary arterial tone following neonatal CH exposure. Future investigation into the link between PKC β and mitoROS occurring in pulmonary VSM of neonates with CH-induced pHTN is important to understand how this pathway is activated and contributes to enhanced pulmonary arterial vasoconstriction.

Conclusions

The aim of this dissertation was to examine mechanisms of enhanced pulmonary vasoconstriction occurring in CH-induced neonatal pHTN. We first investigated the contribution of diminished endothelium-dependent pulmonary vasodilation to enhanced pulmonary vasoconstriction. In contrast to our hypothesis, we found that following CH, vasodilatory responses to endogenous NO were enhanced in a manner dependent on increased eNOS protein expression and Ser1177 phosphorylation. We additionally observed that CH did not alter VSM sensitivity to exogenous NO. We concluded that neonatal CH exposure promotes endogenous NO-dependent pulmonary vasodilation by upregulation of protein expression and Ser1177 phosphorylation of eNOS. Despite this potentially adaptive response, neonates exposed to CH still demonstrated increased baseline PVR, enhanced pulmonary vasoconstrictor sensitivity, and pHTN.

We next evaluated the role of enhanced VSM contraction involving PKC β /mitoROS signaling to mediate enhanced pulmonary arterial vasoconstriction. We found that PKC β activation in neonatal pulmonary VSM leads to mitoROS generation. We additionally observed that PKC β contributes to enhanced basal pulmonary arterial tone and vasoconstrictor reactivity following neonatal CH exposure. ROS signaling similarly contributes to enhanced pulmonary arterial basal tone and agonist-induced vasoconstriction in CH-exposed neonates with mitoROS contributing to enhanced arterial tone development in isolated pulmonary arteries from CH neonates. In conclusion, these data suggest that CH augments pulmonary arterial vasoconstriction in a manner dependent on PKC β /mitoROS and that this response occurs despite a CH-dependent increase in NO-dependent pulmonary vasodilation.

Clinical Significance

Neonatal pHTN is a significant source of morbidity and mortality in infants (5, 246). Research into underlying mechanisms contributing to neonatal pHTN have identified vasoconstriction as an important mediator of the disease (180, 246). Investigation into factors that contribute to enhanced pulmonary vasoconstriction has focused on the role of diminished pulmonary vasodilation to limit vasoconstriction (43, 83, 87). Studying mechanisms of reduced pulmonary vasodilation has yielded clinical interventions for infants with pHTN focused on improving mechanisms of pulmonary vasodilation (5, 246). Evaluation of the effectiveness of current vasodilator therapies demonstrates that these strategies are met with limited success and do not improve mortality or length/cost of hospital stay (160, 273). Therefore, investigation into mechanisms that contribute to enhanced pulmonary vasoconstriction through promotion of enhanced VSM tone and pulmonary vasoconstrictor sensitivity are important to help generate new therapeutics for neonates with pHTN. Through these studies, we have demonstrated that enhanced pulmonary arterial vasoconstriction occurring in neonates with pHTN depends on PKC β , a PKC isoform not previously linked to vasoconstriction in the neonatal pulmonary circulation, and mitoROS signaling. Further, we demonstrated that PKC β can signal to promote mitoROS generation in neonatal PASMCs. The ability to understand these cellular pathways involving PKC β and mitoROS may provide a new means of treating neonatal pHTN. Considering the need for improved understanding of mechanisms responsible for enhanced pulmonary VSM contractility and the subsequent contribution of those factors to enhanced pulmonary vasoconstriction, the findings from this investigation represent a novel step toward improved therapeutic intervention for infants with pHTN.

Future Directions

Although our present findings support a role of PKC $_{\beta}$ /mitoROS signaling to mediate enhanced pulmonary arterial vasoconstriction in neonates with CH-induced pHTN, several questions remain unanswered by the current study. Future studies are necessary to establish the link between CH and PKC $_{\beta}$ activation, PKC $_{\beta}$ and mitoROS generation, and finally the mechanism by which PKC $_{\beta}$ /mitoROS may enhance pulmonary arterial vasoconstriction. In addition, it is important to determine whether the PKC $_{\beta}$ /mitoROS signaling axis contributes to the progression and maintenance of CH-induced pHTN in neonates.

As indicated above, future studies will evaluate the upstream initiator of this pathway following exposure to CH. Considering that ROS are important in the development of neonatal PH (40, 142), our data suggesting that PKC $_{\beta}$ stimulates mitoROS production, and the known role of oxidation to activate PKC $_{\beta}$ (106, 155, 206), we suspect that CH stimulates PKC $_{\beta}$ activity through oxidation of the enzyme in a feed-forward fashion. However, investigation into the impact of neonatal CH exposure on [Ca $^{2+}$] $_i$ in VSM is also important considering that PKC $_{\beta}$ is also sensitive to changes in [Ca $^{2+}$] $_i$ (268).

More investigation is important to better understand how PKC $_{\beta}$ promotes mitoROS generation in PASMCs from neonatal rats. The literature suggests that signaling involving PKC $_{\beta}$ -dependent activation of p66shc and/or mitoK $_{ATP}$ channel opening may contribute to mitoROS generation (16, 58, 252). Additionally, more work is needed to better understand if neonatal CH exposure leads to PKC $_{\beta}$ -dependent mitoROS generation and, if so, how CH may activate this signaling pathway. CH does not appear to impact protein expression of PKC $_{\beta}$ splice variants, but some PKC isoforms translocate from the cell membrane to mitochondria to stimulate mitochondrial O $_2^-$ production (54, 175). It is possible that subcellular localization of PKC $_{\beta}$ may account for

CH-dependent activation of PKC β /mitoROS signaling.

We are also interested in the downstream mechanism of PKC β /mitoROS-dependent vasoconstriction. Dynamic actin cytoskeletal reorganization in VSM is vital to tension generation (258, 259) and early studies from our laboratory in adult rats suggest PKC β /mitoROS signaling contribute to enhanced vasoconstriction in a manner dependent on myofilament Ca²⁺ sensitization reliant on actin polymerization. Classical signaling of PKC to promote Ca²⁺-sensitization in arterial smooth muscle relies on CPI-17-dependent inhibition of myosin light-chain phosphatase (245). Evaluation of the contribution of CPI-17-induced Ca²⁺ sensitization and the contribution of PKC β in this response is important to better understand potential mechanisms by which PKC β leads to enhanced pulmonary arterial vasoconstriction.

Investigation into the potential mechanism by which PKC β /mitoROS leads to enhanced pulmonary arterial vasoconstriction also needs to address which cell type is responsible for observed responses. Our current findings in transiently cultured PSMCs suggest that PKC β /mitoROS signaling is observed in pulmonary VSM. However, studies in endothelium-disrupted, isolated pulmonary arteries are important to determine whether enhanced pulmonary arterial tone observed in CH exposed neonates is a consequence of PKC β /mitoROS acting as endothelium-derived contractile factors.

Finally, increased PKC β and mitoROS signaling may represent early onset responses that initiate the development of pHTN in response to neonatal CH. Furthermore, this mechanism may contribute to the maintenance of pHTN through vasoconstrictor actions. Future studies are necessary to evaluate the contribution of both PKC β and mitoROS to the development of CH-induced neonatal pHTN, and the regression of established pHTN in this model.

In closing, the results from this dissertation as well as potential follow up investigation could identify therapeutic targets for neonatal pHTN. The potential of this

goal is enhanced by the recent use of the PKC β inhibitor, LY-333-351 (13, 239) and the mitochondrial selective ROS scavenger, MitoQ (241, 257) in clinical trials to treat disease in adult humans.

APPENDIX A – ABBREVIATIONS AND ACRONYMS

ACh = acetylcholine
ANOVA = analysis of variance
AVP = arginine vasopressin
BK_{Ca} = Big conductance potassium channel
BSA = bovine serum albumin
[Ca²⁺]_i = intracellular calcium concentration
Ca²⁺ = calcium
CH = chronic hypoxia
DHE = dihydroethidium
eNOS = endothelial nitric oxide synthase
EGTA = ethylene glycol-bis(β-aminoethyl ether)-N,N,N'N'-tetraacetic acid
ET-1 = endothelin-1
FBS = fetal bovine serum
HBSS = Hank's buffered saline solution
5-HT = serotonin
20-hydroxyeicosatetraenoic acid = 20 HETE
i.d. = inner diameter
i.p. = intraperitoneal
iNOS = inducible nitric oxide synthase
L-NNA = N^ω-nitro-L-arginine
LV+S = left ventricle plus the interventricular septum
mitoROS = mitochondrial-derived reactive oxygen species
mitoTEMPO = 2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride
mRNA = messenger ribonucleic acid
N₂ = nitrogen
NADPH = nicotinamide adenine dinucleotide phosphate
nNOS = neuronal nitric oxide synthase
NO = nitric oxide
NOS = nitric oxide synthase
NOX = nicotinamide adenine dinucleotide phosphate oxidase
O₂ = oxygen
O₂⁻ = superoxide anion
P14 = post-natal day 14
P_a = pulmonary arterial pressure
PAF = platelet activating factor
PASMC = pulmonary arterial smooth muscle cell
P_c = microvascular capillary pressure in the lung
PE-20 = polyethylene tubing 20
PE-90 = polyethylene tubing 90
PE-160 = polyethylene tubing 160
pHTN = pulmonary hypertension

PKC = protein kinase C
PMA = phorbol 12-myristate 13-acetate
 P_v = pulmonary venous pressure
PVR = pulmonary vascular resistance
Q = perfusion rate
RNA = ribonucleic acid
ROK = Rho kinase
ROS = reactive oxygen species
RT-PCR = real-time polymerase chain reaction
RV = right ventricle
RVH = right ventricular hypertrophy
RVSP = right ventricular systolic pressure
SEM = standard error of the mean
shRNA = short hairpin ribonucleic acid
SOCE = store operated calcium entry
SOD = super oxide dismutase
SR = sarcoplasmic reticulum
SSRI = selective serotonin reuptake inhibitor
TBS = tris-buffered saline
TEMPOL = 1-oxy-2,2,6,6-tetramethyl-4-hydroxypiperidine
Tiron = dihydroxy-1,3-benzenedisulfonic acid disodium salt
TTBS = tween-20 plus tris-buffered saline
VEGF = vascular endothelial growth factor
VSM = vascular smooth muscle

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