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DOWNREGULATION OF MYOSIN LIGHT CHAIN PHOSPHATASE ACTIVITY: A MECHANISM OF FAILURE OF VASCULAR RELAXATION IN EARLY HYPOXIC PULMONARY HYPERTENSION

Thesis submitted in partial fulfillment of requirements for
Master of Science degree
Department of Physiology

Shyamala Dakshinamurti

(Supervisor: Dr. N. L. Stephens)

Winnipeg, Manitoba
June 2003

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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Downregulation of Myosin Light Chain Phosphatase Activity:

A Mechanism of Failure of Vascular Relaxation in Early Hypoxic Pulmonary Hypertension

BY

Shyamala Dakshinamurti

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Acknowledgements

First and foremost, I am grateful to Dr Newman Stephens for his guidance, generosity and illuminating example, as well as for his patience. The numerous suggestions and refocusing provided by my advisory committee allowed this research project to develop by a more logical path, and I thank Drs Andrew Halayko, Henrique Rigatto and Peter Zahradka for their intellectual rigour.

The techniques of protein analysis and immunohistochemistry, as well as much that I know of practical labsmanship, were learned from Pat Sheppard under the auspices of Dr Dodd. I was materially assisted in phosphatase activity analysis by the technical expertise of Lynne Mellow. I am fortunate indeed to have had their help. My thanks also go to laboratory colleagues and friends, particularly Cheng and Janani, from whom I have learned so much.

Myosin light chain and myosin light chain kinase for phosphatase assay were kind gifts of Dr Mary Pato, Department of Biochemistry, University of Saskatchewan; CPI-17 antibody was a gift from Dr David Brautigan, Center for Cell Signalling, University of Virginia. During my period of fulltime study, I received support from the Duncan Gordon Clinician Scientist Fellowship from University of Toronto. The research was in part funded by a grant from the Manitoba Institute for Child Health.

The members of the section of Neonatology, and in particular Dr Molly Seshia, have been most supportive of my ambition to learn research by the academic method, in tandem with my clinical fellowship; I remain in their debt.

As ever, I am thankful for the energy, good humour, eagle-eyed proofreading and perspective of my husband and family. As I continue in research, I become more conscious of riding, intellectually speaking, upon the shoulders of my father, and I am grateful for his enthusiasm.

"The aim of science is not to open the door to infinite wisdom, but to set a limit to infinite error."

(Berthold Brecht, *Life of Galileo*)

"That which you know is the sand you hold in your hand; that which you do not know is the entire beach."

(Tamil proverb)

ABSTRACT

Background:
Neonatal persistent pulmonary hypertension (PPHN), a disorder of failed circulatory transition and abnormal pulmonary arterial relaxation consequent to perinatal stressors such as hypoxia, is characterized by pulmonary vasospasm and calcium sensitization of vascular smooth muscle, followed by progressive smooth muscle proliferation and eventual vascular fibrosis. Vascular relaxation is mediated by myosin light chain phosphatase (MLCP).

Objective:
To examine regulation of MLCP activity in the early phase of hypoxic PPHN.

Methods:
PPHN was induced in newborn swine (n=11) by maintenance in normobaric hypoxia (FiO_2 0.10) for 3 days. Controls were age matched normoxic swine (n=9), and newborns (n=11). The diagnosis of pulmonary hypertension was established by presence of cardiac right ventricular afterloading. Paraffin sections of en bloc lung were haematoxylin/eosin stained to examine morphometry of size-matched vessels. 2^{nd} to 4^{th} generation pulmonary arteries were obtained by rapid dissection and freezing in dry ice-cooled acetone. Tissue homogenates were compared for protein content by Western blot using antibodies for smooth muscle protein markers. Phosphorylation state of regulatory proteins MYPT and CPI-17 was measured using phosphospecific antibodies, by Western blot. Absolute phosphatase activity was measured in arterial homogenates incubated with ^{32}P-labelled myosin light chain in presence of kinase inhibitors, expressed as percent decrease from initial counts per minute per µg protein; rate of dephosphorylation was also measured.

Results:
Hypoxic animals had significant right ventricular hypertrophy compared to age matched controls ($p<0.05$). Histological evidence of minimal pulmonary arterial medial thickening indicated that vascular wall remodelling was not a major factor in this increase in pulmonary vascular resistance. Contractile phenotype markers smooth muscle myosin heavy chain and smoothelin were increased in hypoxic subjects compared to age-matched controls ($p<0.05$). Myosin light chain kinase and phosphatase protein content were unchanged in hypoxic pulmonary arteries compared to controls. However, an increase in myosin light chain phosphatase activity was seen in day 3 normoxic animals, and this was ablated by hypoxia; phosphatase activity and rate of myosin dephosphorylation were relatively decreased in the hypoxic group (newborn = 8.68; 3d normoxia = 18.65; 3d hypoxia = 10.55 $\%P_i$ release/µg; $p<0.005$). Increased phosphorylation of CPI-17 was seen in the hypoxic group compared to age-matched controls ($p<0.01$). MYPT phosphorylation was unchanged between groups.

Discussion:
In normal newborns, pulmonary arterial smooth muscle dedifferentiates and myosin phosphatase activity increases in the first 3 days of life. Hypoxia increases content of contractile protein markers, and decreases myosin light chain phosphatase activity. Attenuated phosphatase activity, possibly as a consequence of perinatal CPI-17-mediated regulation, may be contributing to calcium sensitization and failure of smooth muscle relaxation in the clinically relevant early phase of increased pulmonary vascular tone in PPHN.

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List of Abbreviations

ATP	Adenosine triphosphate
au	arbitrary units
CPI-17	C-kinase activated phosphatase inhibitor of 17 KDa
DAG	Diacylglycerol
ERK	Extracellular-signal regulated kinase
GTP	Guanosine triphosphate
HIF	Hypoxia-inducible factor
IP_3	Inositol-3-phosphate
JNK	Jun kinase
kDa	Kilodalton
Kdr	Delayed rectifier potassium channel
Kv	Voltage gated potassium channel
MAPK	Mitogen activated protein kinase
MARKS	Myristolated alanine-rich C-kinase substrate
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MYPT	Myosin phosphatase protein targeting subunit
PDK	Phosphodiesterase kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PP-1	Protein phosphatase class 1
PP-1C	Protein phosphatase class 1, catalytic site
PP-1M	Protein phosphatase class 1, muscle isoform
PP-2	Protein phosphatase class 2
PS	Phosphatidyl serine
RhoK	Rho kinase

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Statement of the Problem

Pulmonary hypertension is perhaps better known as a primary fibrotic disease of the adult pulmonary vasculature than it is as a neonatal phenomenon. Persistent pulmonary hypertension of the newborn (PPHN) is, however, one of the most rapidly progressive and potentially fatal of the vasculopathies, stemming as it does from a developmentally crucial moment of pulmonary vascular relaxation in normal perinatal circulatory transition. Adaptation to extrauterine life requires a complex series of structural, morphological and functional changes in pulmonary vascular endothelium and smooth muscle, resulting in precipitous decrease in pulmonary vascular resistance; this is derailed in the context of PPHN [1]. PPHN has significant incidence (reported range from 0.4 to 6.8 per 1000 births [2]) in otherwise healthy term neonates, often due to abnormal environmental factors such as perinatal hypoxia. Current therapeutic agents, including inhaled nitric oxide, are moderately successful in arresting the course of PPHN; approximately one third of patients meeting the criteria for treatment do not respond to these agents, and in this population the disease is often fatal [3]. Survivors of PPHN may have abnormal pulmonary vasoreactivity well into adulthood [4].

It is now becoming apparent that PPHN represents a common pathway of vascular injury activated by a multiplicity of effectors, and that the time course of its progression is variable. Two phases of this disease have been described, which correlate well with clinical experience: an initial phase of increased pulmonary vascular resistance characterized by fluctuating shunt fraction, and failure of vascular relaxation leading to elevated circuit resistance; and a second phase involving vascular medial remodelling and fibrosis, with a fixed increase in pulmonary vascular resistance due to diminished circuit capacity [5]. Studies using a variety of animal models reveal this

endstage pathology is common to all etiologies of PPHN [6], and indeed is little different from the endstage pathologies described in systemic hypertension, atherosclerosis and post-angioplasty restenosis, as well as the related phenomena of airway remodelling and functional smooth muscle change in diseases such as asthma.

Investigation of an endstage phenomenon is useful for revealing concordances of pathophysiology between disparate diseases, and in the delineation of a pathological worst-case scenario; it does not necessarily illuminate causative processes that are important in the early evolution of a disease, nor offer opportunities for early intervention. To this end, this study focuses on the clinically relevant initial phase of hypoxic PPHN, and is limited to an examination of pathways contributing to vascular relaxation failure at the level of the effector organ, pulmonary arterial smooth muscle. This choice is rationalized by the fact that isolated endothelial dysfunction is not always the initiator of the cascade of vascular changes constituting neonatal pulmonary hypertension, and is rarely the limiting factor with respect to treatment failure; smooth muscle is directly responsible for the defining defect in pulmonary arterial relaxation in PPHN, and also for the irreversible vascular remodelling that closes the therapeutic window.

Smooth muscle relaxation is mediated by myosin light chain phosphatase. Diminished myosin light chain phosphatase activity has been described in the terminal stage of a vascular overflow model of PPHN [7]; this is also known to occur in smooth muscle cell culture as a consequence of mechanical strain [8]. Regulation of myosin phosphatase is hence being recognized as a major pathway of smooth muscle calcium sensitization [9]. The onset of PPHN is teleologically linked to impaired developmental regulation of pulmonary circuit-specific vascular relaxation. It is logical, therefore, to pursue the contribution of pulmonary arterial myosin light chain phosphatase regulation to the etiology of hypoxic PPHN.

Persistent Pulmonary Hypertension of the Newborn

Pulmonary hypertension of the newborn (PPHN) is a disorder of vascular transition from fetal to neonatal circulation in the term infant, with an incidence of about 1 in 5000 live term births. It represents a common pathway of injury response following a multiplicity of perinatal stresses, including hypoxia, sepsis and direct lung injury such as meconium aspiration.

Pulmonary hypertension manifests in the term or near term neonate as hypoxemic respiratory failure [3]. It is characterized by high pulmonary vascular tone, such that the oxygen exchange site of the pulmonary capillary bed is bypassed by intracardiac, extracardiac or intrapulmonary shunting towards the systemic circulation [6]. For this reason, it was originally described as "persistent fetal circulation". All etiologies of PPHN result in a critical decrease in tissue oxygen delivery [10]. The etiologic agents are largely perinatal in onset; there is no major genetic risk factor, although some underlying conditions such as maternal diabetes can predispose infants toward PPHN for reasons of altered neonatal pulmonary surfactant physiology.

Distinguishing the Systemic and Pulmonary Circuits

Much of the available knowledge on blood pressure regulation in a muscular circuit is derived from systemic arteries. The systemic circulation, upon dissociation from the placenta, exists in a high-pressure state. Systemic blood pressure, like pulmonary pressure, is a product of cardiac output (stroke volume and heart rate) and vascular resistance [11]. Systemic vascular resistance is controlled locally by nitric oxide/endothelin balance maintained by the endothelium [12], and also controlled hormonally by a variety of neurohumoral mediators (detailed pictorially in Fig 1), including adrenal catecholamines,

the renin-angiotensin system, atrial natriuretic peptide, vasopressin and the kallikrein-kinin system [13]. Systemic hypertension is a chronic disease of multifactorial etiology, generally developing over the course of years. It involves increased arterial wall stiffness due to remodelling and calcification, loss of smooth muscle responsiveness to vasoactive agents with diminished rate of force development and delayed relaxation, and altered intracellular calcium flux [11]. More rapidly progressive hypertensive disorders involving the systemic circuit include post-angioplasty injury, which results in increased coronary vasospastic reponse to agonists and rapid vascular remodelling leading to restenosis. Placental vascular dysfunction, associated with the pre-ecclamptic syndrome of systemic hypertension, may also be considered in this category.

The pulmonary circuit bears some striking dissimilarities from the systemic circuit. The main mechanistic determinant of pulmonary blood flow is the collapsible nature of pulmonary capillaries; surrounded by alveolar pressure, they function as Starling resistors. In other words, when alveolar pressure exceeds pulmonary arterial pressure, which is in turn greater than pulmonary venous pressure, no flow occurs through the capillary; the gradient between arterial pressure, alveolar pressure and venous pressure determines flow. This results in the variable distribution of flow within the three zones of the upright lung [14]. The resistance of the pulmonary circuit is conventionally calculated as the difference between pulmonary arterial pressure and left atrial pressure, divided by the cardiac output. Pulmonary vascular resistance is highly controlled, both in utero and after birth, by redundant systems. Sympathetic adrenergic innervation of the pulmonary vasculature extends from the stellate ganglion, and parasympathetic cholinergic vasodilator fibers from the vagus nerve [15]. However, neurogenic control of the pulmonary circuit is probably slight [16]. In contrast, pulmonary responsiveness to local chemical and blood-borne humoral agents is remarkable, in

most cases the response being the opposite of that in systemic vasculature. Vasoconstriction is produced by a number of systemic vasodilators, including hypoxia, hypercapnia, acidosis, histamine, serotonin and norepinephrine [17]. Responses to vasoconstrictors and vasodilators vary regionally. Endothelin maximally constricts isolated pulmonary arteries of all sizes; conduit pulmonary arteries constrict to serotonin, while resistance arteries do not, and veins dilate. Thromboxane is a potent constrictor of resistance, but not conduit arteries; in contrast, norepinephrine response is progressively attenuated with decreasing arterial diameter. Acetylcholine is a poor vasodilator, while bradykinin relaxes larger arteries and nitric oxide dilates pulmonary arteries and veins [18]. Oxygen is the most powerful vasodilator of the pulmonary circulation, responsible for perinatal vascular relaxation, as well as for regional matching of blood flow with ventilation [19].

The need for rapid decrease in resistance at parturition is a distinguishing feature of the pulmonary circuit, and it may be argued that most mechanisms regulating flow through this vascular bed in the adult, including hypoxic pulmonary vasoconstriction, may be the residua of tightly controlled processes at circulatory transition.

Developing Knowledge of Circulatory Transition

To place the investigation of perinatal pulmonary hypertension into historical context, knowledge of the development of pulmonary circulation in series with systemic circulation, and of its importance for the arterialization of venous blood, is of very long standing; according to a historical review by A.M. Rudolph, it was almost three hundred and fifty years ago when Lower envisioned that fetal pulmonary blood flow was very low, and that it increased greatly at the time of birth [19]. This concept was not further developed, however, until the 1930's when Barclay and colleagues conclusively

demonstrated the increase in pulmonary blood flow after establishment of breathing by angiography [19]. Initial hypotheses regarding circulatory transition focused upon gaseous expansion of the lung as being responsible for decreased pulmonary vascular resistance as a direct physical effect, as well as on the pulmonary vasodilator effects of increased arterial oxygenation [20]. As perinatal endocrine changes were investigated, it became evident that oxygen did not merely act as a direct vasorelaxant, but mediated the release of an intermediate substance responsible for the maintenance of low pulmonary resistance. Bradykinin was an initial candidate for this role, as it is induced by oxygen [21], but it is only briefly elevated in the postnatal infant, and receptor blockade was not seen to alter the course of circulatory transition [22]. Another known pulmonary vasodilator, Prostaglandin D_2 was also explored, but inhibition of prostaglandin synthesis was not sufficient to impede the fall in pulmonary resistance [23]. The obligatory role of the vascular endothelium in acetylcholine-induced vasorelaxation suggested the elaboration of an endothelium-derived relaxing factor important in the perinatal decrease in pulmonary vascular tone [24]; this was later shown to be nitric oxide [25]. This discovery led to a flurry of clinical investigation into the utility of nitric oxide in treating disorders of failed circulatory transition, notably PPHN.

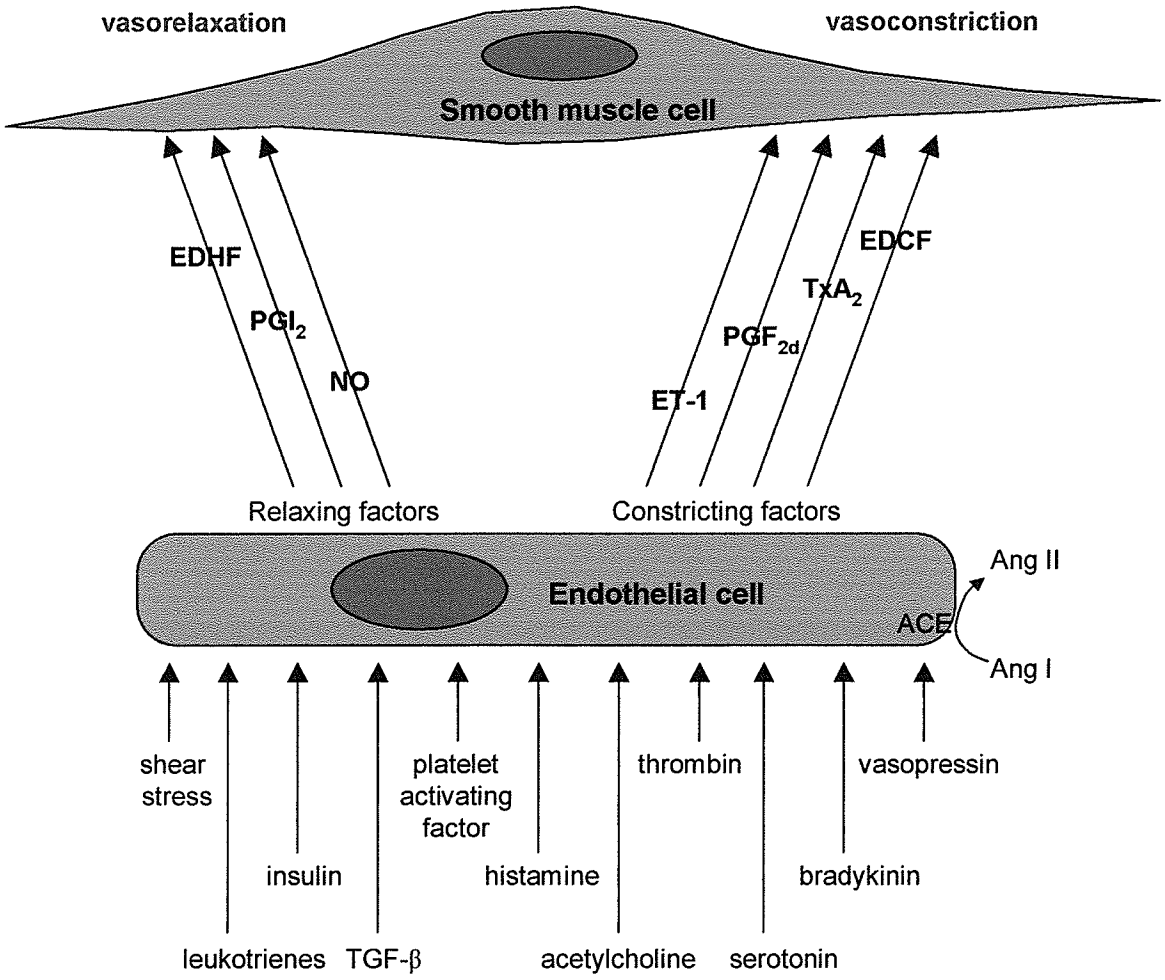
Current therapeutic approaches to PPHN include inhaled gaseous nitric oxide, a short-acting vasodilator that acts selectively on the pulmonary circulation, which has revolutionized the treatment of this condition [3]. However, inhaled nitric oxide has not proven effective in all patients; approximately one third of patients with severe PPHN fail to respond [26]. Early responses are not always sustained; non-responders may have a differing time course of pulmonary pathology, resulting in decreased sensitivity to nitric oxide therapy [27]. Other therapeutic approaches to PPHN have been limited by a relative lack of specificity for the pulmonary circulation, and have received less attention.

Alkalinization appears to act through the same pathway as nitric oxide in achieving local vasodilation [28]. Systemic vasoconstrictors are employed to decrease shunt fraction by increasing systemic blood pressure, but differential effects on pulmonary circuit resistance may result in maintenance or even elevation of the pulmonary to systemic pressure ratio; this approach is falling out of favour [29]. Pharmacologic approaches, including pulmonary surfactant, phosphodiesterase inhibitors, prostacyclin, endothelin antagonists, calcium channel blockers, magnesium sulfate, and tolazoline, have exhibited varying degrees of efficacy in lowering pulmonary vascular pressures in humans and/or animals [30].

While it has been convenient to characterize PPHN as endothelial dysfunction resulting in nitric oxide deficiency, patterns of response to exogenous nitric oxide have called this simplification into question. Consideration of the various mechanisms of vascular injury at play in neonatal pulmonary hypertension reveals a broad range of potential therapeutic targets. As with other multifactorial diseases, a single inciting event may be augmented by multiple concurrent or subsequent phenomena that result in differing courses of disease progression; consequently, it is difficult to target a single effector.

A careful examination of normal perinatal circulatory transition, and the various mechanisms by which this may be disrupted, is essential when commencing investigation of alternative approaches to the management of PPHN.

Fig. 1: Interactions between endothelial cells and smooth muscle cells



Many humoral mediators trigger vasoconstrictive or vasorelaxant signalling from endothelium to smooth muscle, allowing for tight regulation of local vascular tone. Effects of specific humoral agonists often differ between systemic and pulmonary circulations.

(modified from Navar LG, Med Clin NA 1997)

Natural History of PPHN:

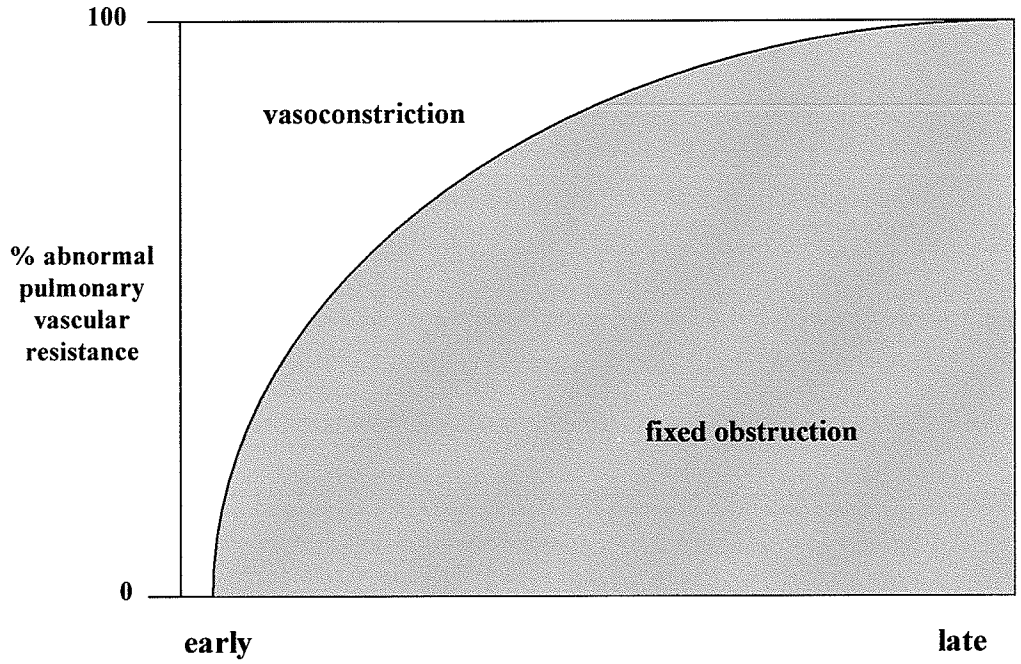
The pulmonary vascular bed in utero receives only about 8% of the cardiac output, as the placenta acts as the organ of gas exchange [6]. Fetal pulmonary vascular resistance is high. Rapidly after birth, the pulmonary circulation must dilate to accommodate the entire cardiac output; triggers for this include tidal expansion of the lungs and increased oxygenation, increased endogenous nitric oxide, PGI_2 and bradykinin, and endocrine changes at parturition [31]. Some of this increase in capacity is accounted for simply by the distension of the lungs with air, and the reduction in turbulence associated with straightening of kinked vessels. Remodelling of the vascular wall also occurs at birth [32]. High fetal pulmonary vascular resistance is ascribed to the shape and arrangement of smooth muscle cells within the vessel wall; in morphometric analysis of human autopsy specimens, fetal arteries are described as lined by brick-shaped smooth muscle cells in a tightly compressed media with a narrow arterial lumen; during transition, these cells are seen to elongate and decrease in thickness to the classical spindle shape, and endothelial cells flatten as the calibre of the lumen enlarges. After a month, remodeling occurs more slowly with growth, involving an increase in wall thickness, connective tissue deposition, and smooth muscle cell maturation [33]. The changes in the vascular smooth muscle cell involve both cytoskeletal reorganization and relaxation of the contractile machinery, which in utero is thought to exist in a low energy-utilizing tonic state [34]. Smooth muscle cell populations with differing proliferative potentials are distinguishable after birth, based on predominance patterns of different cellular markers.

Persistent pulmonary hypertension of the newborn (PPHN) is defined as a failure of normal pulmonary vascular relaxation at or shortly after birth [6], resulting in impedance to pulmonary blood flow which exceeds systemic vascular resistance, such that

unoxygenated blood is shunted to the systemic circulation. Perinatal stressors including hypoxia, hypoglycemia, cold stress, sepsis and direct lung injury alter the course of the transition process.

The initial clinical picture of PPHN is one of dynamic pulmonary vasospasm, with labile flow through the pulmonary circuit and right to left shunting of blood across the ductus arteriosus [6]. The normal postnatal decline in pulmonary vascular tone is absent following exposure to chronic hypoxia [35]. Variable right to left extracardiac shunting and dynamic pulmonary vascular tone, which in some patients responds to local vasodilator therapy, mark the initial stages of pulmonary hypertension. The most severe instances of pulmonary hypertension have been reported to have such severe intracardiac shunting that left ventricular filling is adversely affected, resulting in reversal of aortic arch flow [36]. Late PPHN is typically less amenable to therapeutic intervention, as vasodilator responsiveness is lost at this stage [37]. The histology of end-stage (14 day) pulmonary hypertension is characterized by thickened vascular media, with ultra-structural microscopy revealing hyperplasia and hypertrophy of the vascular smooth muscle layer as well as increased extracellular matrix deposition [38]. The excess collagen deposition increases vascular stiffness and impairs distensibility [39]. This fibrosis results in a fixed increase in pulmonary vascular resistance, which is not reversible. The biphasic course of pulmonary hypertension is delineated in Figure 2; the timeframe of disease progression and hence the slope of the curve separating vasoconstrictive hypertension from fibrotic hypertension varies between different pathophysiologies [40].

Fig. 2: Natural history of pulmonary hypertension



Effect of passage of time in pulmonary hypertension: the hypertensive component owing to vasoconstriction decreases while that due to fixed obstruction increases.

This model is applicable to both neonatal and primary pulmonary hypertension, although the timeframes are disease-specific; days in the former, and years in the latter.

Modified from Barst RJ, Pediatr Clin NA 1999

Mechanisms of Pathogenesis:

Several etiologic mechanisms have been delineated. Particularly in the context of sepsis or meconium aspiration, there is an immediate increase in *circulating vasoactive substances* such as inflammatory cytokines TNF-α, IL-1B, IL-6, IL-10 [41] [42], and an increased thromboxane:prostacyclin ratio [43]. These trigger second messenger pathways favouring contraction and smooth muscle proliferation [44]. Cyclooxygenase pathway metabolites have been implicated in altered vascular responsiveness. Pulmonary cyclooxygenase-2 upregulation in response to endotoxin challenge occurs in a complex, cell-specific manner, including in bronchial epithelium, vascular endothelium and smooth muscle cells [45]. Hypoxic pulmonary vasoconstriction is potentiated by prestimulation with thromboxane mimetic U46619 [46]. Pulmonary acetycholine-mediated vasorelaxation is blunted, although nitric oxide response is unchanged, following short course hypoxia in a newborn swine model. Conversely, hypoxia engenders a constrictor response to cholinergic stimulation, mediated by thromboxane [47]. The early development of thromboxane-mediated constriction is postulated to contribute to the pathogenesis of PPHN. U46619 itself has a calcium sensitizing effect, through pharmacomechanical coupling via a G-protein coupled mechanism, resulting in greater force generation for a given extent of calcium mobilization compared to KCl stimulation [48].

Inflammatory conditions such as sepsis cause induction of endothelial nitric oxide synthesis, attenuating adrenergic and endothelin-mediated vasoconstriction; however thromboxane A_2 responsiveness is unaltered, which may explain the coexistence during sepsis of pulmonary hypertension and lung NOS induction [49]. Enhanced prostanoid generation occurs during endotoxemia and sepsis, consequent to upregulation of cyclooxygenase-2. This endotoxin priming results in enhanced vasoconstrictor responses to secondarily applied stimuli, including the increased level of thromboxane

A_2 [50]. A reduction in cyclooxygenase and prostacyclin synthase enzymes has also been reported in early hypoxic PPHN, suggesting that altered production of arachidonic acid metabolites may contribute to the mechanism of hypoxic vasoconstriction [51].

An etiology of pulmonary hypertension under much scrutiny currently is *endothelial dysfunction*. Nitric oxide functions as a regulator of local vascular smooth muscle tone [52] as well as a modifier of proliferative activation [53]. There is tonic generation of nitric oxide originating within the endothelium which acts postjunctionally to activate a cGMP-dependent pathway within the smooth muscle, with no prejunctional effect [54]. Endothelial nitric oxide synthesis is developmentally regulated during lung embryogenesis, with increased activity of endothelial and inducible isoforms of nitric oxide synthase during the third trimester [55]. The transition from placental supported respiration to air breathing at birth abruptly upregulates NOS activity [56]. NO acts through activation of smooth muscle soluble guanylate cyclase and cGMP, leading to smooth muscle relaxation through regulation of extracellular calcium mobilization [57], as well as through cGMP-independent mechanisms through the enhancement of sarcoplasmic reticulum calcium reuptake [58]. NO also suppresses hypoxic induction of vascular endothelial growth factor (VEGF) and smooth muscle proliferation [59]. Hypertensive lungs have been shown to have diminished immunoreactive endothelial nitric oxide synthase, as well as decreased vascular smooth muscle guanylyl cyclase activity, and a loss of the normal postnatal decline in cGMP-specific phosphodiesterase activity [60]. Exhaled NO is reduced early in the course of hypoxic PPHN, primarily as a result of decreased airway epithelial production [61]; loss of endothelial NOS activity is recorded only after prolonged (two week) hypoxic exposure, in conjunction with a decreased smooth muscle sensitivity to NO, suggesting the impairment of downstream cGMP-dependent pathways [62]. There is increased expression of endothelin-1 [63] and vascular endothelial growth factor-1 [64 65].

Both these growth factors, as well as the loss of nitric oxide's antiproliferative effect, result in activation smooth muscle cell synthetic functions [66]. Impaired endothelium-dependant relaxation is addressed clinically by treatment with exogenous nitric oxide [3]. The recovery phase following NO treatment is characterized by normalization of endogenous NO production, but vascular smooth muscle relaxation lags behind endothelial recovery [67]. Treatment of hypoxic animals with calcium channel blockade ameliorates the development of increased vascular resistance but does not alter the downregulation of eNOS, indicating that NOS expression is directly linked to oxygen tension [37].

Hypoxia is known to be a major player in this pathology. Exposure to chronic hypoxia has been shown to inhibit the postnatal maturation of pulmonary arterial relaxation by both endothelium-dependant and independent means, impairing both nitric oxide release and its downstream effect on vascular smooth muscle [35]. Hypoxia results in attenuated response to pharmacological relaxants, and has a priming effect resulting in exaggerated contraction to subsequent or repeated hypoxic exposure [68]. The low oxygen tension alters the redox state of potassium channel proteins resulting in relative depolarization (this is also the mechanism of hypoxic pulmonary vasoconstriction) [69], and also decreases Kdr channel activity and transcription of Kv channel protein [70]. Differences in mitochondrial function between systemic and pulmonary vascular smooth muscle cells, including mitochondrial membrane potassium current and elaboration of activated oxygen species, are hypothesized to contribute to differences in oxygen sensing [71].

Failure of pulmonary circuit dilation results in diminished capacity, and if subjected to high flow (the unrelieved right ventricular end diastolic volume), significant wall strain. As part of the injury-response programming of the vascular wall, *mechanical*

factors are implicated in the pathogenesis of hypertension. Shear stress due to turbulent, high pressure blood flow results in the transient stretch-activated increase in smooth muscle membrane potassium permeability [70]. Resting preload regulates contractile responsiveness of vascular smooth muscle to various stimulations, mainly by modulating the calcium-force relationship, without affecting the extent of intracellular calcium elevation; this suggests the involvement of calcium sensitization pathways in stretch-dependent regulation of the contractile apparatus [72]. Cyclic strain of smooth muscle in culture increases myosin light chain kinase content, and smooth muscle cell shortening and velocity [73], and also decreases myosin phosphatase activity [8]. Increased vascular constriction in response to increased intravascular pressure may be masked under normal conditions by compensatory endothelial nitric oxide synthesis, but not in PPHN, where nitric oxide synthesis is impaired [74].

Concurrent to calcium sensitization is an increase in contractile cell content, which may arise as a consequence of increased activation of smooth muscle proliferation, or of cellular hypertrophy. A myogenic response has been described in the normal pulmonary circuit in response to strain, resulting in increased vascular contraction [75]. Stretch-induced force generation is increased in perinatal pulmonary circulation [76]. Cyclic strain of vascular smooth muscle in culture results in mitogen activated protein kinase (MAPK) pathway activation, as well as JNK [77]. Shear stress and turbulent high pressure flow induces activation of the protein kinase C (PKC) pathway in smooth muscle cells [78], despite counterregulation via endothelial activation [79]. In the whole animal, high pulmonary blood flow engendered by chronic aortopulmonary vascular graft results in impaired endothelium-dependent relaxation [80 81]. There is also evidence of shear-induced expression of endothelial growth factors such as platelet-derived growth factor [79], and activation of smooth muscle mitotic pathways resulting in

downstream transcription of stress-activated protein kinase and early response gene c-jun [82]. The PKC and RhoA pathways, which are linked to regulation of protein transcription and mitosis, are also connected to calcium sensitization and regulation of MLCP through phosphorylation of the targeting subunit of MLCP and its regulatory protein CPI-17 [83] [84]. These pathways are therefore likely intermediaries connecting regulation of contraction to activation of smooth muscle cell proliferation in the context of mechanical strain.

Altered smooth muscle function has been implicated in the pathogenic process of PPHN, and not just as the effector of endothelial dysfunction. At the level of contractile regulation, it has been noted that endothelium-independent pulmonary vascular relaxation is impaired in PPHN [85]; the finding of decreased vascular myosin light chain phosphatase (MLCP) content and activity after two weeks of flow-induced PPHN also suggests the possibility of slowed myosin light chain dephosphorylation [7]. Calcium sensitization of smooth muscle is directly correlated with increased force accrual to constant stimulus, mediated by change in myosin light chain kinase/phosphatase activity ratio [86]. Knowing that up to one third of patients with PPHN fail to respond to inhaled nitric oxide [26], it is appropriate to focus on smooth muscle as a second level of relaxation dysfunction.

Vasospasm may be a consequence of an altered dynamic equilibrium of contraction and relaxation elements; but this itself may be part of a larger process, involving the inexorable transition of the smooth muscle cell from a contractile phenotype to one capable of mounting the injury response of matrix secretion and proliferation [87]. This type of *phenotypic modulation* is well described in response to intimal injury or following balloon angioplasty [88]. The extremes of the phenotypic continuum are the fully contractile muscle cell with few synthetic organelles and no capability of proliferation,

versus the synthetic smooth muscle phenotype (a term which may overlap with the myofibroblast), which is migratory, secretory and capable of division, but with little contractile machinery [89]. It is known that vascular smooth muscle consists of heterogeneous populations in a range of phenotypes, which expand in response to local factors [90] [91]. Adjacent smooth muscle cell populations in the aortic media display immunohistochemically different profiles of myosin heavy chain isoforms indicating contractile and synthetic phenotypes [92]. Electrophysiologically distinct populations of myocytes have been shown to determine local responses to hypoxia and vasodilator agents [93]. In vascular intimal injury, expression of markers indicating phenotypic modulation precedes intimal smooth muscle proliferation [94]. Phenotypically unique smooth muscle cell subpopulations have been isolated from the inner, middle, and outer layers of the arterial media; they exhibit markedly different growth capabilities, and differences in cell phenotype demonstrated by morphological appearance and differential expression of muscle-specific proteins [95]. Cells may reversibly convert between contractile and noncontractile phenotypes [96]. Serum deprivation of postconfluent cultured smooth muscle results in selection of a population of hypercontractile phenotype characterized by increased myosin kinase content and contraction velocity [97]. Phenotypic modulation of vascular smooth muscle cells in culture is associated with reorganization of contractile and cytosketetal proteins. Contractile cells highly express smooth muscle-specific markers compartmentalized in the contractile apparatus, including α-sm-actin, sm-myosin heavy chain, and calponin. Structural proteins α-actinin and vinculin are concentrated at the cell periphery, reflecting importance in cell stabilization and adhesion. Synthetic smooth muscle cells have decreased expression of contractile and adhesion proteins, including regulatory enzymes such as the smooth muscle isoform of myosin light chain kinase, and have a concomitant increase in the cytoskeletal proteins non-muscle actin and vimentin; the

distribution of structural proteins also alters, without the distinct compartmentalization apparent in functionally contractile cells [98]. A similar alteration in protein expression profile is borne out in examination of airway smooth muscle phenotypic plasticity [87].

Phenotype is developmentally regulated. The smooth muscle myosin heavy chain isoform SM-B, likely indicating a highly contractile phenotype, is not seen in rat fetal pulmonary vascular smooth muscle until birth, when it first distributes to trachea and bronchi of saccular lung. As development proceeds, it spreads distally through airways, expressed heterogeneously in the smooth muscle population. Blood vessels do not express this marker until adulthood, and then only in small resistance arterioles; it is hypothesized that the phasic contractile phenotype conferred by this myosin isoform is relevant to ventilation/perfusion matching [99]. The normal course of human neonatal cardiovascular transition involves a rapid change in smooth muscle cytoskeletal architecture in the pulmonary vascular media, from brick-shaped and dense to elongate in shape [1]. Within the first week of life, a transient reduction in immunohistochemical expression of smooth muscle myosin heavy chain (MHC) isoform and vinculin is reported in the pulmonary vascular media, associated with a transient increase in cell proliferation. The actin content also decreases perinatally, followed by a postnatal, permanent increase in proportion of monomeric to filamentous actin, involving first the alpha then the gamma isoform [100]. The relative proportions of smooth muscle and non-muscle MHC isoform expression do not change between birth and adulthood in elastic pulmonary arteries, but in muscular arteries the smooth muscle isoforms increase after the second week of life. Hypoxic pulmonary hypertension prevents both the postnatal burst of medial smooth muscle replication, and the transient postnatal reduction in actin content and myosin isoform switching. These findings suggest that rapid remodelling of the actin cytoskeleton and phenotypic modulation is an essential prerequisite of the

normal postnatal fall in pulmonary vascular resistance, and that this progression is fundamentally altered by hypoxia [100]. The time course of hypoxic exposure is key; in animals hypoxic from birth, medial thickness is increased in resistance (but not elastic) arteries, because endothelial and smooth muscle cells retain their fetal shape, position, overlap, interdigitation and the low surface to volume ratio characteristic of fetal life. Perinatal hypoxia also results in increased smooth muscle myofilament density, without concomitant smooth muscle hypertrophy or hyperplasia. Animals exposed to hypoxia after two weeks of life have normal postnatal maturation of pulmonary vascular smooth muscle shape and surface to volume ratio. This indicates that short periods of exposure to neonatal hypoxia impairs adaptation and appears to potentiate contractile capacity in stiff-walled arteries; this response is less marked after two weeks of age [101].

Hypoxia itself favours both contraction consequent to increase in IP_3 [102], and proliferation through protein kinase C (PKC) activation [103] and elaboration of the transcription factor HIF-1α [104] [105], which in turn regulates transcription of many genes involved in smooth muscle growth including VEGF [106]. HIF-1α is critical for hypoxia-induced reduction in K^+ current density, and smooth muscle hypertrophy [107]. It is tightly coupled to oxygen tension, typically induced by tissue oxygen concentrations below 4% [108]; however, this may in fact be more stringent than the level of hypoxia experienced by pulmonary vascular smooth muscle in PPHN, given the small alveolar/arterial diffusion gradient. Hypoxia activates stress-activated protein kinase (JNK), extracellular signal-regulated protein kinase (ERK), and p38 kinase in pulmonary arteries [82]. Cultured pulmonary arterial smooth muscle cells exposed to hypoxia for short periods of time display no alteration in phenotype, although endothelial cells in the same conditions do decrease their myofibrillar density and actin content [109]. Prolonged hypoxia has been shown to stimulate the proliferation of a population of cells from the vascular media

which stain negatively for smooth muscle myosin heavy chain (sm-MHC), grow rapidly and are capable of secreting mitogens [110]. Late stage PPHN is characterized by a predominance of myofibroblasts in the vascular media and adventitia [111] [112]. The elevation of pulmonary vascular resistance in prolonged hypoxia is largely due to remodelling and fibrosis mediated by these synthetic cells, with a minimal component of smooth muscle contractile function, as demonstrated by failure of vascular relaxation on treatment with papaverine [85].

The pathophysiology of neonatal pulmonary hypertension involves multiple pathways of injury, from altered circulating agonist balance, to endothelial dysfunction, to smooth muscle dysfunction and phenotypic change. Analysis of the time course of the disease process suggests that any or all of these factors may be operative in a single patient, as additional effector pathways are activated in a time-sensitive fashion. It is thus difficult to identify a single crucial element in the pathogenesis of this disease, the relief of which would uniformly reverse its progression.

In this research project, the focus has been limited to smooth muscle dysfunction as many of the pathways of injury discussed above converge upon this point; amelioration of excessive vascular smooth muscle contraction is most important to the current therapeutic approach to PPHN, while widening of the therapeutic window for any such interventions would result from a delay in activation of vascular smooth muscle proliferation.

Regulation of Contraction in Vascular Smooth Muscle

Smooth muscle has been described as "strong, slow and economical", such that physiologic functions involving sustained, tonic contraction may be carried out with least expense of cellular energy [113]. It also carries out more rapid contractile activity in tissues characterized by rapid force development; this phasic smooth muscle is capable of spontaneously generated contraction, while tonic smooth muscle is not capable of spontaneous activity and has slower contraction kinetics [114].

Smooth muscle activation is dependent upon structure; most visceral smooth muscle is single unit, while most vascular smooth muscle is in multi-unit arrays. Single unit smooth muscle is activated by neuronally-independent myogenic mechanisms, by virtue of spontaneously arising or pacemaker-driven membrane depolarizations that are propagated through the functional syncytium via gap junctions. Multi-unit smooth muscle is made up of functionally discrete units as in skeletal muscle, which must be activated independently by innervation at sympathetic and parasympathetic nerve terminals [115]. Neural control of smooth muscle contraction is well studied in the context of hypertension, where sympathetic overactivity contributes to increased vascular smooth muscle tone via central neural α_2 adrenergic receptors which modulate sympathetic neural outflow, and peripheral excitatory discharge of postganglionic α-adrenergic sympathetic fibers [116]. Neurotransmitter release causes electrical depolarization of smooth muscle, involving the opening of voltage-operated calcium channels, followed by repolarization of the cell by rectifier potassium channels. Neuroendocrine mechanisms of smooth muscle contraction are also important, with sympathetic outputs resulting in altered hormonal homeostasis, such as the renin-angiotensin system in vascular smooth muscle.

The structure and function of the contractile unit of smooth muscle can be modulated dramatically by phenotypic shift toward more synthetic, less contractile states [89], or by more subtle changes toward faster or slower contractile phenotype, in response to hormonal stimulation or agonist-induced calcium sensitization [113].

Phosphorylation of the 20 kDa subunit of myosin plays a central role in regulation of smooth muscle contraction. Phosphorylation of both smooth muscle and non-muscle myosin promotes molecular assembly into filaments and actin interaction [117]. In vitro experiments have demonstrated that phosphorylation of myosin light chain increases actin-associated myosin ATPase activity in a graded manner [118]. Myosin light chain phosphorylation at the serine 19 and threonine 18 residues enhances both velocity and force of the actomyosin crossbridging cycle as a consequence of increased Mg^{2+} ATPase activity of myosin [119]. Force is mainly regulated by slowly cycling crossbridges, which have lower levels of myosin light chain phosphorylation. Following electrical stimulation of airway smooth muscle, there is a 500msec period of latency; thereafter there is a linear relation between tissue stiffness and myosin phosphorylation during activation, followed by a slower accrual of isometric force [120].

The main pathway of activation of smooth muscle is calcium binding to calmodulin, which activates the enzyme myosin light chain kinase (MLCK). Upon cell surface receptor activation, cleavage of membrane phospholipids by phospholipase C results in formation of intermediary molecule IP_3, which rapidly induces calcium-mediated calcium release from sarcoplasmic reticulum stores by binding to IP_3 receptors on the SR. to bind calmodulin; the binary complex, consisting of four calcium molecules with each calmodulin effector, then binds to MLCK to form an activated ternary complex, which phosphorylates the 20 kDa myosin light chain. Monophosphorylation of the serine 19 site on myosin regulatory light chain by myosin light chain kinase is the primary

phosphorylation that is associated with activation of smooth muscle contraction. It appears that the rate of conversion of myosin light chain kinase from an inactive to an active enzyme by the calcium/calmodulin complex may be the rate limiting step for the initiation of myosin light chain phosphorylation [120].

Yamashita and colleagues have described calcium availability in smooth muscle at three locations: bound calcium on the external surface of the cell membrane at sialic acid residues, calcium localized to the membrane inner surface, and calcium in intracellular stores such as the sarcoplasmic reticulum. Depolarization-induced contraction due to potassium requires influx of extracellular calcium; calcium stored intracellularly may be mobilized by the action of certain hormonal and chemical agonists [121]. Potassium-mediated contraction of smooth muscle cells is entirely dependent on function of voltage-gated calcium channels; antagonists of these channels attenuate but do not block G-protein-coupled receptor-mediated reactivity, as mobilization of intracellular calcium may be sufficient for contraction [122]; potassium can also release neurotransmitters from synaptic terminals by nerve depolarization, which is the probable route of potassium-mediated contraction in the whole tissue. The pressor response to thromboxane depends upon both intracellular and extracellular calcium sources to achieve calmodulin-dependent vasoconstriction, unlike other prostanoids which are independent of calmodulin activation [123]. Agonist sensitivity, measured by extent of maximal calcium mobilization in response to thromboxane receptor agonist or KCl, also varies inversely with resting smooth muscle load, so that mural remodelling and stiffening may eventually attenuate agonist response [124]. Contraction of normally polarized smooth muscle can be regulated through pharmacomechanical coupling, which is independent of membrane voltage and voltage-gated calcium channels. Pharmacomechanical regulation of cytosolic calcium is largely, though not solely,

controlled by the phosphatidylinositol cascade and the calcium pumps of the plasma membrane and sarcoplasmic reticulum [125]. Upon activation of cell surface receptors by neurotransmitters or hormones, phosphoinositide metabolism is stimulated to form IP_3. The IP_3 binds to sarcoplasmic reticulum receptors and causes calcium release, which then binds to calmodulin. As described above, the calcium/calmodulin complex activates myosin light chain kinase [126].

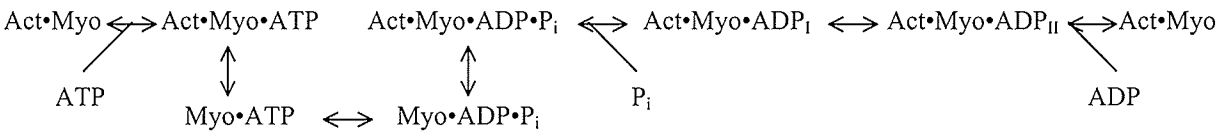
Myosin light chain kinase activation is responsible for the rate-limiting step of myosin phosphorylation in smooth muscle. The enzyme is found tightly bound to actomyosin-containing filaments, as a consequence of targeting of the C-terminal domain, or of its independently expressed congener telokin, to unphosphorylated myosin [127]. The MLCK catalytic domain contains residues on its surface that bind a regulatory segment, resulting in autoinhibition. When bound to calcium/calmodulin, the regulatory segment is displaced from the catalytic cleft, allowing phosphorylation of myosin regulatory light chain. Kinase activity depends upon calcium/calmodulin binding to the calmodulin-binding sequence, and also to additional interactions between calcium/ calmodulin and the catalytic core [128].

Two major isoforms of MLCK have been characterized. The 220kDa non- muscle MLCK isoform is widely expressed in smooth muscle and non-muscle tissues, colocalizing with interconnected bundles of F-actin. The 130 kDa smooth muscle isoform is also highly expressed in many adult tissues, including skeletal and cardiac muscle [129] as well as smooth muscle, and is also is detectable during embryonic development [130], colocalizing with nonmuscle myosin IIA but not with myosin IIB or F-actin [131]. Downregulation of smooth muscle MLCK expression in cultured smooth muscle cells results in attenuated tension development on agonist stimulation; additional

downregulation of non-muscle MLCK does not cause further decrease in tension, indicating no role for the non-muscle isoform in contraction [132].

MLCK is known to be activated by cytosolic calcium levels higher than 10^{-7} M, in a logarithmic manner. Most tissue MLCK is activated upon electrical stimulation of airway smooth muscle, resulting in calculated maximal cellular rates of light chain phosphorylation similar to measured values. The temporal correlation between light chain phosphorylation and maximal velocity of shortening supports the hypothesis that myosin phosphorylation in smooth muscle functions in regulating cross-bridge cycling rates [133]. Shortening velocity of skinned smooth muscle is dependent on the degree of activation of myosin light chain kinase (MLCK), as determined by calcium availability; lower calcium levels are associated with low ATPase activity, low shortening velocity and low force generation. Variations in myosin phosphatase (MLCP) activity by inhibition with okadaic acid also change force and shortening velocity, showing that extent of myosin light chain phosphorylation, rather than calcium level alone, is responsible for the modulation of cross bridge kinetics [113].

Crossbridge interaction between myosin and actin is described as:



(rapidly cycling crossbridges) (slowly cycling crossbridges)

(from Arner and Malmqvist, 1998 [113])

Actomyosin crossbridges in smooth muscle exist in a weakly bound state (Act•Myo in schematic above) when in low cellular calcium concentration at rest, and transition to a

strongly bound conformation upon activation, which is then responsible for fibre shortening. These structural changes have been demonstrated by Xray diffraction studies [134]. Upon contractile activation by myosin phosphorylation, crossbridges initially are rapidly cycling (Act•Myo•ATP to Act•Myo•ADP•P_i above) followed by prolonged slow crossbridge cycling (Act•Myo•ADP_I to Act•Myo•ADP_{II} above) which lasts for the duration of the cellular calcium transient [135]. The rapid crossbridges utilize more ATP, have faster cycling velocity and are hence associated with faster velocity and extent of shortening; slowly cycling crossbridges, or latch bridges, form in the presence of the dephosphorylated myosin, utilize little energy, but generate higher force [113].

According to the latch bridge model, recruitment of latch bridges should be dependent on MLCK and MLCP activities, where slowly cycling crossbridges are recruited at low levels of activation by dephosphorylation of attached states [113]. This latch bridge hypothesis is not entirely consistent with the previously cited result of inhibition of myosin dephosphorylation with okadaic acid; the two models are reconciled by separating the functions of length reduction and force generation, so that the main population of phosphorylated, rapidly cycling crossbridges subserve shortening while phosphorylation above a basal level may not be necessary for force development by slowly cycling crossbridges [118]. An analysis of the time course of dephosphorylation of myosin light chain shows that MLC_{20} phosphorylation falls to near basal levels while force relaxation is still in a plateau phase [136]; however, in vivo, agonist sensitization of smooth muscle to cytoplasmic calcium is modulated through changes in light chain phosphorylation, with inhibition of phosphatase activity converting phasic calcium responses to tonic ones [86]. Shortening and velocity of shortening of smooth muscle are closely temporally associated with phosphorylation state of myosin, in a linear relationship [137] [138].

In applying these models to vascular smooth muscle in vivo, it should be noted that changes in vascular lumen diameter are primarily accomplished by smooth muscle shortening, which occurs during rapid crossbridge cycling [139]; maximum shortening capacity may be increased in sensitized tissue even while magnitude of isometric tension remains unaltered [140]. Force generation may have a role in economical, long-term maintenance of tone [141] or in resistance to wall strain, but is not the key element in vascular hypertension. The earliest change in mechanical properties of arteries from spontaneously hypertensive rats is an increase in maximum shortening ability; changes in maximal isometric tetanic tension are late indices of disease. This increased shortening is associated with increased maximal velocity of early cross-bridges, whereas latch bridge activity is normal. Since hypertension must result from narrowing of blood vessels, isotonic shortening is the important variable; changes in force contribute to increased vascular wall stiffness but do not directly account for increased resistance [139]. The cause of the increased cycling rate of crossbridges is probably increased myosin ATPase activity following myosin light chain phosphorylation. As such, the regulation of myosin light chain phosphorylation by the dynamic equilibrium of MLCK and MLCP activity is crucial to the contribution of smooth muscle to hypertensive pathologies. The major mechanism of physiological calcium sensitization is through inhibition of smooth muscle myosin light chain phosphatase; major mechanisms of calcium desensitization include inhibition of myosin light chain kinase and activation of phosphatase [125].

Myosin Light Chain Phosphatase and Regulation of Relaxation

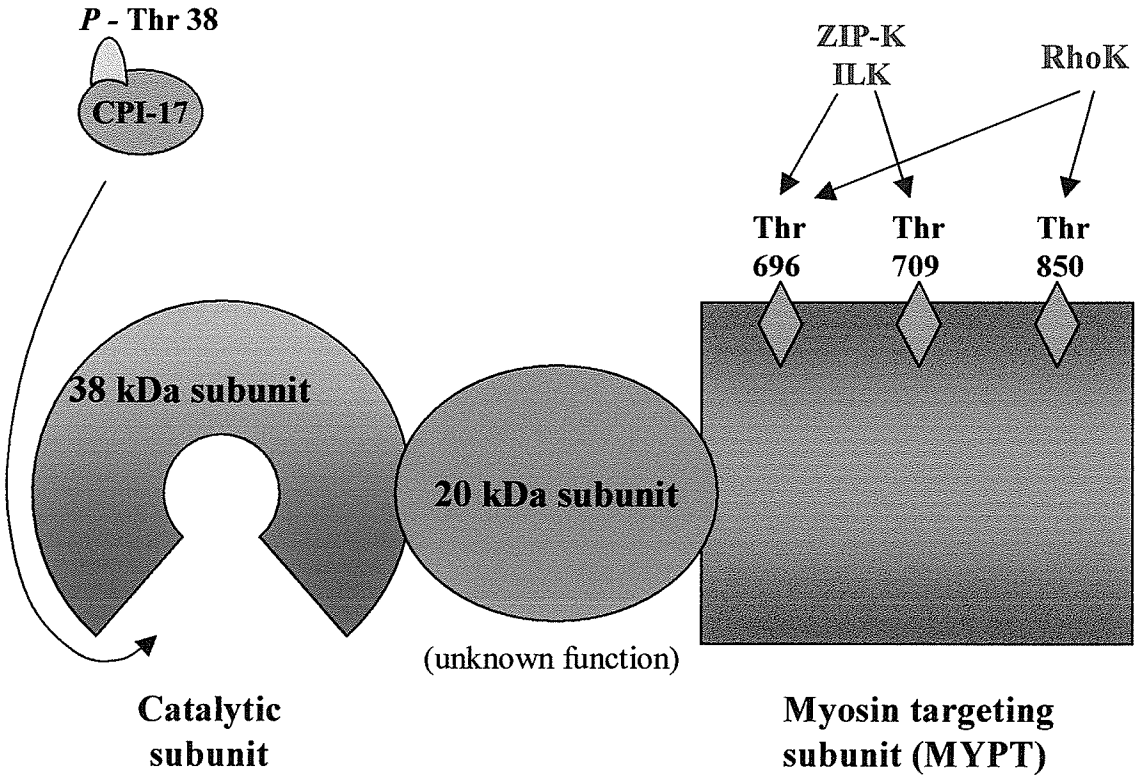
Failure of arterial smooth muscle relaxation has long been known to be a key contributor to the development and maintenance of arterial hypertension [142]. Regulation of the calibre of the vascular lumen determines resistance to flow, and is determined by shortening or elongation of helically arrayed vascular smooth muscle [143]. The relaxation index proposed by Liu and colleagues describes a three-stage mechanism, on the basis of crossbridge cycling velocity at zero load (V_O): (i) an initial concave downward, curvilinear slope of isotonic relaxation associated with a declining muscle activation state, and mediated by myosin dephosphorylation; (ii) a linear phase of passive recoil of the series elastic component, with static activation state; and (iii) a final convex downward, curvilinear phase marked by a small increase in spontaneous smooth muscle activation and V_O, indicating resumption of crossbridge cycling due to myosin rephosphorylation. The initial relaxation is felt to be a function of MLCP activity, while the terminal reactivation is due to reactivation of MLCK, presumably for the purpose of slowing the extent of cellular elongation [143], and thus minimizing turbulence of blood flow. This index has also been validated in airway smooth muscle, where the stoichiometry of myosin light chain phosphorylation confirms these mechanisms [144]. Failure of relaxation may thus occur as a consequence of prolongation of any of these phases. Since the active relaxation phase responsible for the greatest proportion of tissue re-elongation is Phase I [143], this discussion will focus on the Phase I regulatory enzyme, myosin light chain phosphatase.

MLCP dephosphorylates myosin light chain at serine 19 and threonine 18, resulting in inactivation of actin-activated myosin Mg-ATPase activity [145]. Hormonal or agonist-mediated control of actomyosin contractility involves a reduction in the

requirement for calcium, or calcium sensitization [86]; first thought to involve changes in MLCK activity, this phenomenon is now believed due to alteration in MLCP activity [9]. In sensitized airway smooth muscle, relaxation failure has been ascribed to both increased MLCK activity [146], and decreased MLCP activity [144]. Calcium sensitization of vascular smooth muscle, in particular through inhibition of myosin phosphatase, is being recognized as a common point of downstream signalling independent of causative factors, and a critical component of the pathogenesis of hypertension [147]. Excitation-contraction coupling in pulmonary vascular smooth muscle, underlying adrenergic and prostanoid-mediated responses, involves agonist-specific activation of a tyrosine kinase-linked pathway as well as RhoA [148], Rho kinase [149] and protein kinase C [150], leading to enhanced calcium sensitivity of the contractile apparatus. Since these pathways are also implicated in the response to hypoxia, the addition of stimulation by circulating contractile agonists may have a greater-than-additive effect on smooth muscle. In an endstage overflow model of neonatal pulmonary hypertension, Belik and colleagues [7] have reported unchanged MLCK activity, but decreased MLCP activity, which leads to further consideration of phosphatase activity as a determinant of pulmonary vascular relaxation failure in hypoxia. However, hypoxia has also been reported to increase myosin phosphatase activity in adult aortic smooth muscle, resulting in impaired aortic vasoconstriction [151]. This implies a more complex regulation of phosphatase activity with developmental and tissue specificity in hypoxic conditions, such that relaxation may be impaired in one tissue while being augmented in another, and one might presume that this regulation may be of functional significance during perinatal circulatory transition.

Fig. 3 : Heterotrimeric structure of Myosin Light Chain Phosphatase (PP1-M)

Multiple sites of phosphatase activity regulation, by inhibitory phosphorylation of MYPT subunit or by catalytic site inhibition, are responsible for smooth muscle calcium sensitization in vivo.



- Catalytic site is active only when associated with myosin targeting subunit
- *P*-CPI-17 competitively inhibits MLCP by binding catalytic site
- *P*-CPI-17 is 1000 x more potent than CPI-17, functions as rapid on-off switch for MYPT catalytic activity

- Responsible for attachment to myosin light chain (also known as myosin binding subunit, or MBS)
- 130 and 133 kDa isoforms
- 3 known inhibitory phosphorylation sites
- Phosphorylation of MYPT by RhoK causes dissociation of targeting subunit from holoenzyme and inactivation of phosphatase

MLCP Structure and Function:

The family of cellular protein phosphatase enzymes is divided into type 1 (PP-1) and type 2 (PP-2) classes, with specific cell type and subcellular distributions as well as individualized substrate specificities within each class. All the cytosolic protein phosphatase isoforms active in smooth muscle, designated as PP-1M, PP-2A and PP-2C, are capable of dephosphorylating myosin light chains in vitro; but only the myofibrillar phosphatase PP-1M is active towards whole myosin, and is able to carry out the function of myosin dephosphorylation in vivo [152]. PP-1M is identified by subcellular fractionation as the phosphatase associated with the cytoskeletal compartment [153]. In migratory fibroblasts, myosin phosphatase subunits localize predominantly to the tailing edge of the cell, and are depleted from the leading half of the cell, with the catalytic subunit also found in the nucleus and in focal adhesions; because the tailing edge of migrating cells is known to contain phosphorylated myosin, inhibition of myosin phosphatase may be necessary for cell motility [154]. Microinjection of PP-1 class phosphatase into the cytoplasm of isolated fibroblasts leads to rapid disassembly of the actin microfilament network and dephosphorylation of myosin light chain; PP-2A injection does not have this effect, supporting the notion of substrate specificity of protein phosphatases [155].

Myosin light chain phosphatase (depicted in Fig 3) is a heterotrimeric protein consisting of three subunits: the 37 kDa δ isoform of type 1 protein phosphatase, containing the catalytic site (PP1C); a 130 kDa regulatory subunit known as MYPT (for myosin protein targeting subunit), responsible for targeting the holoenzyme to myosin; and a 20 kDa subunit of unknown function [156]. PP1C is positioned to hydrolyse phosphoryl groups from proteins targeted by the MYPT subunit. The association of the catalytic subunit with the targeting subunit allosterically regulates the kinetic properties of

PP1C; the measured K_M of PP1C with MYPT is lower, and V_{max} higher, than that of the PP1C monomer alone [157].

In vitro activity of MYPT can be inhibited by calyculin A and okadaic acid, although there is some functional overlap with dose ranges effective to inhibit PP-2A type protein phosphatases [158]. Myosin phosphorylation in skinned smooth muscle is markedly inhibited and force generation enhanced by okadaic acid treatment, suggesting significant downregulation of PP-1 activity [159]. Type 2-C phosphatases are resistant to okadaic acid. Distinction between type 1 and 2A phosphatases is best achieved by use of inhibitor 2 [160], a catalytic site-specific intrinsic inhibitory factor of PP-1 [161]. Overall, myosin phosphatase activity in tonic smooth muscle is less sensitive to phosphatase inhibitors than that in phasic muscle, which may contribute to the slower rate of relaxation in tonic muscle [162].

Regulatory phosphorylation sites have been discovered on the MYPT subunit, which modulate phosphatase activity of the holoenzyme. Phosphorylation of threonine 696 of MYPT is found to markedly repress MLCP activity. This residue is amenable to phosphorylation by Rho kinase [163], as well as integrin-linked kinase (ILK) [164], and the MLCP-associated enzyme ZIP kinase [165] [166]. ILK produced an intermediate level of inhibition, and RhoK a marked inhibition of phosphatase activity, through MYPT phosphorylation at this site. The adjacent serine 694 may also be phosphorylated by cAMP-dependent protein kinase (PKA), but with no effect on phosphatase activity [164]. Another MYPT residue, threonine 850, is phosphorylated by Rho kinase [167] resulting in dissociation of the holoenzyme from myosin, and thus inhibition of phosphatase activity [168]. Other putative phosphorylation sites on MYPT include threonine 709, which has been shown to have little inhibitory effect on catalytic activity [164], and serine 430 which is known to cause phosphatase activation by phosphorylation with an unknown kinase

during mitosis [169]. The functional relevance of these phosphorylation sites is unclear, although cell cycle dependent redistribution of PP-1 has been demonstrated in mammalian cells with specific localization to metaphase chromosomes, where PP-1 slows the cell cycle in G_2/M phase to allow DNA repair [170]. The activation of myosin phosphatase during mitosis would enhance dephosphorylation of the myosin regulatory light chain, suggesting a role in cytokinesis by leading to the disassembly of stress fibers during prophase [169]. Major inhibitory phosphorylation sites on the MYPT subunit are summarized in Fig 3.

The MYPT subunit has two isoforms that differ by a central insert of 56 residues [171], which lies near a regulatory phosphorylation site [172]. This insert contains a C-terminal leucine zipper, which is important for interaction with cGMP-dependent protein kinase (PKG). Isoform switching is tissue-specific and developmentally regulated, as a consequence of cassette-type alternative splicing of a 31-nucleotide exon, with tonic smooth muscle expressing preferentially leucine zipper positive (M133) MYPT, and mature phasic smooth muscle MYPT (M130) lacking the leucine zipper insert. Fetal phasic muscle is leucine zipper positive until roughly time of birth, which corresponds to the time of development of a cGMP-resistant phenotype, unable to dephosphorylate myosin and relax in response to 8-bromo-cGMP. This isoform switch is not known to occur in tonic arterial smooth muscle [173]. The capability for agonist-induced calcium sensitization is also associated with the insert-positive isoform, and following switch to an insert-negative isoform, phasic smooth muscle is no longer sensitive to force enhancement by GTPγS. It is postulated that MYPT phosphorylation is unnecessary for calcium sensitization by this method [172]. The rapidity with which isoform switching occurs is not yet established, nor whether this mechanism may contribute to vascular hypertensive pathologies, especially in the neonate. While it is a very intriguing prospect,

just for temporal reasons it is unlikely to be responsible for the early loss of relaxation in hypoxia; MYPT isoform switching may well be a component of the later phase of pulmonary hypertension.

The physical relationship between subunits is relevant for regulation of phosphatase activity. Confocal localization studies show that in contractile smooth muscle cells, the catalytic and targeting subunits of MLCP are dissociated from each other in an agonist-specific manner, causing a slower rate of myosin light chain dephosphorylation, and hence facilitation of cellular contraction. This dissociation is preceded by targeting of the holoenzyme to the cell membrane, where the MYPT subunit is phosphorylated by Rho kinase [174]. This mechanism of regulation of phosphatase activity is conserved in other cell lines. In neurons, PP-1 is involved in cytoskeletal dynamics and inhibition of stress fiber formation, and is downregulated by phosphorylation by the actin-binding protein neurabin at the actin cytoskeleton [175].

MLCP Regulation By Rho Kinase:

The two arms of phosphatase regulation distinguished in this project are, as illustrated in Fig 4, (i) inhibitory phosphorylation of MYPT, via the Rho kinase pathway among others, and (ii) catalytic site inhibition by CPI-17 via the PKC pathway. When searching for potential mechanisms of myosin light chain phosphatase activity regulation in hypoxia, it is reasonable to begin by examining effectors of phosphatase phosphorylation at known inhibitory sites, and then to relate these intermediates with smooth muscle regulation in hypoxia. The Rho kinase pathway of phosphatase regulation links the small GTPase Rho, a subgroup of the Ras superfamily of 20-30 kDa GTP binding proteins involved in a wide variety of cytoskeleton-related cellular functions such as cell adhesion, motility, cell growth and cytokinesis [176], and regulation of cell

contraction. One of its main effectors, Rho kinase, appears to play a key role in the regulation of force and velocity of actomyosin crossbridging in smooth muscle and non-muscle cells by inhibiting MLCP-mediated myosin light chain dephosphorylation [177].

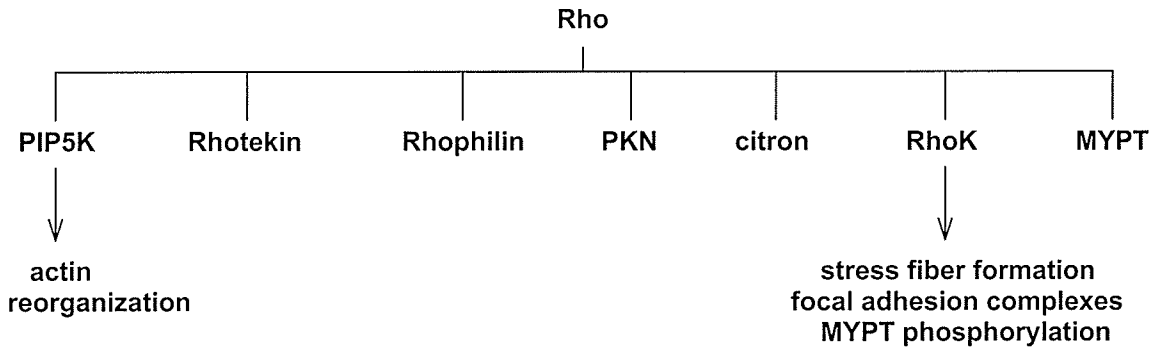
Rho and its congeners link plasma membrane receptors to the assembly and organization of the actin cytoskeleton, including stress fiber formation [178], ruffling and lamellipodia [179]. Activity of Rho GTPases is determined by the ratio of their GTP- to GDP-bound forms. This ratio is regulated by the opposing effects of guanine nucleotide exchange factors, which enhance the exchange of bound GDP for GTP; GTPase-activating proteins, which increase the rate of GTP hydrolysis; and guanidine dissociation inhibitors, which inhibit both GTP hydrolysis and GDP exchange [180].

Various extracellular stimuli trigger the activation of hierarchical GTP cascades involved in cytoskeletal organization of fibroblasts. CDC42 is activated by bradykinin; CDC42 is responsible for filipodia formation, and activates the downstream signalling protein Rac [181]. Rac may also be independently activated by agonists such as insulin and PDGF [179]. Activated Rac is in turn involved in lamellipodia formation [178], and in the activation of Rho. Rho is involved in cytokinesis, polymerizing actin in late anaphase to produce the contractile ring machinery [182]. Stress fibers are formed through non-muscle myosin phosphatase phosphorylation and increased actomyosin association in fibroblasts and other non-muscle cells, analogous to calcium sensitization in smooth muscle [183]. The involvement of Rho in stress fiber formation has been demonstrated by expression of active Rho mutants, resulting in appearance of focal adhesions and stress fiber induction; this pathway is also activated independently by lysophosphatidic acid [178], linking stress fiber formation to injury response mechanisms. Rho kinase is also independently activated by arachidonic acid [184] [185], resulting in PPIC dissociation from the MYPT subunit [186].

Expression of Rho is associated with the contractile smooth muscle phenotype; cultured vascular smooth muscle in a synthetic state has low levels of transcription of Rho and low protein abundance, but when transfected with constitutively active Rho, reorganizes its cytoskeletal architecture to resemble a contractile phenotype [187]. Agonist-induced, dose-dependent translocation of RhoA to the cell membrane activates this pathway, and plays a causal role in calcium sensitization of force in vascular smooth muscle, with a time course of Rho recruitment and translocation paralleling the time course of contraction. Calcium sensitization could be thereafter reversed without a decrease in membrane-fraction Rho [188].

RhoA effector mutant analyses suggest that simultaneous activation of multiple effectors, including Rho kinase, is required for formation of stress fibers. A separate downstream pathway connects RhoA to activation of serum response factor (SRF) and hence phenotypic transformation; transformation through another GTPase (Rac1) correlates neither with cytoskeletal reorganization nor Rho kinase activation [189].

Mammalian targets of Rho: *(from Van Aelst and D'Souza-Schorey 1997 [176])*



Rho kinase phosphorylates MYPT at threonines 696 and 850, causing decreased phosphatase activity [168] and membrane translocation of the myosin targeting subunit, dissociating it from the catalytic subunit [174]. Rho kinase inhibition in smooth muscle has

been shown to reduce myosin light chain phosphorylation and result in relaxation of force generation. Addition of exogenous constitutively active RhoA to permeabilized smooth muscle results in increased myosin phosphorylation and force at constant calcium concentrations [183]. Further light is shed on the relationship between the Rho kinase regulatory pathway and the genesis of vascular hypertension by Uehata and colleagues [190], by means of specific inhibition of Rho kinase in permeabilized arterial strips with pyridine derivative Y27632. This preparation inhibited GTPgammaS-induced calcium sensitization, with no effect on calcium-induced contraction, suggesting independence of this pathway of regulation from membrane ion channel- or phospholipase C-mediated contraction. In vivo, the reduction of systemic blood pressure obtained with Y27632 in spontaneously hypertensive rats is significant; little reduction is seen in the blood pressure of normotensive animals, implying increased importance of this mode of relaxation failure in pathophysiologic states [190].

Not all agonist-mediated calcium sensitization can be explained by this route, however. In an examination of multiple smooth muscle agonists, contraction mediated by GTPγS and thromboxane analogue U46619 is reduced by Rho kinase inhibition, implying MYPT phosphorylation via this pathway is required for these agonists; arachidonic acid- and phorbol ester-induced force was not relaxed by Rho kinase inhibition, suggesting a dual mechanism of calcium sensitization for arachidonic acid (one of which, through its metabolite thromboxane, involves Rho kinase), and a Rho kinase-independent mechanism of phorbol ester-induced calcium sensitization, which is known to involve protein kinase C (PKC) [191].

The vasodilator action of cGMP-dependent kinase PKG, which decreases calcium sensitivity of contraction of smooth muscle through stimulation of MLCP by unknown mechanisms [192], as well as decreasing cytosolic calcium, causes inhibition of

RhoA-induced calcium sensitization in vascular smooth muscle. Insulin utilizes this route to achieve a vasodilator effect, inhibiting Rho and Rho kinase to activate myosin phosphatase [193]. By increasing nitric oxide synthesis and activating cGMP signalling, insulin decreases site-specific phosphorylation of MYPT at its threonine 695 residue, and decreases actin fiber reorganization in response to agonist challenge [194]. Activation of RhoA is also decreased by the nitric oxide donor sodium nitroprusside [195]. In considering the etiology of calcium sensitization in nitric oxide-resistant hypoxic pulmonary hypertension, the importance of Rho kinase must be mitigated by its susceptibility to nitric oxide therapy. Other routes of calcium sensitization, not amenable to nitric oxide, are likely to be crucial. Additionally, the Rho pathway links agonist-mediated activation with stress fiber formation, which may be presumed to be a feature of the contractile smooth muscle phenotype. Increased smooth muscle proliferation in PPHN would have a different etiologic pathway.

MLCP Regulation by Protein Kinase C:

A major mechanism of myosin phosphatase regulation, which does not involve MYPT inhibitory site phosphorylation, is catalytic site inhibition. This recently discovered pathway involves a small protein phosphatase pseudosubstrate that is activated upstream by protein kinase C, and is gaining importance in the current understanding of regulation of vascular relaxation. This arm of phosphatase downregulation will now be considered in detail (see Fig 4).

The PKC family is a ubiquitous set of phospholipid-dependent serine/threonine kinases involved in many interrelated mitotic signalling pathways. All PKC enzymes are assembled by cassette-type splicing of three distinct functional domains, labelled C1 (activated by diacylglycerol), C2 (activated by calcium), and C3 (responsive to

phosphatidylserine) [196]. Based on their composition, PKC isozymes are divided into three classes: *conventional*, including PKC-α, -β (βI and βII), and -γ, which include all three cassettes and can thus be activated by phosphatidylserine (PS) at C3, diacylglycerol (DAG) or phorbol esters by binding to the C1 domain, and calcium binding to the C2 region; *novel* PKC-δ, -ε, -η and -θ, lacking the C2 domain, and thus calcium independent, but still PS, DAG and phorbol ester-responsive; and the *atypical* PKC-λ/ζ and -ξ, which lack both the C2 region and the C1 domain for a functional DAG-binding site, and thus are responsive only to PS but not to DAG, phorbol esters or calcium [197].

PKCs reside in the cytosol in an inactive conformation, and translocate to the cell membrane [198] or other subcellular sites [199] upon activation. When inactive, the catalytic site is sterically blocked by the arm of a hydrophobic pseudosubstrate domain. Three distinct phosphorylation sites have been identified on what is referred to as the activation loop, amenable to phosphorylation by phosphoinositide-dependent kinase (PDK) which links the membrane phosphoinositide pathway and IP_3 to PKC; this step is necessary prior to DAG binding [200]. When the enzyme is activated, phosphorylation of the activation loop renders the enzyme catalytically competent [201]; then autophosphorylation of the pseudosubstrate domain causes a conformational change such that the catalytic site is laid open for substrate binding. This activation route holds true for conventional [202], novel [203] [204] and atypical [205] PKCs. Once activated, PKC enzymes modify many cellular functions through the phosphorylation of target proteins. Varying autophosphorylation domains on the different PKC isoforms may be important for subcellular localization of the enzyme [206].

Several actin-binding and modulating proteins have been identified as PKC targets in vitro and in vivo, including talin [207], vinculin [208], moesin [209], fascin [210], ezrin [211] and MARCKS [212]. The relevance of many of these interactions for actin cytoskeletal

reorganization in smooth muscle remains unclear. Recently, Brandt and colleagues postulated a pathway of conventional or novel PKC-induced actin stress fiber reorganization in aortic smooth muscle via a Src- and Rho-dependent pathway [196].

The various PKC isoforms are differentially distributed in various tissues, and activation may have tissue-specific implications. Expression pattern may also be species-specific. Human vascular smooth muscle cells express PKC isoforms α and βI in the cytosol, with translocation to membrane on activation by mitogens; βII, δ and ε are always found in the particulate layer on subcellular fractionation [213]. Porcine coronary artery smooth muscle contains α-, δ-, ε-, and ζ-PKC [214]. Vascular smooth muscle cell migration, proliferation and extracellular matrix protein production are key steps in vascular medial fibrotic change. Activation of PKC in vitro increases cellular proliferation; downregulation of PKC activity diminishes agonist-stimulated proliferation. Investigations using isoform-specific activators and inhibitors of PKC suggest these differential effects of PKC on vascular cells may be mediated through PKC-α, -βI, δ and/or -ε isoforms [215].

Contractile smooth muscle cells isolated from intact tissues are known to have a limited PKC expression pattern, including PKC-α, δ and ζ; following cell culture, these cells additionally express mRNA for PKC-ε. PKC-δ and ε isoenzyme expression appears to vary with state of smooth muscle differentiation, with PKC-ε expression increasing and δ isoform disappearing as cells become proliferative [216]. PKC activity and PKC-α expression decreases during rapid vascular smooth muscle growth, increasing again in confluent cells. Dedifferentiated cells express increased amounts of smooth muscle actin when injected with active PKC-α; retinoic acid-induced phenotypic differentiation is associated with an increase in PKC-α expression. Subcultured, rapidly growing vascular myocytes from spontaneously hypertensive rats have diminished PKC-α expression

when compared with cells from control animals, implying an enduring alteration in smooth muscle PKC activity in chronic hypertensive pathologies [217].

Pulmonary hypertension of the newborn is marked by two significant alterations of the pulmonary vascular environment: tissue hypoxia, often as a consequence of ventilation/perfusion mismatch and further perpetuated by shunting, and mechanical wall strain due to increased circuit pressure. Both of these phenomena are known to affect activation state of the smooth muscle PKC pathway.

In large pulmonary blood vessels there is now convincing evidence that the medial layer is made up of many different subpopulations of smooth muscle cells, which respond differently to hypoxia. Some cell populations proliferate and increase matrix protein synthesis, while in other cell populations no apparent change in the proliferative or differentiation state of the cell takes place [218]. The earliest smooth muscle proliferative changes in response to hypoxia occur in the outer vascular media. The heterogeneous growth capacity of pulmonary vascular medial arterial smooth muscle in hypoxia governs, at least in part, the pattern of abnormal smooth muscle proliferation observed in vivo. Outer medial cells have greater total cellular activity, expression, and hypoxia-induced activation of PKC (α isozyme in particular) than cells isolated from the middle sector of the media [219]. The predominant proliferative changes occurring in peripheral vessels in the pulmonary circuit in response to hypoxia take place in certain fibroblast populations of the adventitial layer rather. PKC is known to activate mitogen-activated protein kinases (MAPK) through activation of Raf and MAPK activator MEK [220]. Hypoxia, in the absence of serum or mitogens, specifically activates PKC isozymes, as well as members of the mitogen-activated protein kinase family of proteins. This selective activation appears to take place in response to hypoxia only in those cells exhibiting a proliferative response, and antagonists of this pathway inhibit the response [218]. Hypoxia

stimulates increases in DNA synthesis and growth in a subset of quiescent neonatal vascular adventitial fibroblasts in the absence of exogenous mitogens, and also markedly augments serum-stimulated growth responses, through G protein α-mediated activation of a complex network of MAP kinases, including ERK1/2, JNK1, and p38 MAP kinase [221].

Mechanical force is an important modulator of cellular morphology and function in a variety of tissues, and is particularly important in cardiovascular systems. Mechanical stresses can directly stretch the cell membrane and alter receptor or G protein conformation, initiating mitotic signalling pathways. Cellular strain rapidly induces phosphorylation of platelet-derived growth factor (PDGF) receptor, activation of integrin receptor, stretch-activated cation channels, and G proteins, which might serve as mechanosensors. Once mechanical force is sensed, PKC and thence MAPK cascades are activated, leading to increased c-fos and c-jun gene expression and enhanced transcription factor AP-1 DNA-binding activity [222]. Smooth muscle cell phenotype can be altered by physical forces, as demonstrated by cyclic strain-induced changes in proliferation, orientation, and secretion of macromolecules. Mechanical deformation causes rapid activation of PKC isoforms associated with the contractile phenotype. Smooth muscle cells express PKC-α and -ζ predominantly in the cytosolic fraction; application of cyclic mechanical strain to cultured cells translocates PKC-α and -ζ to the membrane fraction with a corresponding decrease in cytosolic fraction [223]. In keratinocytes, cyclic strain causes translocation of PKC α and δ, but not ζ isoforms, to the membrane. PKC inhibitors prevent strain-induced PKC activation and proliferation, but do not block the effects of strain on cellular morphology or alignment [224]. In vascular smooth muscle, inhibition of PKC prevents neither strain-induced realignment nor proliferation, suggesting the multifactorial nature of these responses [225].

Modification of myosin ATPase activity by phosphorylation of myosin light chain is a significant mode of regulation of contractile activity of unique importance in smooth muscle, particularly in regard to the generation of phasic contractions and the initial development of tonic contractions. There is an important role for PKC in the maintenance of agonist-induced smooth muscle tone [226]. Vasoconstrictors, in addition to the mobilization of calcium through activation of membrane phospholipase C and calcium channels, are also thought to activate a distinct cellular mechanism for downregulating myosin light chain phosphatase activity, involving Rho p21 and PKC, and resulting in an increase in the calcium sensitivity of myosin phosphorylation and contributing to the maintenance of agonist-induced contraction [227]. Basal myosin light chain phosphorylation in intact arterial tissue is ascribed to myosin light chain kinase, but receptor-initiated contraction is mediated by both MLCK and PKC. Detergent disruption of the sarcolemma causes quantitative loss of PKC enzyme and results in dissociation of the steady state myosin phosphorylation to muscle tension relationship [228].

Various PKC isoforms have been implicated in the vascular contractile response. Introduction of a constitutively active PKC catalytic fragment, or of calcium-independent isozyme PKC-ε, induces slow, sustained contraction in permeabilized aortic smooth muscle. PKC-ε is activated upon stimulation of cells with phenylephrine, suggesting a role in smooth muscle contraction; PKC-ζ also translocates, but moves from a perinuclear localization to the interior of the nucleus, suggesting a role other than contraction [229]. PKC-α and -δ are implicated in the development of vasospasm following subarachnoid hemorrhage [230]. PKCα is a positive mediator of smooth muscle proliferation and activation of the mitotic MAP kinase pathway; however, it is a negative regulator of migration, and has no effect on extracellular matrix synthesis [231]. Confocal microscopic evidence may support a role for PKC-α in tyrosine kinase receptor-mediated

growth factor response, with immediate translocation to cytoskeletal structures followed by movement to the nucleus [232]. In synthetic smooth muscle cells, PKC-δ is seen to rapidly translocate to the membrane on stimulation of smooth muscle by platelet-derived growth factor, which stimulates smooth muscle migration; this is blocked by the mitogen transforming growth factor β [233]. Use of specific PKC-δ inhibitor rottlerin reveals a specific role for this isozyme in mediating the in vivo pressor response of the pulmonary vascular circuit to agonists, via calcium sensitization; immunocytochemistry of cultured pulmonary artery smooth muscle cells further demonstrates PKC-α and -δ localization in response to angiotensin II [234]. Additional evidence links the δ isoform to direct modulation of smooth muscle contraction; in freshly isolated canine pulmonary arterial smooth muscle, confocal microscopy shows PKC-α movement to the nucleus, but PKC-δ localization to myofilaments, triggered by activation with angiotensin II [199].

PKC-Activated Inhibitor CPI-17

The nature of PKC-induced calcium sensitization by inhibition of myosin light chain phosphatase has been illuminated by the discovery of a protein intermediate, which interacts with both molecules. CPI-17 (for protein kinase C-potentiated inhibitory protein of 17 kDa) is a smooth muscle-specific protein expressed in both tonic and phasic muscle, although at markedly greater concentrations in the former. The relative CPI-17 and MLCP levels correlate with the capability of the tissue to be calcium-sensitized by PKC activation. PKC stimulation of arterial smooth muscle with a high CPI-17 to MLCP ratio results in greater myosin light chain phosphorylation and force generation than stimulation of tissue with low CPI-17 content, implicating CPI-17 as a signaling intermediate modulating vascular tone [84]. MLCP is specifically inhibited by the interaction of CPI-17 with its catalytic subunit (shown in Fig 3). Phosphorylation of CPI-17 at the threonine-38 residue increases its inhibitory potential over 1000-fold; the

central domain molecular sequence, including this site of phosphorylation, is necessary for recognition by myosin phosphatase [235]. The solution NMR structure of CPI-17 reveals a phosphorylation-induced conformational change which is responsible for its interaction with the MLCP catalytic site, where it acts as a molecular switch [236]. A return to the previously cited experiment examining reconstitution of PKC-induced calcium sensitization in demembranated arterial smooth muscle reveals a requirement for activated CPI-17 for reconstitution of myosin light chain phosphorylation, implying that loss of this small regulatory molecule by transmembrane leakage is the cause of attenuated PKC sensitivity in demembranated tissue. Unphosphorylated recombinant CPI-17 alone induces a small but significant contraction at constant calcium concentration. Isoform-selective PKC inhibitors inhibit unphosphorylated but not pre-thiophosphorylated CPI-17-induced contraction [237]. In response to histamine stimulation, PKC-α and -δ are known to mediate the phosphorylation of CPI-17. Purified PKC-δ is highly reactive with CPI-17; PKC-α has a lesser effect [238]. Other smooth muscle kinases including Rho kinase [238], integrin-linked kinase [239], p21-activated kinase [240], and ZIP kinase [241] also phosphorylate CPI-17 in vitro. Specific inhibition of PKC α/β inhibits phorbol ester-induced arterial contraction but incompletely, while general PKC inhibition ablates contraction, suggesting a significant role for PKC-δ in CPI-17 activation [238]. The connection between the PKC pathway, CPI-17 activation, and regulation of smooth muscle relaxation via downregulation of MLCP activity is delineated in Fig 4.

Agonist-induced contraction of intact and permeabilized smooth muscle strips shows a time course of increased CPI-17 phosphorylation that corresponds to the time course of increase in myosin light chain phosphorylation. In the same preparation, a significant proportion of MYPT subunit of MLCP is phosphorylated at rest, at the threonine-695 residue known to be phosphorylated by Rho kinase. Removal of agonist

markedly diminishes CPI-17 phosphorylation and myosin regulatory light chain phosphorylation, but does not alter level of MYPT phosphorylation; this suggests that CPI-17 plays the greater role in agonist-induced downregulation of myosin light chain phosphatase activity [242]. This is further delineated in a study examining the role of MYPT phosphorylation and CPI-17 phosphorylation in various tonic and phasic smooth muscle tissues, where tissues with high CPI-17 concentrations, specifically tonic muscle (such as in femoral artery) and certain phasic muscles (such as portal vein), rely to a greater extent on CPI-17-mediated inhibition of MLCP activity, while tissues with low CPI-17 content utilize other routes of inhibition, such as MYPT phosphorylation. Phosphorylation of MYPT at threonine 695 appears to be independent of G-protein-mediated or PKC-mediated stimulation in phasic muscle, but MYPT threonine 850 and CPI-17 threonine 38 are phosphorylated in response to agonist stimulation, and dephosphorylated by inhibition of Rho kinase and PKC respectively [243]. The speed of this regulation may be a key to its functional utility. The MYPT subunit is relatively resistant to dephosphorylation, remaining unchanged on removal of agonist, and making it less likely to play a role in rapid modulation of smooth muscle relaxation; on the other hand, when agonist is removed, phosphorylated CPI-17 is promptly susceptible to protein phosphatases 2A, 2B and 2C (but not PP1), indicating its suitability for regulating rapid changes in level of myosin light chain phosphorylation [244].

In the carotid artery, nitric oxide is known to increase myosin phosphatase activity concurrent with cGMP-mediated decreases in calcium and force generation, by means of CPI-17 phosphorylation (Etter et al, 2001). The pathway of this activation is not delineated, but protein kinase G, a downstream effector of nitric oxide, directly associates with the MYPT subunit of MLCP, and this interaction may be necessary for cGMP-mediated dephosphorylation of myosin light chain (Surks et al, 1999).

There is no data published to date on the abundance or functional role of CPI-17 in the pulmonary vasculature, especially in neonatal circulatory transition. The rapid oscillations of pulmonary vascular resistance seen in early pulmonary hypertension, manifested clinically as fluctuating shunt fraction with any external or environmental stimulus, indicate dynamic changes in smooth muscle tone. Intuitively, a slower acting modulator of calcium sensitivity such as MYPT phosphorylation is less likely to mediate these fluctuations, than the rapid inhibition and disinhibition of phosphatase activity engendered by CPI-17, which seems to function as an 'on-off switch', rather than merely a steady source of basal tone. In addition, activation of the PKC pathway by hypoxia and mechanical wall strain is an attractive route for this regulation in PPHN, as PKC is also linked to activation of smooth muscle proliferation, which is the hallmark of the later stage of this disease. It is worth noting here that Rho kinase can also activate CPI-17 [83]; this pathway is associated with cytoskeletal strain response and calcium sensitization, but not directly with myocyte proliferation. Since Rho kinase alone also phosphorylates MYPT, differential phosphorylation of these two intermediates may be used to identify the upstream kinase involved.

In summation, relaxation of smooth muscle is a highly regulated process, with recent revelations of multiple pathways impinging on activation state of the primary relaxant enzyme in the contractile machinery, myosin light chain phosphatase. Smooth muscle calcium sensitization is likely to involve impairment or prolongation of relaxation, by pushing the kinase/phosphatase balance towards the side of cell contraction. MLCP catalytic activity can be regulated at the level of subunit assembly, of targeting subunit isoform, by phosphorylation of targeting subunit via the Rho/Rho kinase pathway, among others, and through competitive inhibition by activated CPI-17 in the PKC pathway. The most rapidly responsive of these mechanisms of regulation seems to be CPI-17, which is

enriched in tissues where it plays an important regulatory role, and can be quickly phosphorylated and dephosphorylated. Regulation of MYPT phosphorylation is slower, and may play a key role in agonist-induced tone; this also appears to determine subunit assembly and localization. MYPT isoform switching is developmentally regulated, especially in phasic smooth muscle, but there is no etiologic evidence for this in acute hypertensive disorders, perhaps because of the length of time necessary for this alteration.

Vascular relaxation is known to be impaired in neonatal pulmonary hypertension, particularly in its early stages. Much of the data on calcium sensitization of vascular smooth muscle derives from systemic hypertension, and knowledge of the extent or role of MLCP regulation in pulmonary hypertension, especially in the neonatal period, is scant. As this disorder is engendered at a developmentally crucial time where timely, circuit-specific vascular smooth muscle relaxation is a factor of life and death, it is logical that regulation of relaxation may be awry in PPHN. Both the Rho kinase pathway and the PKC pathway are potential pathways by which this may occur; both have significant implications for other downstream signaling events that may be activated by these pathways, concurrently to smooth muscle relaxation failure, which may control the further natural history of the disease. Elucidation of the activation state of these pathways is therefore useful to understanding the pathophysiology.

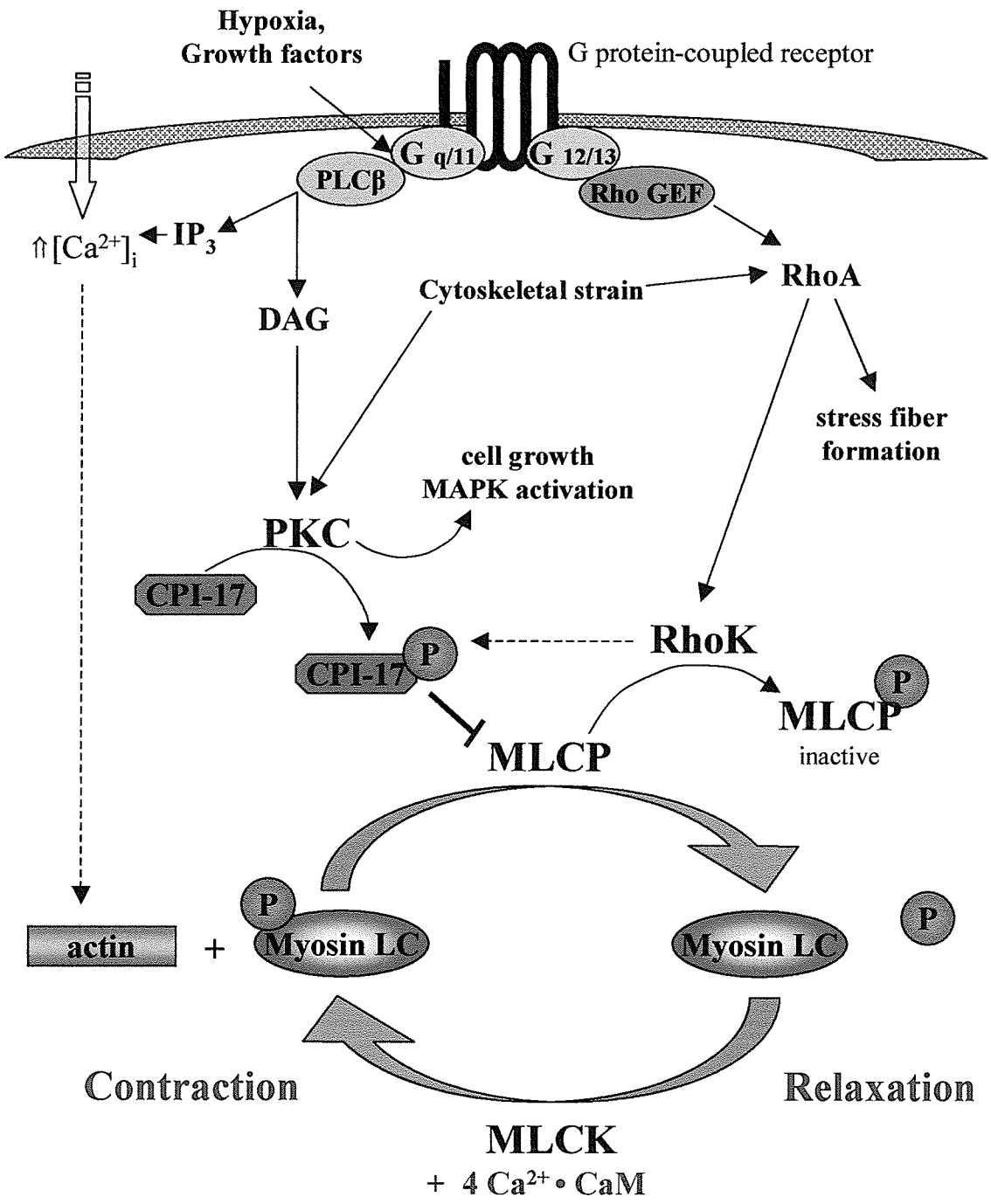


Fig. 4 : Pathways of MLCP regulation

Two main pathways are known to converge on regulation of myosin light chain phosphatase activity: Rho kinase and PKC. Both are activated by cytoskeletal strain and growth factors; PKC is also responsive to hypoxia. Multiple levels of crosstalk exist between the two pathways.

Modelling Neonatal Pulmonary Hypertension

Animal models of disease are crucial to understanding specifics of causation and pathophysiology. PPHN is studied in a number of different animal models, and it is apropos to review the various possible approaches to this problem.

Primary pulmonary hypertension, a chronic adult disease, is marked by vascular remodeling, including abnormal deposition of matrix proteins [245]. The fawn-hooded rat tends to develop primary pulmonary hypertension with minimal hypoxia, which is histologically of a fibrotic type with no early phase vascular hyperreactivity [246]. This type of pulmonary hypertension is also induced by the anorectic agent dexfenfluramine, which inhibits serotonin transport [247]. It is considered a good model of the genetically determined pulmonary hypertension seen in Down syndrome, or in primary adult disease [248], but not of the neonatal disease process.

Pharmacological maneuvers to induce pulmonary hypertension include exposure to monocrotaline, which induces progressive fibrotic pulmonary hypertension in adult animals, similar histologically to the effects of chronic hypoxia [249]. Interestingly, it is less effective in neonates. However, this model has led to the discovery of some crucial factors regulating vascular wall remodeling in neonatal pulmonary hypertension, including matrix metalloproteinases, the matrix glycoprotein tenascin, and endogenous vascular elastase, the inhibition of which prevents hypertensive remodelling [250].

Meconium aspiration is one of the most frequent causes of ventilation/perfusion mismatch leading to respiratory failure and PPHN in newborns. This has been replicated in animal models by intratracheal administration of collected meconium [251] [252]. The method of preparation of meconium suspension varies, and this may be responsible for

variation in efficacy; in addition, as in the clinical setting, fewer than 10% of infants with true meconium aspiration develop significant PPHN, resulting in great variability in induced disease state [253]. It has been postulated that meconium aspiration is ineffective in producing acute pulmonary injury unless combined with perinatal asphyxia; however, a trial combining meconium instillation with acute asphyxia in a baboon model resulted in only moderate PPHN, similar to that produced by meconium alone [254].

A more reliable method of inducing pulmonary hypertension uses a model of neonatal sepsis, which produces known hemodynamic effects in the pulmonary as well as systemic and splanchnic circulations [255]. Administration of live or heat-denatured Group B streptococci or its endotoxin causes acute, biphasic pulmonary hypertension, systemic hypotension and decreased cardiac output [256] [257], primarily through activation of the arachidonic acid pathway [258]. The effect of nitric oxide inhalation has been well studied in this model, particularly in the neonatal piglet [259] [260]. The disadvantage of this model is the multiplicity of signaling pathways and secondary processes activated by circulating inflammatory mediators and hypotensive shock, which complicate both acute hemodynamic management and later interpretation of vascular findings.

Clinical studies showing hypertensive remodeling of pulmonary arteries in newborns dead due to PPHN on the first day of life, have led to the suggestion that intrauterine events may cause PPHN [261]. Chronic intrauterine hypoxia has been attempted by maternal hypoxic exposure; limitations of this include greater susceptibility of the adult to prolonged hypoxia, leading to loss. However, maternal hypoxia did not result in any structural or functional alteration in the pulmonary vasculature of neonatal guinea pig [262]. Embolization of the placenta has been tried in sheep, with better pathologic effect, but high fetal mortality [263]. Pulmonary hypoplasia is an intrauterine event resulting in muscularization of resistance pulmonary vessels due to overflow

through a limited capacity circuit; this has been documented in autopsy case studies [264]. This has prompted the development of a fetal model of pulmonary hypertension by partial or complete ligation of the ductus arteriosus [265] [266]. Surgical ligation of the ductus arteriosus in the in utero fetus results in shear stress due to pulmonary overperfusion, and changes of PPHN similar to that seen in pulmonary hypoplasia or hypertension, including elevated pulmonary vascular resistance due to abnormal vasoreactivity and hypertensive remodeling, and right ventricular hypertrophy [267]. Fetal sheep are large enough to be easily instrumented for in situ ductal ligation. Preclinical trials of inhaled nitric oxide were performed using this model [268]. Performed in near term sheep, the procedure still, however, carries a high risk of fetal loss [253]. Surgical models of congenital diaphragmatic hernia also approach the problem of overperfusion of a hypoplastic vascular bed causing pulmonary hypertension [269]. An aorta-lobar pulmonary artery shunt produces striking but localized vasculopathy, with development of severe pulmonary hypertension within a short time frame; low mortality makes this model highly attractive for investigation of mechanisms that underlie pulmonary hypertension in high flow states [270] [81]. Systemic hypertension caused by unilateral fetal renal artery constriction has also been reported to produce chronic pulmonary arterial hypertension due to increased muscularization of resistance vessels, suggesting either an alteration in pulmonary to systemic blood flow ratio, or circulating smooth muscle mitotic activators, as pathogenic mechanisms [271].

Environmental induction of PPHN is one of the most reliable methods, using either normobaric or hypobaric hypoxia [47] [272]. Histological evidence of PPHN is demonstrated after 3 days of FiO_2 0.10, with progressive fibrotic changes after longer courses of hypoxia [273]. Since these phenotypic alterations are related teleologically to hypoxia during circulatory transition, this is considered the most clinically faithful model

of PPHN. Hypoxic gas mixtures are reliably provided by mixing nitrogen and room air in a circuit, with no adverse clinical effects of increased nitrogen concentration [274].

We have selected the normobaric hypoxia model of PPHN as preferable to the others described, in view of its ease of reproduction with similar pathophysiologic and histologic findings in exposed animals, and in that abnormalities identified in these animals may be readily applied to the clinical scenario of perinatal hypoxic pulmonary vasospasm. The reported natural history of pulmonary hypertension in these animals most closely approximates the typical course of human neonatal pulmonary hypertension, in which hypoxia alone is the most common initiating factor. Additional sources of acute lung injury, with associated inflammation, may reduce the clarity of disease progression, and add potential sources of error. The reported loss rate for hypoxic neonates is relatively low, and invasive instrumentation is not necessary. Unlike septic models of PPHN, the range of applied stimuli to the vascular smooth muscle in normobaric hypoxia is limited, so that mechanistic investigation can be attempted without too great a list of potential variables.

This study of PPHN employs the hypoxic piglet model. The hypoxic piglet model of pulmonary hypertension has been extensively studied, and is well characterized. The piglet pulmonary vasculature is accepted as being structurally and histologically similar to the human neonate [275]. Circulatory transition in the newborn piglet is described as similar to the human infant down to the histological level [275] [276]. Ultrastructural studies show thinning of the piglet pulmonary arterial wall at birth due to reduction in smooth muscle overlap, and postnatal remodeling [277] with a time course comparable to that described in human infants [33]. Examination of isolated porcine pulmonary arteries show relaxation of myocytes, as attested by elongation and acquisition of fusiform shape, in a developmentally regulated manner within the first two weeks of life [276]. The size of the

neonatal piglet also makes it appropriate for study, as resistance pulmonary vessels can be dissected without too much difficulty. If it has received an initial feeding of colostrum, the piglet can be maintained up to a week without significant bacterial disease or need for antimicrobial prophylaxis. The effect of hypoxia on perinatal structural adaptation of the pulmonary circulation has been studied extensively in piglets by Haworth and colleagues, who apply quantitative morphometric techniques to injected lung specimens. Animals exposed to hypoxia from birth demonstrate an abnormal circulatory transition; the normal perinatal reduction in arterial muscularity is arrested, followed by a secondary increase in muscularity. In animals allowed to adapt fully to extra-uterine life and first exposed at 14 days, arterial muscularization proceeds with increase in wall thickness and extension of muscle into smaller peripheral arteries, similar to that reported in hypoxic adults [273]. Timing of hypoxic exposure is key in determining the nature of pulmonary vascular effect. Induction of beta-adrenoceptors in the pulmonary circulation, a developmental change associated with circulatory adaptation, is attenuated by perinatal hypoxia, but not by hypoxia after the third day of life [278].

To date in the literature, most of the changes of pulmonary hypertension have been studied in end-stage PPHN, where the final common pathway of vascular injury repair is medial thickening due to increased secretion of matrix and muscular hyperplasia; the obstruction to pulmonary blood flow is at this point fixed and fibrotic [85], and has been described as the end product of remodelling in response to increased wall stress [279]. In fact all etiologies of pulmonary hypertension, including chronic overflow states such as Eisenmenger physiology and pulmonary hypoplasia, are histologically identical at this stage [280 281]. Lately, greater research interest has been focused earlier in the natural history of the disease, where clinical intervention has a hope of averting the accelerated vascular fibrotic response.

Specific Objectives

This research project addresses the following specific objectives:

- To characterize pulmonary hypertension resulting from short course normobaric hypoxia in a neonatal piglet model
- To examine regulation of myosin light chain phosphatase activity in the early phase of hypoxic PPHN

Hypotheses

It is hypothesized that:

- Pulmonary hypertension can be induced in newborn animals by exposure to moderate hypoxia (FiO_2 0.10) starting within the first day of life. This will serve as a model for PPHN.
- Myosin light chain phosphatase activity is downregulated by short course (3 day) neonatal hypoxia
- Regulation of MLCP activity is linked to smooth muscle proliferative activation by hypoxia via the PKC signalling pathway, and its intermediate CPI-17

In formulating these hypotheses, certain points discussed at length in the literature review have been considered as crucial. While velocity and extent of contraction are unaffected by age, the distinguishing feature of neonatal pulmonary vascular smooth muscle is slow relaxation [34]; this may contribute to maintenance of high fetal tone, and also be etiologic in abnormal circulatory transition. Neonatal pulmonary arteries, down to the level of resistance vessels are particularly prone to hypoxic

constriction [5]. Early PPHN is characterized by dynamically fluctuating pulmonary vascular tone, and prolongation of relaxation has been described in pulmonary arteries of animals exposed to short course hypoxia [35] [28]. Increased smooth muscle tone is known to be the primary source of elevation of pulmonary arterial pressure in short course hypoxia, unlike prolonged hypoxia where morphometric changes contribute to increased resistance [85]. Acute lung injury also results in selective impairment of pulmonary vasorelaxation [282]. Calcium sensitization in smooth muscle occurs through activation of signalling intermediates including Rho kinase and protein kinase C, which are sensitive to hypoxia. Both of these pathways cause calcium sensitization by modulating activity of myosin light chain phosphatase. Hypoxia is known to alter myosin phosphatase activity in systemic arteries [151]. Therefore it is hypothesized that relaxation failure in hypoxic pulmonary vascular smooth muscle may be mediated by altered myosin phosphatase regulation.

In view of the function of the protein kinase C pathway in mitotic signalling, and knowing that smooth muscle proliferation is a key feature of the later stages of hypoxic pulmonary hypertension, the linking of the two phenomena of relaxation failure and proliferative activation through a single pathway is an attractive application of Ockham's razor. Hence it is further hypothesized that PKC-mediated downregulation of myosin phosphatase, via the regulatory protein CPI-17, may be etiologic in pulmonary vascular relaxation failure in this model of hypoxic pulmonary hypertension.

Materials and Methods

Induction of Hypoxic Pulmonary Hypertension:

All experiments were performed on farm-bred piglets, made available from a local swine farm. All conditions of transport, care, feeding and experimental treatment were approved by the Central Animal Care Committee (Protocol # 99-067), with further advice of veterinarians Dr Tim Snyder at Heartland Vet, Winnipeg, and Dr Kathy McCutcheon at the Bannatyne Campus Cental Animal Care Facility, University of Manitoba. Animals were transported to the Central Animal Care facility of the University of Manitoba Bannatyne campus in a warm vehicle, enclosed in a cardboard carrying container, and provided with a towel rubbed on the sow for maternal scent. Only one animal was transported at any given time. After an hour of quiet adjustment post transport, animals being housed for environmental manipulation were transferred to a prewarmed, thermoregulated isolette (Small ICS warmer with dome cover, Thermocare, Nevada) with tenderfoot flooring.

All animals received a minimum of 6 hours of maternal colostrum, which is felt to be sufficient prophylaxis to prevent enteric bacterial infection during commercial swine transport (private communication, Mr Don Down, Elite Swine, Winnipeg), prior to parting from the sow. Newborn animals were obtained at between 8 and 24 hours of age; 3 day old controls were obtained from the farm on the day of experimentation. For newborn animals housed for environmental manipulation, swine artificial milk replacer (WetNurse, from Feedrite) was mixed according to package instructions, and initial feedings were done by hand using syringe or bottle with crosscut nipple. Feeding was carried out every 2 to 3 hours until oral intake was well established. Once piglets displayed an ability to

feed *ad libitum*, they were returned to the isolette, and its edges sealed with tape to enclose its internal environment. One animal was housed in the isolette at any time.

In the hypoxic group, pulmonary hypertension was induced in newborn (8-24 hr age; n=11) piglets from the first day of life, by exposure to normobaric hypoxia using nitrogen washout technique, method per Shime et al [274] (FiO_2 0.10, achieved by mixture of N_2 [approximately 6 L/min] and room air [approximately 2 L/min] in a sealed plexiglass isolette; total continuous gas flow rate 8 L/min) for 65 to 70 hours. Environmental gas concentrations (PO_2 and PCO_2) were sampled at a minimum of every 6 hours and routinely monitored by analysis in a blood gas machine (ABL 500, Radiometer, Copenhagen), and gas flows adjusted to keep PO_2 from 55 to 65 mm Hg, and PCO_2 under 0.4 mm Hg. All animals were kept in a temperature and humidity controlled environment, with appropriate diurnal cycling; temperature was servo-controlled at 80F, and humidity kept between 55 to 70%. Weight and respiratory status were monitored closely, and animals unable to maintain adequate oral intake were gavage fed (the maximum permitted cumulative weight loss was 150g, or approximately 10% of arrival weight). The isolette was opened no more than 1 hour per day for cleaning and animal care (methodology as established by Fike and Kaplowitz [85]). Animals were euthanized after completing 3 days hypoxic exposure by pentobarbital overdose (euthanyl 2 ml/kg). Comparable normoxic control piglets were obtained and euthanized on the first (n=11) and third (n=9) days of life.

Gross dissection was performed by parasternotomy incision followed by identification and clamping of the trachea. Pericardium and pleural membranes were divided prior to breaching of blood vessels. Heart and lungs were removed en bloc and immediately placed in ice-cold calcium-free Krebs Henseleit physiological buffer (in mM: 115 NaCl, 25 $NaHCO_3$, 1.38 NaH_2PO_4, 2.51 KCl, 2.46 $MgSO_47H_20$, 5.56 dextrose).

Abdominal aorta was also obtained, for use as an internal control. Cardiac ventricular afterloading was estimated by relative ventricular weight (blotted tissue, ratio of LV + septum to RV).

A total of between 200 to 300mg of pulmonary arterial tissue was dissected from each piglet (methodology as described below). Tissues obtained from the initial cohort of 14 animals (5 = newborn; 5 = hypoxic day 3; 4 = normoxic day 3) were utilized for protein content analysis and histology. A subsequent cohort of 16 animals (5 = newborn; 6 = hypoxic day 3; 5 = normoxic day 3) was dedicated to phosphoprotein analysis and phosphatase activity assay.

A pictorial summary of treatment groups and experimental methods used in this study is given in Figure 5.

Histology:

Tissue blocks derived from left lower lung lobes of the first cohort of piglets were fixed in acid alcohol (1% acetic acid in 95% ethyl alcohol) for15 hours, then paraffin embedded. 5 μm thick sections of whole lung, serially cut by microtome onto coated slides, were deparaffinized, hydrated and stained with haematoxylin and eosin [283], then mounted with permount. Randomly stratified slides were examined under a light microscope, and photographs of representative sections (20x magnification) made using digital image capture software. Comparisons were made qualitatively.

Protein Analysis:

Under a dissecting microscope, 2^{nd} to 4^{th} generation pulmonary arteries were removed from lungs, cleaned of adventitia and stored at –70C. Dissection time was not monitored for this procedure (first cohort of piglets). Tissue was minced and pulverized

at 4°C in homogenization buffer of composition 0.1% SDS, 1% Triton X, 1% NP-40, 1mM EDTA, 1mM PMSF, 5μM aprotinin and 10μM leupeptin, using a mechanical rotary blade homogenizer (4 ml buffer/g wet weight tissue). Homogenates were centirifuged at 16000*g* for 15 minutes to remove particulate matter, and protein concentration of supernatants assayed using the Bradford assay kit [Biorad, Hercules, CA].

20μg protein from each sample, as well as appropriate molecular weight marker in a total volume of 25μl, were loaded and run on SDS-PAGE gels, using Laemmli loading buffer and standard technique [284]. Separated proteins were transferred onto nitrocellulose membrane overnight at 4°C in transfer buffer containing 20% methanol. The nitrocellulose blots were then blocked overnight at 4C in Tris-buffered saline containing 0.1% Tween (TBS-T) and 4% (w/v) skim milk powder. Membranes were probed with primary antibody for 2 ½ hours at room temperature, followed by species-specific biotinylated secondary antibody for 1 hour, and streptavidin/horseradish peroxidase-labelled tertiary conjugate for 40 minutes (dilution 1:5000 [Amersham, Piscataway, NJ]); between steps, blots were washed for 3 times 10 minutes in TBS-T with 1% skim milk. Antibody sources and working dilutions are detailed in table 1. Visualization of protein bands was carried out using enhanced chemiluminescence (ECL) according to manufacturer instructions [Amersham] followed by autoradiography, and quantification. Quantification of data obtained on film was accomplished using a CCD camera connected to digital imaging densitometer, under nonsaturating conditions. Multiple film exposures were acquired, to ensure the absence of film saturation by comparing numerical results from a number of exposure times (best visual appearance selected for presentation in experimental figures; unsaturated film for data analysis). Background subtraction was achieved by reading the absorbance of an equal sized

region directly adjacent (above or below) to the band. Graphical data are presented as means ± SD for a minimum of three replicate samples.

Proteins quantified by Western blot were MLCK, sm-MHC, nm-MHC, sm-α-actin, β-actin, smoothelin [antibodies from Sigma, St Louis, MO]; MLCP (MYPT subunit) [antibodies from Upstate Biotech, Waltham, MA].

Phosphoprotein Analysis:

The second cohort of piglets was utilized for this experiment, and for the subsequent phosphatase assay. Using a dissecting microscope, 2^{nd} to 4^{th} generation pulmonary arteries were rapidly removed from lungs, cleaned of adventitia and immediately frozen in dry ice-cold acetone (total dissection time < 1 hour). Half of the obtained arterial tissue was cooled in liquid N_2, pulverized mechanically with a glass dounce homogenizer and then sonicated for 3 times 5 minutes at 4°C to obtain a tissue homogenate for phosphoprotein analysis, in an extraction buffer (protocol from Kinetworks [Kinexus Bioinformatics, Vancouver, BC]; 4 ml/g wet weight tissue) containing 20 mM MOPS (pH 7.0), phosphatase inhibitors 2 mM Na_3VO_4, 30 mM NaF, 40 mM β-glycerophosphate and 10 mM Na pyrophosphate, ion chelators 2 mM EGTA, 5 mM EDTA, detergent 0.5% NP-40, and protease inhibitors 3 mM benzamidine, 1 mM PMSF, 5 μM pepstatin A, 10μM leupeptin. Homogenates were centirifuged at 16000*g* for 15 minutes to remove particulate matter, and protein concentration of supernatants assayed using Bradford assay kit [Biorad]. Phosphoprotein analysis was carried out by Western blot of arterial homogenates as described above, using phosphospecific antibodies for MYPT, *P*- MYPT (thr 696), *P*- MYPT (thr 850) [antibodies from Upstate Biotech]; CPI-17 [gift of Dr. D. Brautigan]; *P*- CPI-17 (thr 38) [antibody from Santa Cruz Biotechnology, Santa Cruz CA]).

Myosin Phosphatase Activity Assay (MLC Dephosphorylation):

The remainder of the arterial tissue (second piglet cohort) was pulverized and then homogenized on ice in extraction buffer (6 ml/g wet weight tissue) containing 50 mM KCl, 1.125 mM EGTA (as kinase inhibitor), 1 mM DTT, 1.25 mM benzamidine, 5 µM pepstatin A, 10µM leupeptin in 50 mM Tris HCl (pH=7.0). Homogenates were centrifuged to remove particulate matter, and protein quantified as previously described.

Myosin light chain was labelled with ^{32}P by method of Pato [285]. Briefly, myosin light chain (MLC; derived from rabbit smooth muscle [Sigma]) in myosin labelling buffer (0.25mM $CaCl_2$, 10mM $MgCl_2$ in 50mM Tris buffer, pH 7.4) was incubated with smooth muscle MLCK (derived from chicken gizzard; gift of Dr. M. Pato), calmodulin [Sigma] and ^{32}P-ATP for 90 minutes at room temperature, using usual precautions for radioactivity. Labelled MLC was injected into a 10,000 MW dialysis cassette and dialyzed against 0.5M KCl dialysis buffer at 4C for 3 hours, removing unbound ^{32}P. 0.5M KCl dialysis buffer was replaced, and MLC redialyzed for 3 hours, followed by a final dialysis against 50mM KCl buffer for 15 hours at 4C. Washed MLC was removed from the dialysis cassette, and 5 µl MLC added to 100 µl 17.5% trichloroacetic acid, 100 µl 1M bovine serum albumin (BSA) and 5 ml BCS scintillation cocktail. Radioactivity of labelled MLC was counted in this sample in a digital plate reader using an isotope-specific program. Efficiency of labelling was estimated on the basis of available counts (calculated from dated ^{32}P-ATP radioactivity), and MLC was considered to be sufficiently phosphorylated for experimental purposes if greater than 5000 counts per minute were recorded per µl labelled MLC. This value was designated as total available phosphate for the purposes of myosin light chain phosphatase activity assay.

Phosphatase activity assay reaction was started by adding ^{32}P-MLC to serial dilutions of arterial homogenates in extraction buffer (1, 1:5, 1:25, 1:125; total reaction volume 50 µl) and incubating at 30℃ for 15 minutes. The reaction was stopped by addition of 100 µl 17.5% TCA and 100 µl 1M BSA and incubation on ice for 15 minutes. The precipitate was sedimented by centrifugation (1 minute at 15600g) and radioactivity present in the supernatant (unbound ^{32}P) counted. Spontaneously released radioactivity, representing spontaneous release of labelled phosphate in absence of myosin phosphatase, was also counted in control samples (without addition of arterial homogenate) at every time point measured, and this number was subtracted from total radioactivity measured for each sample to obtain a value for labelled phosphate release specifically due to phosphatase activity in each sample. Phosphate released by this calculation was standardized to protein concentration of initial homogenate, and expressed as a percentage of total available labelled phosphate.

Rate of myosin light chain dephosphorylation was measured in tissue from the three treatment groups by quantifying phosphatase activity at serial time intervals (0, 5, 10 and 20 minutes reaction time). Inhibition of phosphatase activity by incubation with okadaic acid was also measured, with the intent of narrowing down the specific protein phosphatase class active in these homogenates. 1 nM okadaic acid was added to each arterial homogenate sample prior to addition of labelled myosin light chain; incubation of reaction mixture at 30°C for 15 minutes and subsequent measurement of released phosphate was performed as described above.

Absolute phosphatase activity was calculated as percent decrease from initial counts per minute (CPM) per µg protein:

$$100 \times \frac{\text{CPM release @ 15 minutes reaction time} - \text{spontaneous CPM release @ 15 minutes}}{\text{µg protein in sample}} \Big/ \text{total available CPM}$$

= % P_i release / µg total protein @ 15 minutes reaction time

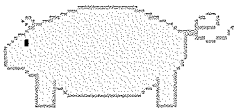
Statistical Analysis:

Quantitative data was analysed by means of analysis of variance, or in the case of subgrouped data, multiway analysis of variance, with individual comparisons made using Tukey's test with Bonferroni correction. All quantitative data is presented in experimental figures as mean +/- SD, the latter being represented in graphical data by error bars. A p value of <0.05 was considered significant.

Fig. 5: Summary of Methods

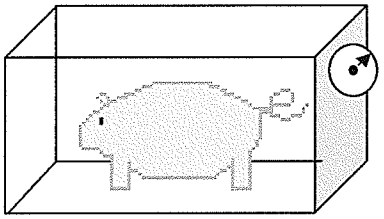
3 treatment categories:

Newborn



Day 0 Control
Grp 1 n = 5
Grp 2 n = 5

Normobaric Hypoxia
day 0 to day 3
FiO_2 0.10 (N_2 + R/A)



Day 3 Hypoxic
Grp 1 n = 5
Grp 2 n = 6

Farm bred, age-matched
Normoxic 3 day old

Day 3 Control
Grp 1 n = 4
Grp 2 n = 5

General Methods:

- Pentobarbital euthanasia
- PPHN dx by cardiac ventricular weight ratio
- Rapid dissection of 2^{nd} – 4^{th} generation pulmonary arteries

Tissue from Grp 1 animals:

- Histology of paraffin-embedded tissue(H+E stain)
- Protein analysis by Western blot

Tissue from Grp 2 animals:

- Phosphoprotein analysis by Western blot
- Myosin phosphatase assay = dephosphorylation of ^{32}P labelled myosin light chain (%P_i release per μg total protein)
- Time course of myosin light chain dephosphorylation
- Phosphatase activity in presence of protein phosphatase inhibitor okadaic acid

Table 1 Antibody sources and working dilutions

Antigen	Type	Source	Mol Wt by SDS-PAGE	Dilution (Western blot)
sm- α- actin	mouse monoclonal	Sigma	43 KDa	1: 1000
β- actin	mouse monoclonal	Sigma	42 KDa	1: 5000
CPI-17	rabbit polyclonal	Gift, Dr.D Brautigan	22 KDa	1: 5000
Phospho-CPI-17 (thr 38)	rabbit polyclonal	Santa Cruz	25 KDa	1: 1000
nm- myosin heavy chain	rabbit polyclonal	Sigma	200 KDa	1: 1000
sm- myosin heavy chain	mouse monoclonal	Sigma	204 KDa	1:1000
sm-MLCK	mouse monoclonal	Sigma	108 KDa	1: 1000
MYPT	rabbit polyclonal	Upstate Biotech	130 KDa	1: 10,000
Phospho-MYPT (thr 696)	rabbit polyclonal	Upstate Biotech	130 KDa	1: 2000
Phospho-MYPT (thr 850)	rabbit polyclonal	Upstate Biotech	130 kDa	1: 2000
Smoothelin	mouse monoclonal	Chemicon	59 KDa, 100 KDa	1: 1000
mouse IgG (2°)	sheep polyclonal, biotinylated	Amersham	n/a	1: 2500
rabbit IgG (2°)	sheep polyclonal, biotinylated	Amersham	n/a	1: 2500

Results

Establishment and Characteristics of Pulmonary Hypertension

Piglets were successfully raised in the hypoxic environment, with one mid-trial loss due to sepsis (tissue was not collected from this animal, and it was not included in the sample size). Age-matched controls were not dissimilar in terms of body weight; expressed as mean ± standard deviation, newborns weighed 1400 ± 51g, 3 day normoxic controls 1756 ± 350g, and 3 day hypoxic animals 1633 ± 197g; p=0.125 (Table 2). Hypoxic environment was maintained in the range of 9.96 ± 1.37 % FiO_2.

The diagnosis of pulmonary hypertension was made on the basis of cardiac right ventricular afterloading, using the blotted tissue weight ratio of left ventricle plus septum to right ventricle to determine relative right ventricular hypertrophy (Table 2; Fig 6). Newborn piglets had a low ventricular ratio indicating right ventricular dominance, which is consistent with high prenatal pulmonary circuit resistance (LV+S: RV = 1.271; $p<0.01$ when compared to 3 day normoxic group). By the third day of life, normoxic piglets achieved an increase in ventricular weight ratio, as left ventricular weight increased relative to right (LV+S: RV = 1.746). In hypoxic animals, this shift to left ventricular dominance did not occur, and cardiac ventricular ratio was significantly lower (LV+S: RV = 1.170; $p<0.01$ when compared to 3 day normoxic group), largely due to increased right ventricular weight; cardiac ratio in the hypoxic group was not significantly different from newborns. Mild left ventricular hypertrophy was also seen in the hypoxic group (7.178 ± 1.232g, $p < 0.05$ compared to newborn group), and was tentatively ascribed to the effects of hypoxic myocardial dysfunction.

After three days hypoxic exposure, haematoxylin/eosin staining of randomly stratified sections from paraffin embedded blocks of whole lung tissue revealed no qualitative difference in vascular medial thickness between hypoxic and control animals (Fig 7). There was no visible difference in tissue edema or in inflammatory cell infiltration.

Further characterization of the pulmonary vascular effects of short course hypoxia in this animal model involved examination of predominant medial smooth muscle phenotype. Protein homogenates from cleaned 2^{nd} to 4^{th} generation (conduit and resistance) pulmonary arteries of hypoxic, control and newborn piglets were compared for abundance of marker proteins associated with contractile or synthetic smooth muscle phenotype, by means of Western blot analysis. As an initial experiment, smooth muscle and non-muscle myosin light chain kinase content was compared between small (diameter < 3mm) and large (diameter > 3mm) pulmonary arteries, and no difference was found in abundance of either isoform between the two arterial size groups (data not shown). This permitted us to include both conduit (elastic) and resistance (muscular) arteries in homogenated tissue samples; while proportions of large and small vessels were kept approximately equivalent in each sample, it was felt that contributions from different sized arteries would not unduly influence predominant smooth muscle phenotype.

Myosin heavy chain (smooth muscle isoform) was increased in hypoxic animals compared to age matched controls (optical density standardized to newborn control = 1 arbitrary unit; 3 day normoxic 0.416 ± 0.025; 3 day hypoxic 2.015 ± 0.722 relative density expressed as mean ± SD; $p<0.05$). There was no significant difference in abundance of the more ubiquitous non-muscle isoform of myosin heavy chain (newborn 1 ± 0.256 au; 3 day normoxic 1.682 ± 1.969; 3 day hypoxic 1.401 ± 0.636, p=NS; Fig 8).

Smooth muscle α-actin content was also not different between newborn, 3 day normoxic and 3 day hypoxic animals (optical density 1 ± 0.204 vs 0.799 ± 0.174 vs 0.979 ± 0.157 arbitrary units (au) respectively; Fig 9). However the intermediate filament-associated protein smoothelin, which is known to be increased in the contractile muscle phenotype, remained elevated in hypoxic pulmonary artery in comparison to age-matched normoxic controls, where smoothelin content decreased from birth (optical density 1 ± 0.540 arbitrary units in newborn vs 0.322 ± 0.155 in 3 day normoxic vs 1.109 ± 0.356 in 3 day hypoxic group, 3 day hypoxic groups > 3 day controls, $p<0.05$; Fig 9).

Table 2 Animal weights, cardiac morphometry and oxygen exposure data

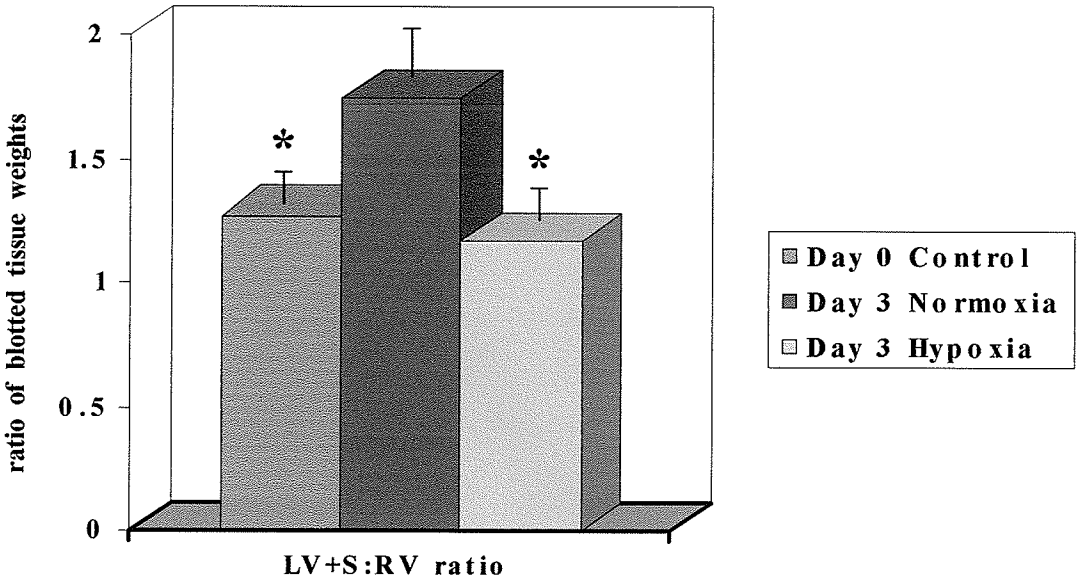
Treatment group	N	Final weight	RV weight (g)	LV+S weight (g)	Ratio LV+S: RV	FiO_2
Day 0 Control	11	1400 +/- 51g	4.279 +/- 0.876	5.414 +/- 1.061	1.271 +/- 0.108	21 % (R/A)
Day 3 Normoxia	9	1756 +/- 350g	3.923 +/- 0.691	6.842 +/- 1.432	1.746 +/- 0.221 #	21 % (R/A)
Day 3 Hypoxia	11	1633 +/- 197g	6.295 +/- 1.689 #*	7.178 +/- 1.232 #	1.170 +/- 0.166 *	9.96 +/- 1.37 % #*

significantly different from Day 0 Control group, $p < 0.05$
* significantly different from Day 3 Normoxic group, $p < 0.05$

Piglets in all three treatment groups, whether farm-raised or institutionally raised, had similar weights at time of euthanasia. The hypoxic group was maintained in a tightly controlled environment, with oxygen tension in the range of 9 - 11% for 65 - 72 hours.

Pulmonary hypertension was diagnosed by decreased cardiac left ventricle plus septum to right ventricle blotted tissue weight ratio in the hypoxic group compared to age-matched normoxic controls; the hypoxic group had a similar cardiac weight ratio to neonatal controls. The majority of this change was contributed by right ventricular hypertrophy. Mild left ventricular hypertrophy was also seen in some hypoxic animals, possibly as a result of hypoxic myocardial dysfunction.

Fig. 6: Morphometry I: Right Ventricular Hypertrophy

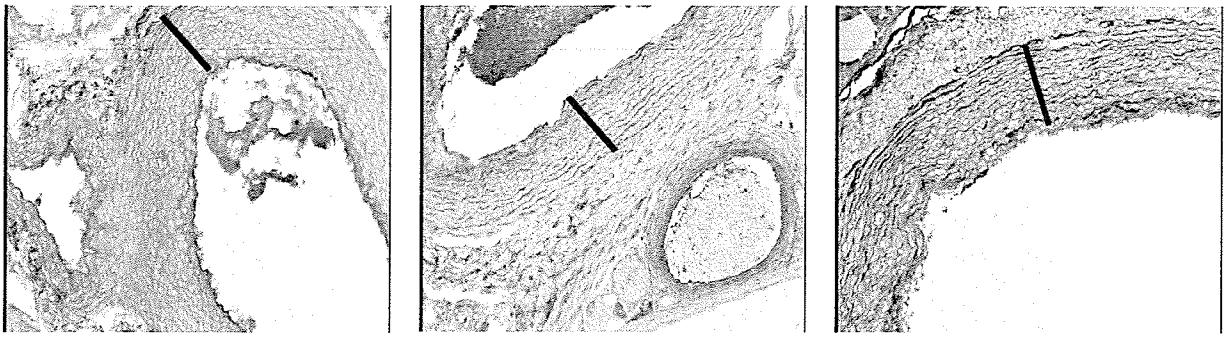


Bars represent standard deviation

* $p < 0.01$ compared to Day 3 Normoxia grp

Blotted tissue weight ratio of cardiac left ventricle and septum to right ventricle. Cardiac ventricular weight ratio indicates right ventricular dominance in normoxic neonatal piglets, with an increase in ratio by the third day of life, likely related to enlargement of left ventricle and septum. Hypoxic piglets had relative right ventricular hypertrophy on the third day of life; this is significantly different from age-matched normoxic controls.

Fig. 7: Morphometry II: Histology (H+E stain)



Day 0 Control **Day 3 Normoxia** **Day 3 Hypoxia**

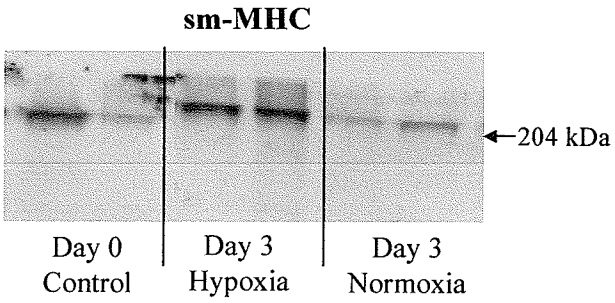
(Black bar = tunica media)

Representative of serial examination of a minimum of 15 tissue sections per treatment group, randomly stratified within paraffin-embedded tissue blocks; 20x magnification; qualitative analysis, medial thickness not quantified.

No apparent difference in vascular medial thickness between control (newborn), 3 day normoxic and 3 day hypoxic piglets, suggesting the absence of structural remodelling after short course hypoxia.

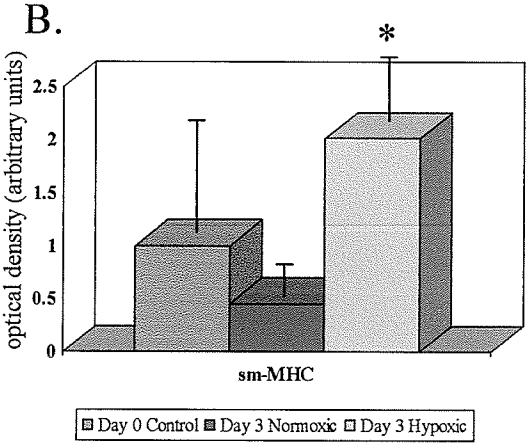
Fig 8: Smooth muscle and non-muscle isoforms of myosin heavy chain

A.



Representative Western blot,
pulmonary arterial homogenate,
20 µg protein per lane

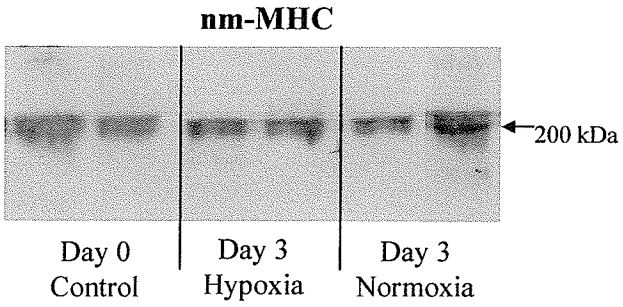
B.



Densitometry, ratiometric to Day 0 controls
Bars represent standard deviation

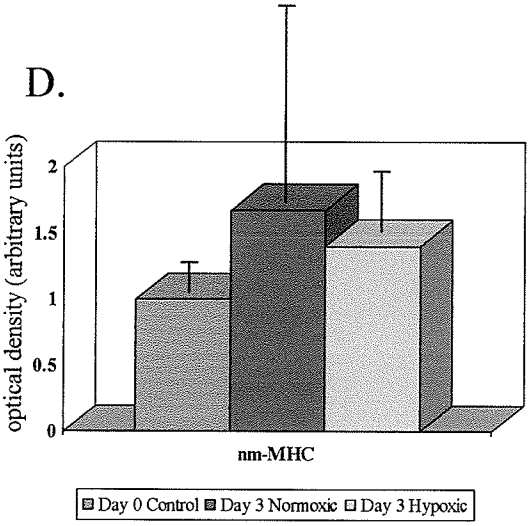
* $p < 0.005$ compared to Day 3 Normoxia grp

C.



Representative Western blot,
pulmonary arterial homogenate,
20 µg protein per lane

D.



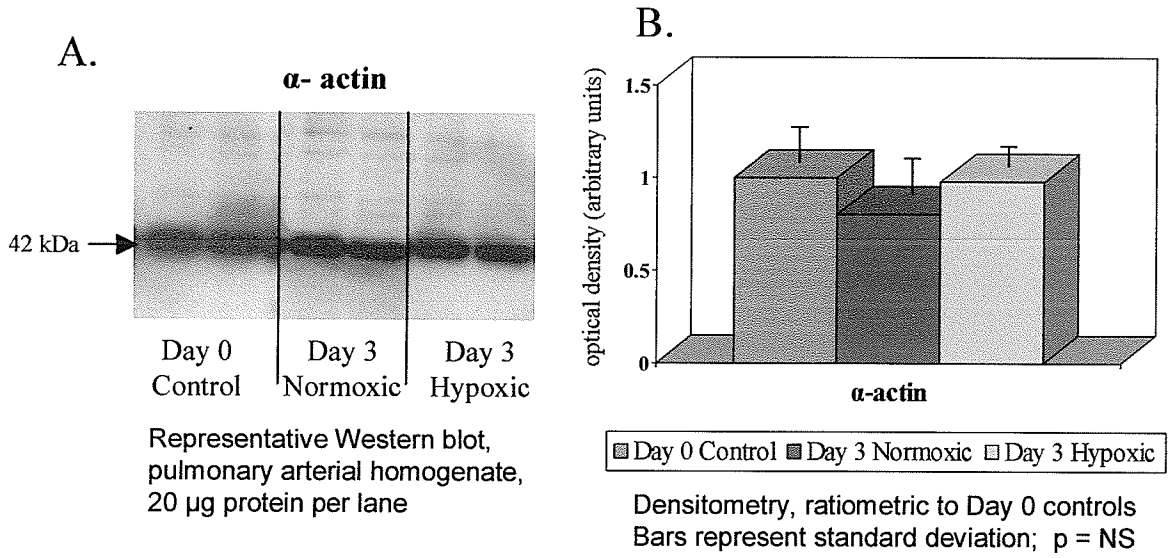
Densitometry, ratiometric to Day 0 controls
Bars represent standard deviation; p = NS

Pulmonary arterial homogenates; representative Western blots and densitometry (Day 0 control n = 5; Day 3 normoxic n = 4; Day 3 hypoxic n = 5)

A, B: Smooth muscle isoform of MHC decreases by the third day of life (this decrease is not statistically significant). In contrast, hypoxia results in an increase in sm-MHC content, significantly greater than in age-matched controls, indicating hypoxia induces increase in differentiated smooth muscle phenotype.

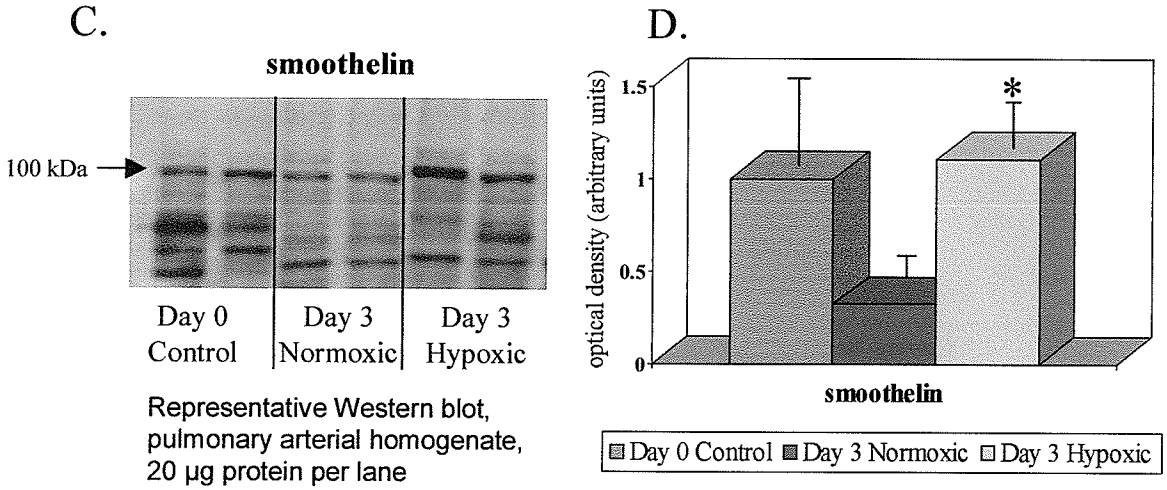
C, D: Non-muscle isoform of MHC is unchanged between treatment groups, although within-group variation is very large.

Fig 9: Smooth muscle isoforms of actin and smoothelin



Representative Western blot, pulmonary arterial homogenate, 20 µg protein per lane

Densitometry, ratiometric to Day 0 controls
Bars represent standard deviation; p = NS



Representative Western blot, pulmonary arterial homogenate, 20 µg protein per lane

Densitometry, ratiometric to Day 0 controls
Bars represent standard deviation

* $p < 0.05$ compared to Day 3 Normoxic grp

Pulmonary arterial homogenates; representative Western blots and densitometry (Day 0 control n = 5; Day 3 normoxic n = 4; Day 3 hypoxic n = 5)

A, B: Smooth muscle α-actin was unchanged between treatment groups.

C, D: Intermediate filament protein smoothelin (vascular isoform) was higher in neonates and decreased on the third day of life; hypoxic animals had significantly higher smoothelin content than age-matched controls, indicating a relative increase in smooth muscle contractile phenotype.

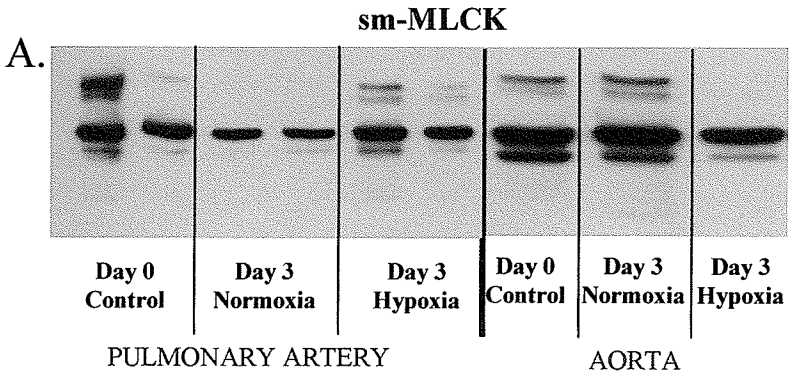
Myosin Light Chain Phosphatase Content and Activity

Tissues from a total of 16 animals (5 day 0 controls; 5 day 3 normoxic; and 6 day 3 hypoxic) were examined in the following experiments. Myosin targeting subunit content was utilized as an indicator of myosin light chain phosphatase content, as one MYPT subunit is known to be required for each active MLCP holoenzyme molecule, and an antibody targeted to MYPT is commercially available. Pulmonary arterial MLCP protein abundance, as indicated by MYPT content, was similar in newborn, 3 day control and 3 day hypoxic animals (optical density as mean ± SD: 1 ± 0.172 vs 0.890 ± 0.232 vs 0.727 ± 0.205 arbitrary units, respectively). Smooth muscle myosin light chain kinase content was also unchanged between the three groups tested (newborn 1 ± 0.079; 3 day normoxic 1.039 ± 0.330; 3 day hypoxic 1.476 ± 0.064 au; Fig 10).

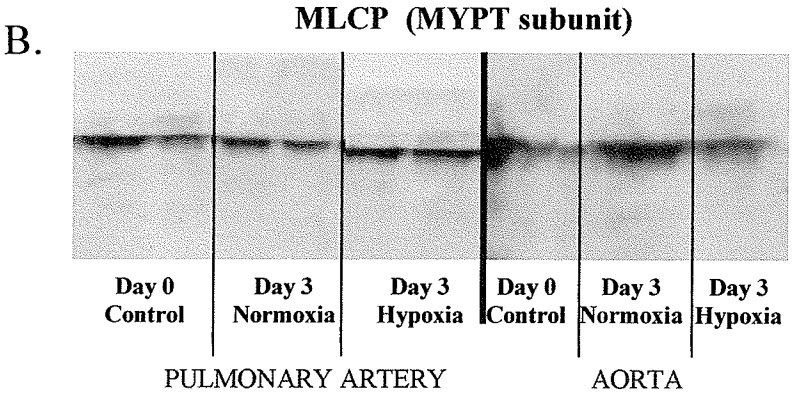
Myosin light chain phosphatase activity was measured in pulmonary arterial homogenates in the presence of a myosin kinase inhibitor (calcium chelator EGTA), using labelled myosin light chain as substrate, and expressed as emitted counts per minute per microgram total protein, as a proportion of available labelled substrate (Fig 11). Phosphatase activity was low in newborn animals (8.675 ± 1.103% P_i release/μg total protein), and showed a pronounced increase by the third day of life in normal animals (18.65 ± 4.64 μg P_i release/μg total protein; $p<0.005$ compared to newborn group). Hypoxic animals did not develop this age-related increase in phosphatase activity (10.55% P_i release/μg total protein; $p<0.005$ compared to 3 day normoxic group); rate and extent of myosin light chain dephosphorylation were decreased in this group to newborn levels (Fig 12; 3 day normoxic group significantly higher P_i release/μg total protein than newborn control and hypoxic groups at 5, 10 and 20 minutes, $p<0.005$).

Phosphatase activity was measured in the presence of the protein phosphatase inhibitor okadaic acid, and was found to be proportionally diminished in all treatment groups; nanomolar concentrations of okadaic acid caused a decrease in extent of myosin light chain dephosphorylation to approximately 30% of starting values for all pulmonary artery homogenates; newborn 5.13 ± 1.35% P_i release/μg total protein (mean reduction 74.53 ± 12.69% from initial phosphatase activity), 3 day normoxic 10.43 ± 2.85% P_i release/μg total protein (mean reduction 68.53 ± 8.2% from initial activity), 3 day hypoxic 5.79 ± 1.9% P_i release/μg total protein (mean reduction 75.79 ± 7.5% from initial value); a comparison of relative reductions in measured phosphatase activity by okadaic acid revealed no difference between three treatment groups (p=NS; Fig 13).

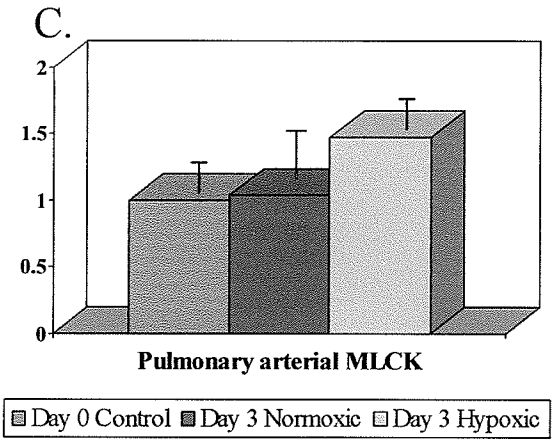
Fig. 10 Protein content of MLCK and MLCP



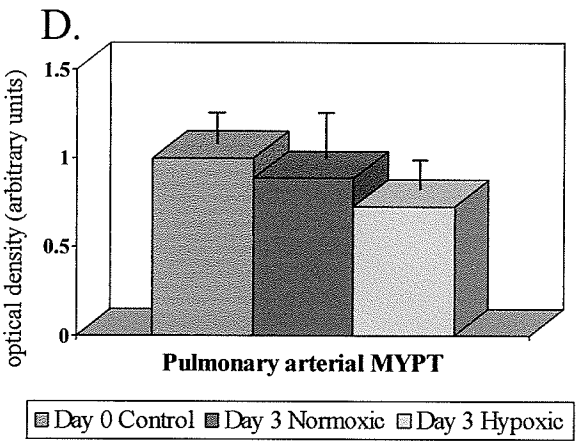
Representative Western blot, pulmonary arterial homogenate, 20 µg protein per lane



Representative Western blot, pulmonary arterial homogenate, 20 µg protein per lane



Densitometry, ratiometric to Day 0 controls
Bars represent standard deviation; p = NS

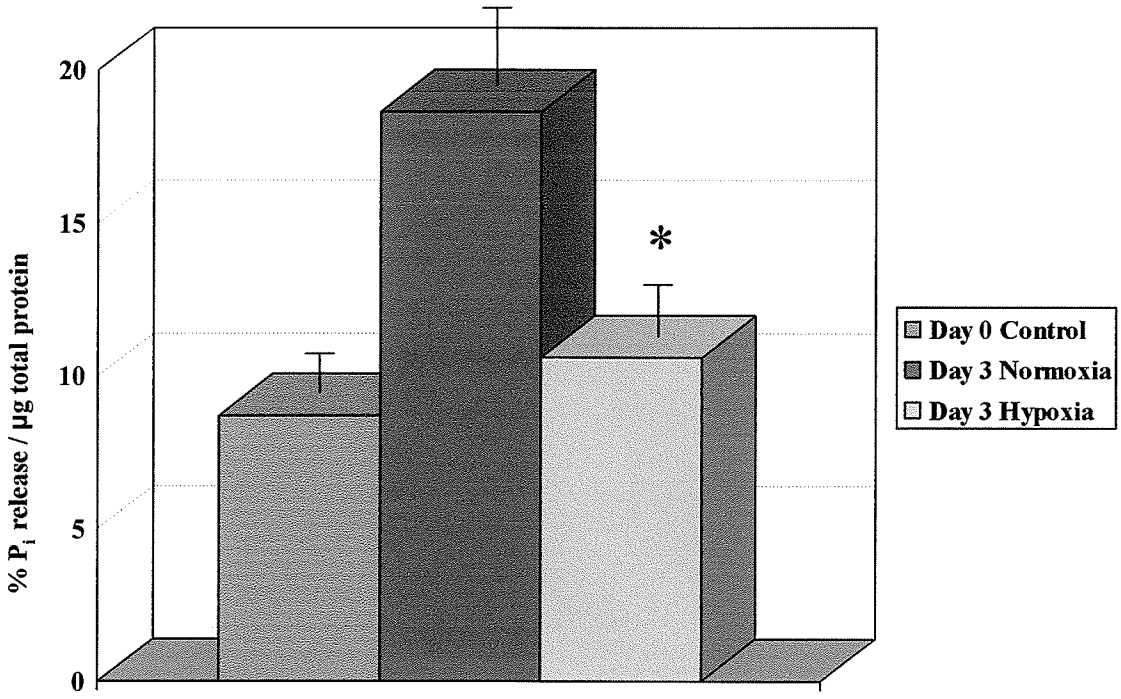


Densitometry, ratiometric to Day 0 controls
Bars represent standard deviation; p = NS

Pulmonary arterial homogenates; Aortic homogenates shown as negative control. Representative Western blots and densitometry (Day 0 control n = 5; Day 3 normoxic n = 5; Day 3 hypoxic n = 6)

A, C: MLCK is equally abundant in all treatment groups
B, D: MLCP (as estimated by quantification of MYPT subunit) is equally abundant in all treatment groups

Fig. 11: Myosin Light Chain Phosphatase activity



Bars represent standard deviation

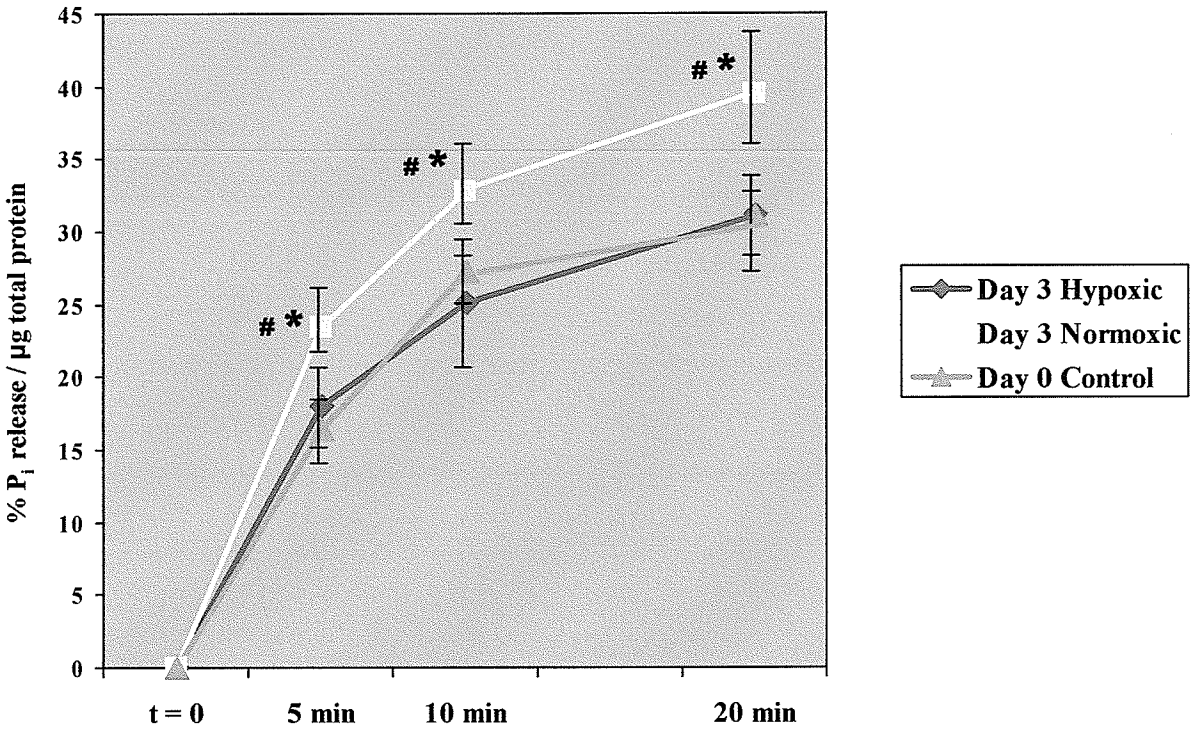
* $p < 0.005$ compared to Day 3 Normoxia grp

(Day 0 control n = 5; Day 3 normoxic n = 5; Day 3 hypoxic n = 6; four samples per animal)

Phosphatase activity increases over the first 3 days of life in pulmonary arterial homogenates from normoxic animals, likely representing a maturation of pulmonary phosphatase activity during normal circulatory transition.

Pulmonary arterial smooth muscle from hypoxic piglets had significantly lower myosin phosphatase activity (decreased P_i release per gram total protein) compared to age-matched normoxic animals. This was not associated with a relative change in phosphatase content (Fig 10), and therefore is reported as decreased enzyme activity.

Fig. 12 Rate of myosin light chain dephosphorylation



Bars represent standard deviation

* $p < 0.05$ compared to Day 0 control group

\# $p < 0.05$ compared to Day 3 hypoxic group

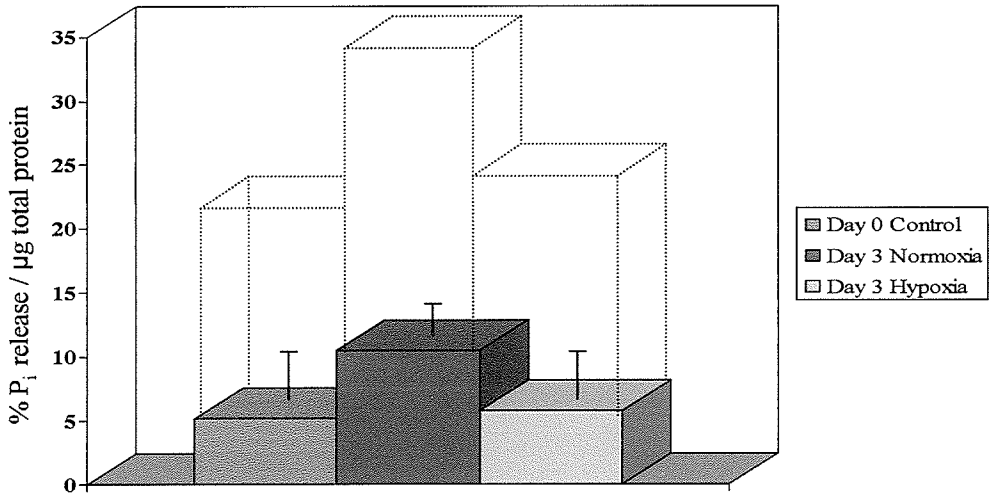
Rate of dephosphorylation of myosin light chain by tissue homogenates from newborn control, (Day 0 control n = 5; Day 3 normoxic n = 5; Day 3 hypoxic n = 6; three samples per animal per time point)

Day 3 normoxic and Day 3 hypoxic piglets shows significant upregulation of activity in normal aniamls by third day of life, which is ablated in hypoxic animals.

Measured phosphatase activity is significantly different in Day 3 normoxic group compared to newborn and hypoxic groups at all point along curve, with the majority of difference in activity achieved by 5 minutes reaction time.

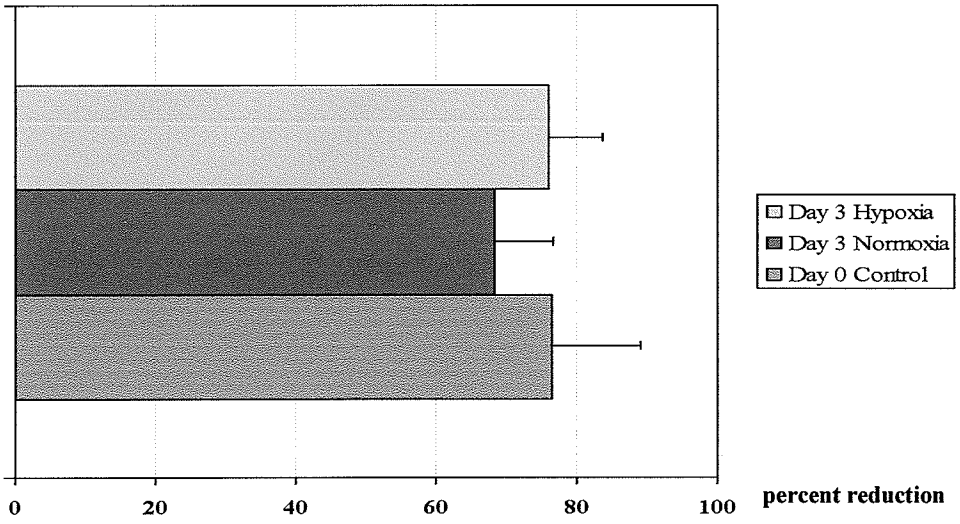
Fig. 13 Reduction of phosphatase activity in presence of 10^{-9} okadaic acid

A. Absolute Phosphatase Activity



Dotted columns show initial phosphatase activity measured in each treatment group; Coloured columns are absolute phosphatase activity after treatment with 10^{-9} okadaic acid. Bars represent standard deviation.

B. Relative Reduction of Measured Myosin Light Chain Dephosphorylation



Proportionate reduction of phosphatase activity in presence of okadaic acid (comparison of paired samples). Bars represent standard deviation; no significant difference between groups.

A: Phosphatase activity is decreased in all treatment groups by okadaic acid

B: Measured phosphatase activity is equally attenuated in all treatment groups by protein phosphatase inhibitor okadaic acid, relative to measured activity in samples unexposed to okadaic acid. Profile of protein phosphatases present in newborn, normoxic and hypoxic pulmonary artery is therefore similar.

Upstream Regulation of Phosphatase Activity

Pathways that may contribute to myosin phosphatase downregulation in hypoxia were investigated by examining the activation state of upstream signalling molecules. Tissues from a total of 16 animals (5 day 0 controls; 5 day 3 normoxic; and 6 day 3 hypoxic) were examined in the following experiments. Phosphorylation of the myosin targeting subunit (MYPT) of phosphatase was determined using phosphospecific antibodies, in a protein homogenate, the homogenization buffer containing both phosphatase inhibitors (including sodium orthovanadate) and kinase inhibitors (calcium chelators) so as to maintain in vivo tissue phosphorylation state. Neither the threonine 696 residue (optical density of bands expressed as mean +/- SEM: newborn 1 +/- 0.790; 3 day normoxic 2.322 +/- 0.856; 3 day hypoxic 2.241 +/- 0.996 au; p=NS) nor the threonine 850 residue of MYPT (optical densities 1 +/- 0.196 vs 1.010 +/- 0.055 vs 0.978 +/- 0.197 arbitrary units for newborn, 3 day control and 3 day hypoxic group respectively, p=NS) was differentially phosphorylated in hypoxic pulmonary artery compared to controls (Fig 14). The abundance of MYPT phosphorylated at the threonine 696 site increased from birth to the third day of life in both normoxic and hypoxic groups, although this numerically fell short of statistical significance; this trend suggests that MYPT threonine 696 phosphorylation may be regulated developmentally by factors other than hypoxia. A secondary band was noted on the Western blots of phospho-MYPT-thr696, migrating just below the 130 kDa band (estimated molecular weight 120 kDa); the identity of this protein band is not clear. The density of this band varied considerably within groups, and the variation between treatment groups did not achieve significance, so it was not further pursued.

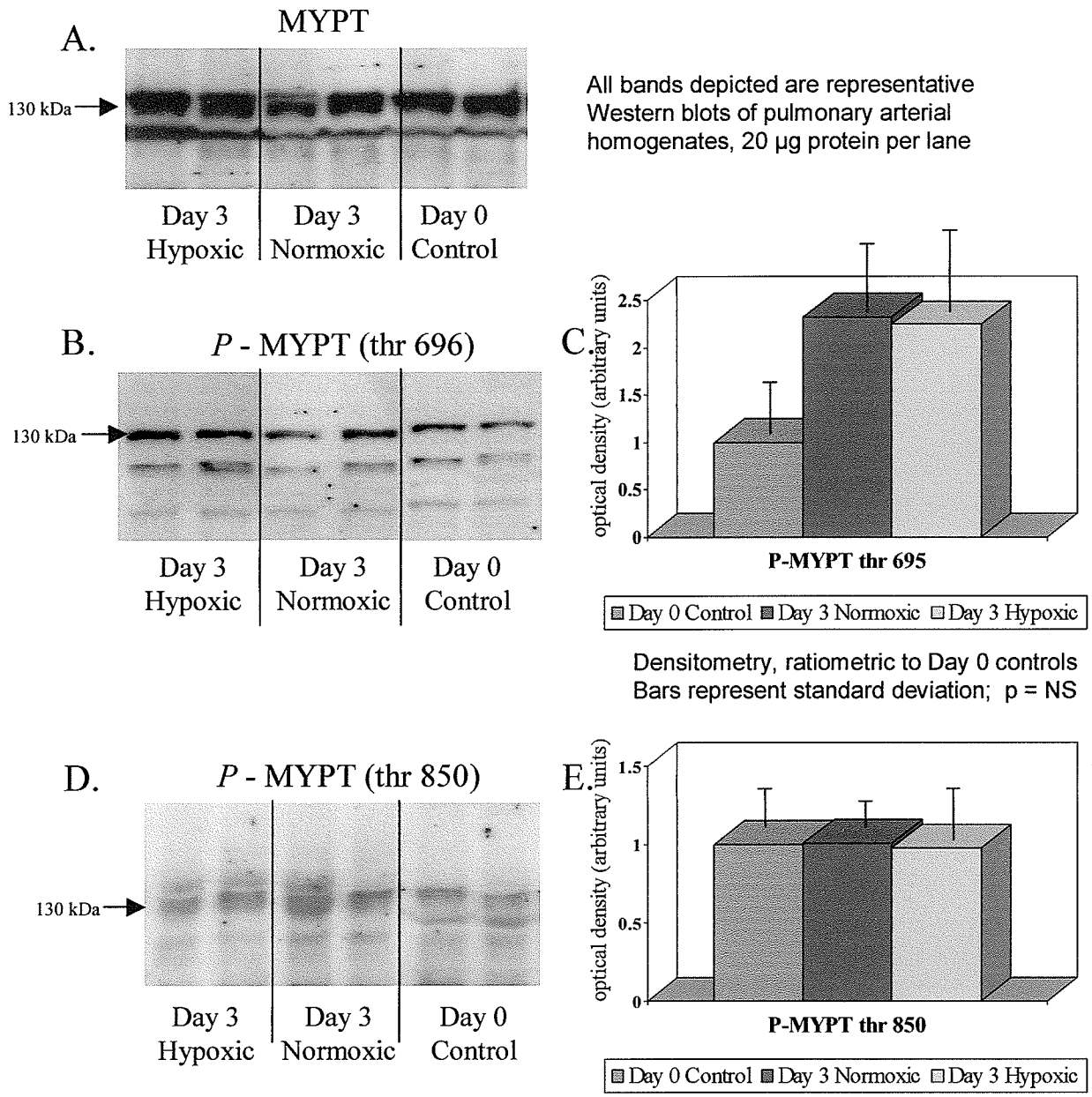
Abundance of CPI-17, a pseudosubstrate inhibitor of myosin phosphatase that competes for its catalytic site, was also determined by Western blot; activation state of

CPI-17 was estimated by threonine 38 residue phosphorylation (Fig 15). Native CPI-17 content was greatest in newborn pulmonary artery (optical density 1 +/- 0.434 au), and appeared to decline by the third day of life in normoxic animals (optical density 0.471 +/- 0.278 au). Hypoxic animals had a greater abundance of CPI-17 than normoxic animals (density 0.735 +/- 0.318 au), although this difference did not achieve statistical significance. Phosphorylated CPI-17 was greater in newborn pulmonary artery (equated to 1 +/- 0.299 au) compared to artery from normoxic 3 day olds (density 0.593 +/- 0.164 au); in hypoxic animals, CPI-17 phosphorylation, and hence activation state, was increased (density 0.951 +/- 0.130 au, significantly different from age-matched normoxic controls, $p<0.01$).

The ratio of native to phosphorylated CPI-17 was approximated by comparing band density of paired samples, normalized to newborn controls (Fig 16). While not indicative of actual fractional phosphorylation due to differing primary antibody affinities, this ratio was indicative of relative proportion of CPI-17 phosphorylation between treatment groups, and revealed no significant differences in proportionate CPI-17 activation; in other words, amounts of phosphorylated and total CPI-17 were increased in equal proportions in newborn and hypoxic animals compared to 3 day normoxic controls (expressed as mean +/- SEM, ratio 1.375 +/- 0.305 in newborns, 2.188 +/- 1.312 in 3 day controls and 2.245 +/- 1.68 in 3 day hypoxic animals, p=NS). Total CPI-17 was compared to MYPT abundance in a similar ratio of band density of paired samples (Fig 17). Pulmonary arterial CPI-17 to MYPT ratio was essentially no different between groups, owing to a wide standard deviation in all groups (ratio in newborns 1.422 +/- 0.493, 3 day normoxics 0.884 +/- 0.656, 3 day hypoxics 1.455 +/- 0.572). In both of these ratiometric comparisons, a very wide standard deviation prevented any useful interpretation of results, and this threw into doubt the utility of such empiric comparisons

of optical densities using unrelated antibodies, in determining relative protein expression. A much larger sample size would probably be necessary to address these questions in the whole animal model; alternatively, direct measurement of protein abundance in cellular or tissue homogenates by immunoprecipitation might be a method less prone to intrinsic error.

Fig. 14 : MYPT subunit regulatory site phosphorylation

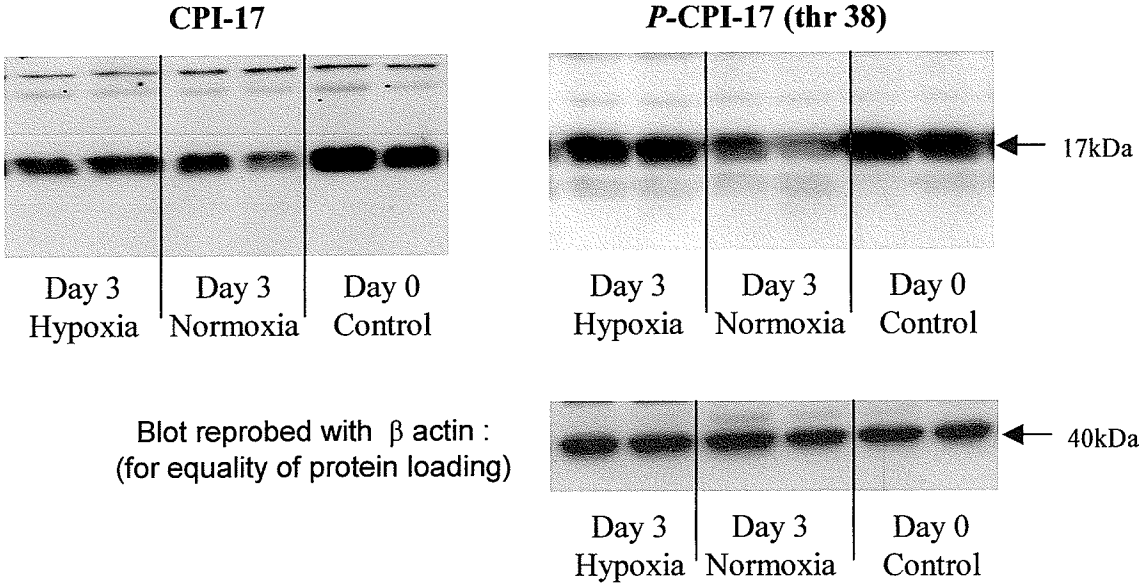


Pulmonary arterial homogenates; representative Western blots and densitometry (Day 0 control n = 5; Day 3 normoxic n = 5; Day 3 hypoxic n = 6)

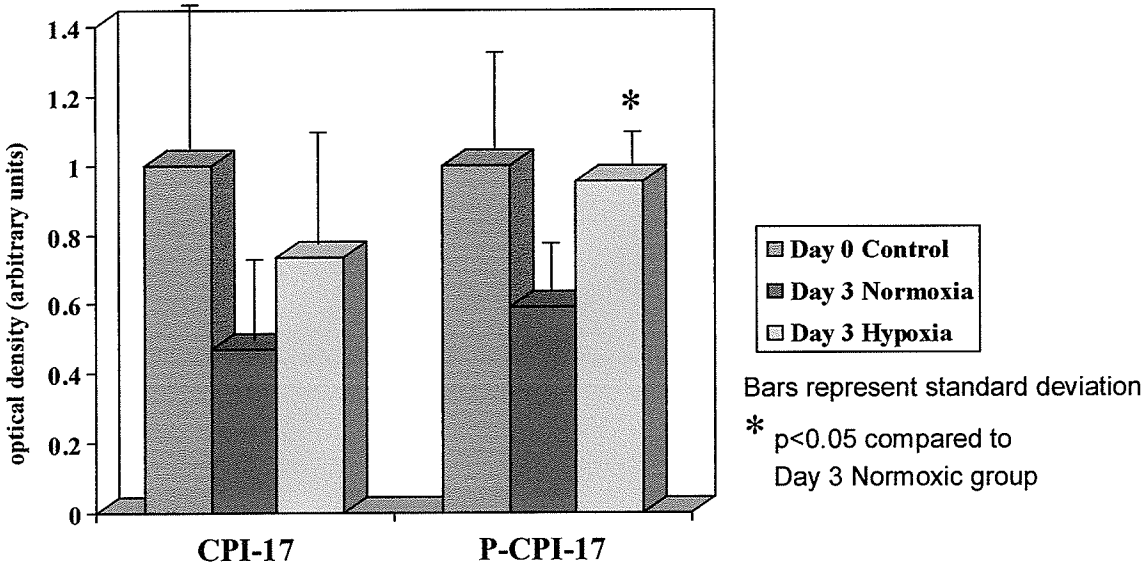
A: MYPT content is constant in all three treatment groups.
B, C: Phosphorylation of MYPT at threonine 696 is qualitatively increased in both 3 day old groups over newborns, suggesting possible developmental control (not statistically significant). MYPT phosphorylation is unaltered by perinatal hypoxia.
D, E: Phosphorylation of MYPT at threonine 850 is comparable between all treatment groups.

Fig. 15 : Phosphorylation of CPI-17

A. Representative Western blots of pulmonary arterial homogenates, 20 µg protein per lane

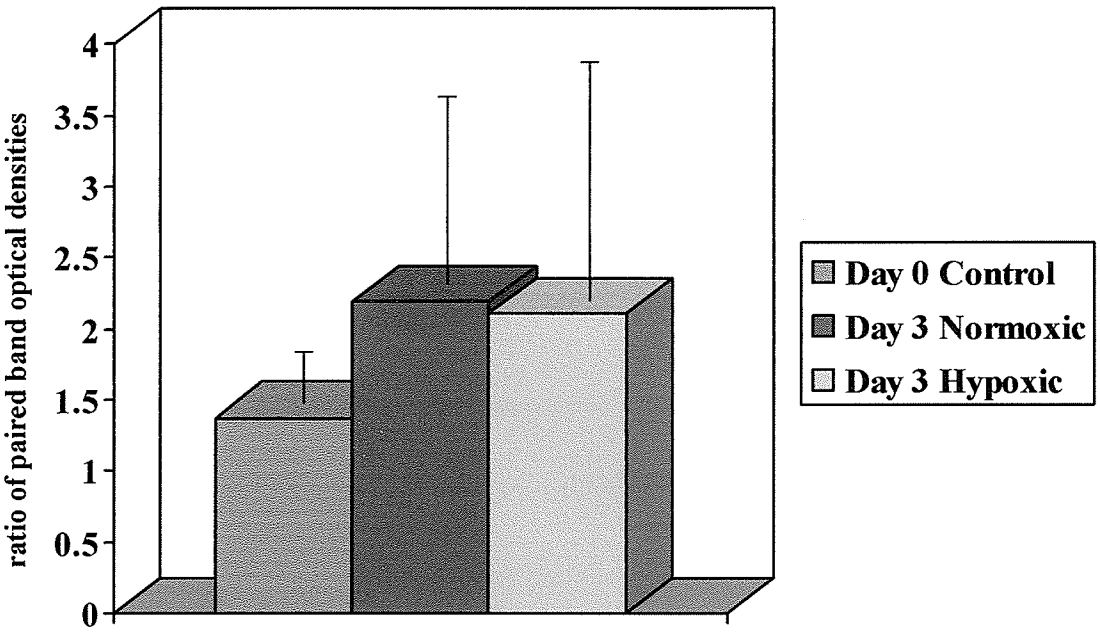


B. Densitometry, ratiometric to Day 0 controls



A, B: Regulatory protein CPI-17 appears more abundant in normal newborns, albeit with wide within-group variability; CPI-17 content is relatively decreased on the 3rd day of life in both normoxic and hypoxic groups, although this did not reach statistical significance.A significant increase in CPI-17 threonine 38 phosphorylation is seen following 3 day hypoxia, compared to age-matched normoxic controls, resulting in increased inhibitory activity.

Fig. 16: Relative band density ratio of P-CPI-17 : CPI-17

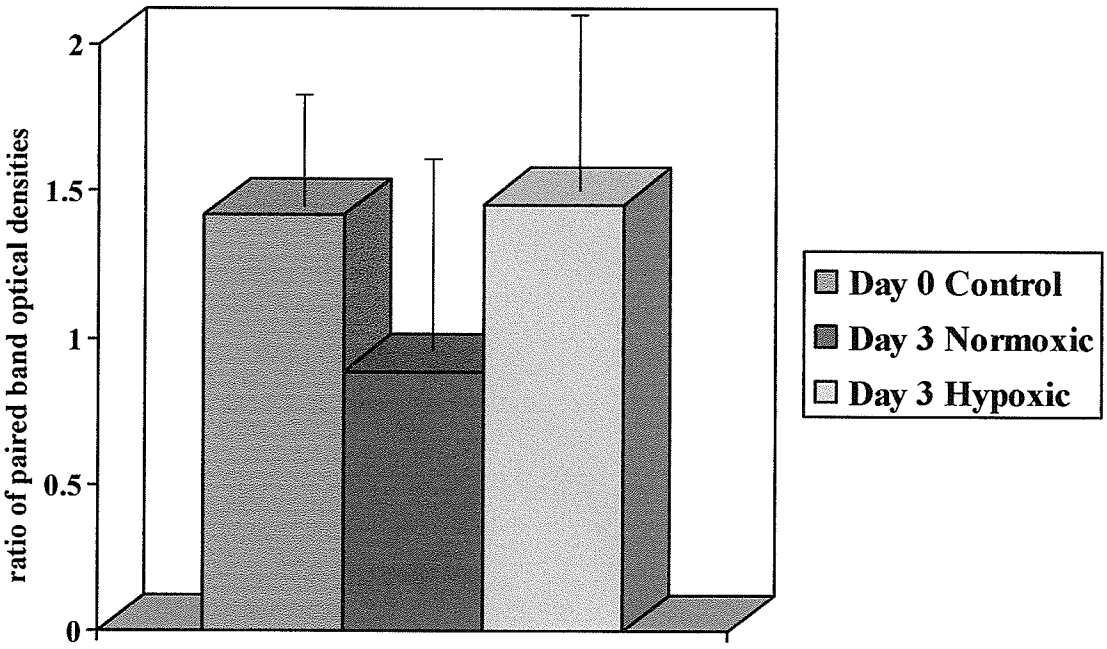


Bars represent standard deviation; p = NS

Paired band density ratio, used as an indicator of proportional phosphorylation, although not numerically equivalent due to differing antibody affinities.

A wide within-group variation exists in this calculated ratio. No significant differences can be detected between treatment groups in relative CPI-17 phosphorylation, suggesting that at least part of the observed increase in phospho-CPI-17 content is due to an overall increase in CPI-17 abundance, as well as increased upstream kinase activity.

Fig. 17: Relative band density ratio of CPI-17 : MYPT



Bars represent standard deviation; p = NS

Paired band density ratio, used as an indicator of proportional content, although not numerically equivalent due to differing antibody affinities.

No significant difference in CPI-17 to MYPT ratio was detected between treatment groups. A wide within-group variation exists in this calculated ratio.

Conclusions and Discussion

The following conclusions are drawn from the results detailed above:

- Pulmonary hypertension develops as a consequence of three days exposure to hypoxia, which in the absence of significant vascular wall remodelling may be ascribed to increased muscular contraction.
- Hypoxia causes an increase in the expression of smooth muscle myosin heavy chain and smoothelin in the pulmonary arterial wall, which are markers associated with a contractile phenotype of pulmonary arterial vascular smooth muscle; hypoxia does not change expression of α-actin, which is a more general smooth muscle marker.
- There is no change in either pulmonary arterial myosin light chain kinase or phosphatase protein content following three days neonatal hypoxic exposure.
- Myosin light chain phosphatase activity, and the rate of in vitro myosin light chain dephosphorylation, is lower in newborns and increases over the first three days of life in normoxic neonates; hypoxic exposure ablates this increase.
- Phosphatase activity is attenuated in the normal newborn, and is correlated with an increased content of protein phosphatase inhibitor CPI-17; CPI-17 content decreases during the course of normal circulatory transition.
- Hypoxia affects the relationship between CPI-17 and MLCP activity. In our model of hypoxic pulmonary hypertension, CPI-17 activity is increased at three days, and this is associated with concurrent downregulation of myosin phosphatase activity. Phosphorylation of myosin targeting subunit by Rho kinase is not found at this time. These data suggest the possibility of PKC pathway involvement in pulmonary vascular smooth muscle functional alterations following three days neonatal hypoxic exposure.

The establishment of the diagnosis of pulmonary hypertension by non-invasive measurements is difficult, particularly in moderate neonatal hypoxia where fetal hemoglobin is oxygen-avid, the brain is relatively resistant to hypoxic damage, and where both hemodynamic and behavioural changes in hypoxic PPHN are subtle. The use of ventricular tissue weight ratio as an indicator of right ventricular hypertrophy due to increased distal circuit pressure has been well validated [7], in particular in the hypoxic piglet model [273]. That three days exposure to an FiO_2 of 10% results in increased pulmonary vascular pressure was determined in this study on the basis of persistence of right ventricular dominance. Hypoxic animals had a decreased weight ratio of left ventricle and septum to right ventricle, likely due to right-sided afterloading, related to elevated pulmonary arterial resistance compared to systemic resistance. Other groups using short-term normobaric hypoxia have also demonstrated this [7] [101], with corroborative arterial pressure measurements in the isolated perfused lung [85]. The lack of visible pulmonary arterial remodelling at this stage suggests that increased circuit resistance may be due to muscular contraction rather than increased mural thickening. This is again in keeping with findings reported elsewhere for this phase of PPHN [286]. The role of smooth muscular contraction in the maintenance of perinatal pulmonary vascular tone is well known [287]. Lack of early hypoxic remodelling is consistent with the experience of other groups using this model [288], where significant remodelling is a later developing phenomenon than is increased pulmonary vascular contractile capacity [101], and where increased pulmonary vascular tone engendered by short course, but not long course hypoxia, may be fully ablated by papaverine [85]. Loss of pulmonary vasodilator responsiveness is described following prolonged hypoxia, when remodelling also occurs [289]. The normobaric hypoxia model must be seen as more reflective of the clinical condition of PPHN than are monocrotaline-induced or overflow models, which are

characterized by immediate loss of vascular contractility and rampant mural remodelling, and therefore reflect a slightly different chain of pathophysiological events.

Expression of contractile smooth muscle markers (smooth muscle myosin heavy chain, smoothelin) is high in newborn pulmonary artery, and decreases during the course of circulatory transition to lower levels by the third day of life. Hypoxia is associated with increased abundance of these two contractile phenotype markers in whole tissue homogenates. Non-muscle myosin heavy chain is unchanged in this timeframe. In reviewing this group of Western blots, the significant differences are seen between age-matched normoxic and hypoxic groups, and only in markers that are abundant in contractile phenotype smooth muscle [290], which decrease during the first three days of life in normal animals, but remain at newborn levels in hypoxic subjects. Two possible interpretations of these data, that may in part underlie the differences seen between treatment groups, are: (i) that the subpopulation of smooth muscle cells exhibiting a contractile phenotype is selectively expanded by hypoxic stimulation, resulting in an increase in smooth muscle specific markers without significant change in undifferentiated muscle markers; or (ii) that the perinatal burst of smooth muscle replication and arterial wall remodelling is associated with dedifferentiation of the predominant smooth muscle phenotype or increase in non-muscle elements in the normal 3 day old, and that this normally occurring phenotypic modulation is altered by hypoxia. Other groups have reported the latter explanation, along with the finding that pulmonary arterial smooth muscle apoptotic changes are not induced by hypoxia [33 286].

The degree of elevation of smooth muscle specific isoforms can be considered in context of the model proposed by Stenmark and Frid, that the vascular wall is composed of a very heterogeneous population of smooth muscle cells at different points on the phenotypic continuum, only some of which will proliferate in response to hypoxia [112]. If

this model is applicable, what appears to be a small protein increase in a whole tissue homogenate could represent a dramatic increase in contractile protein expression in that group of cells whose phenotype is altered by this stimulus. The observed increase in smoothelin and smooth muscle myosin heavy chain is not accompanied by decreased non-muscle myosin heavy chain, suggesting that the amplification of the measured contractile phenotype-specific markers might be due to expansion of a subpopulation of contractile cells rather than differentiation of existing synthetic cells, which would presumably be associated with a decrease in non-muscle markers. Hypoxia-induced pulmonary arterial remodelling has also been reported to involve mobilization of circulating bone marrow-derived vascular progenitor cells, which may differentiate to augment synthetic or contractile smooth muscle cell populations, as well as into endothelial cells [291]. Since it is clear that myocytes are not the only cell type found in the vascular wall, any changes we report in smooth muscle phenotype markers may actually reflect relative changes in other vascular medial cells. This would need to be further evaluated by immunohistochemistry. The more ubiquitous α-actin is unchanged between treatment groups, but since this isoform of actin has been reported, albeit in lower amounts, in myofibroblasts [290] and endothelial cells [292] as well as in fully differentiated smooth muscle, it is not a marker of great specificity. The inhomogeneity of smooth muscle response to hypoxia reported in immunohistochemical analyses elsewhere, may also explain the wide standard deviation seen in calculated tissue protein content, and has led to the hypothesis that factors other than hypoxia, including mechanical wall strain, may be driving these phenotypic alterations [293].

Pulmonary arterial smooth muscle differentiation is developmentally regulated in the fetus, and is fully competent for contraction at term [90]. In human neonates, the normal course of circulatory transition is known to involve architectural remodelling of

pulmonary arterial smooth muscle [33], and probable changes in phenotype. A postnatal burst of pulmonary smooth muscle proliferation is reported, associated with transient decrease in smooth muscle-specific markers and an increase in monomeric actin in normoxic subjects, and prevented by short course hypoxia [100]. Circulatory transition is therefore postulated to require time-sensitive phenotypic alteration, as an adjunct to mechanical pulmonary expansion and myocyte cytoskeletal reorganization, which is derailed by perinatal hypoxic exposure. Our results support these hypotheses, as some smooth muscle markers increase following short course hypoxia, and this could be associated with a change in contractile function; while total α-actin content is unchanged in our model, ratio of filamentous to monomeric actin was not determined, and might have been more revealing of architectural changes.

Hypoxia does have an established effect in cultured smooth muscle as a promoter of cell growth. However, hypoxia-inducible factor (HIF), the primary mitotic signalling agent in hypoxia, is not elaborated above oxygen tensions of 4% [108]; the moderate hypoxia in our model, and indeed the moderately hypoxic environment in the pulmonary hypertensive vascular bed, may not have a sufficiently low oxygen tension for the classic growth response. In addition, Stenmark and colleagues have described hypoxic proliferation in only certain cells from medial and adventitial compartments, while other populations of cells decrease proliferation; the heterogeneity of this hypoxic response may be more apparent in whole tissue, where contractile cells usually lost in the process of tissue culture can still be evaluated [95 218]. We have not measured proliferation in this study, and such an assessment (immunohistochemically in whole tissue, or in hypoxic smooth muscle cells in culture) would improve our understanding of the specific effects of hypoxia on smooth muscle phenotype and proliferative activation. Additional forces, with the notable example of mechanical wall strain consequent to

perfusion of a constricted circuit, may be responsible for the myogenic response in the hypoxic pulmonary vasculature [75], and may thus explain the biphasic course of PPHN, as these forces may have serial rather than parallel effects. Contractile smooth muscle cells themselves have been reported to exhibit exaggerated proliferative responses in the context of certain types of injury. In culture, phenotype dictates the growth response of smooth muscle cells to mechanical force, with the more differentiated cells displaying increased proliferation in response to chronic increases in pulse pressure [78]. This finding may be of relevance in PPHN, as hypoxia appears to induce a perinatal increase in contractile marker expression (as seen in this study), as well as a perhaps paradoxical increase in smooth muscle proliferation (reported elsewhere [286]), which may be additionally due to mechanical forces.

The extent to which phenotype switching of distinct subpopulations of smooth muscle cells in the tunica media will alter the behaviour of the whole tissue is not clear. Individual smooth muscle tissues can alter their contraction velocity under physiological conditions, which may be a consequence of long-term modulation of phenotype [113]. Cellular determinants define phasic and tonic muscle contractile properties; arterial smooth muscle is almost uniformly tonic, and as such has slow velocity of shortening but high force maintenance [294]. One could make the case that any change in cumulative contractile protein content appreciable in a whole tissue homogenate must represent a significant phenotypic alteration, the impact of which is likely to be felt in tissue contractile function. The isolation of hypercontractile smooth muscle cells on withdrawal of serum is a culture phenomenon [97]; the contribution of such cells to in vivo contractility has yet to be elucidated. Functional interconnection of smooth muscle cells via gap junctions could result in a few cells influencing contraction of many surrounding cells. An increase in contraction velocity or calcium sensitization in a few cells in the vascular

media may thus alter the balance of tone toward contraction in the whole tissue, but this hypothesis has not been validated. Cell migration and growth may certainly be affected by phenotypic change, and this may alter the structure of the tissue as a whole, over time; for example, the accumulation of medial myocyte synthetic phenotype markers is associated with intimal plaque formation in atherosclerosis [295].

Short course hypoxia does not, in our model, alter protein expression of the regulatory enzymes MLCK and MLCP. Any change in isolated muscle function known to occur at this point is therefore likely to be due to modulation of enzyme activity, as myosin phosphorylation remains the rate-limiting step in muscle contraction and relaxation [86]. There is literature evidence for diminished myosin phosphatase content at the two- week point, in an overflow model of PPHN, with no alteration in kinase content or activity. Phosphatase activity was not assayed in that experiment [7]. More recently, phosphatase activity and content, as determined by abundance of myosin targeting subunit, was found to be influenced by hypoxia in systemic vascular smooth muscle; hypoxic aorta had increased phosphatase activity [151], which is consistent with the clinical finding of decreased systemic to pulmonary resistance ratios in PPHN. Mechanical strain of cultured cells has also resulted in altered contractile enzyme content, with increased MLCK content and activity, and decreased MLCP activity, in cell lysates [8]. Changing enzyme content may represent a late response to cellular stressors, while activity may be modulated earlier through the action of signalling intermediates.

Since kinase activity has elsewhere been established as unchanged in PPHN [7], we specifically examined myosin phosphatase activity in early hypoxia. Based upon the finding of low MLCP activity in the newborn, with upregulation of activity in normal animals on the third day of life, and knowing that vascular relaxation is the crucial element of decreasing pulmonary vascular tone [287], we concluded that phosphatase

activity is developmentally regulated as part of normal circulatory transition. We also found that perinatal hypoxia decreases measured pulmonary arterial myosin phosphatase activity to newborn levels, either by preventing or downregulating the postnatal rise in phosphatase activity. This is a finding of significance, since after hypoxic pulmonary vasoconstriction, it is the only developmentally specific alteration in smooth muscle function identified in PPHN. That it occurs quite early in the course of the disease makes phosphatase downregulation a probable etiologic agent in hypoxic pulmonary relaxation failure, rather than merely a consequence of endstage changes in smooth muscle. In addition, this might explain the fact that a portion of PPHN patients treated with inhaled nitric oxide for endothelial dysfunction fail to respond to this therapy, implying a downstream defect [27]. Altered contractile enzyme balance in the effector organ of vascular hypertension would be a powerful source of intractable increase in vascular resistance in such a context.

The identity of the myosin dephosphorylating enzyme in our preparation as a cellular protein phosphatase is based on its inhibition by okadaic acid. Classically, nanomolar concentrations of okadaic acid tend to inhibit type 2 protein phosphatases, while type 1 phosphatases are inhibited by higher concentrations [158]. This dose response separation is not absolute [160], so the specific type of protein phosphatase activity measured in this assay remains to be determined, despite its partial inhibition by low concentration okadaic acid. Given the overlap of okadaic acid inhibitory ranges for protein phosphatase subgroups, the classes of protein phosphatase present in measured tissue homogenates cannot be surmised on this basis. We do conclude that, as phosphatase activity decreases equally across all treatment groups in the presence of okadaic acid, the profiles of protein phosphatases present in normoxic and hypoxic pulmonary arterial tissues are unlikely to differ substantially. The degree of phosphatase

inhibition we observed is consistent with the extent of smooth muscle phosphatase inhibition by low dose okadaic acid reported elsewhere [144]. There are numerous phosphatases present in smooth muscle; however as the major phosphatase in arterial smooth muscle is known to be PP-1M (MLCP) [151], it is reasonable to speculate that the myosin light chain phosphatase activity we were measuring was due to the enzyme myosin light chain phosphatase. This will need to be confirmed using more specific inhibitors of PP-1.

Two possible pathways of MLCP downregulation are MYPT phosphorylation by Rho kinase and CPI-17 phosphorylation. MYPT phosphorylation does not appear to be regulated at threonines 696 or 850 during the neonatal period, as no difference was seen in phospho-MYPT abundance between newborns and normal three-day-old animals. In short course hypoxia, we also did not see differential phosphorylation of either threonine site, suggesting that this pathway is not important in the developmental regulation of pulmonary vascular phosphatase activity in early hypoxia. There is no change in MYPT subunit phosphorylation at either threonine 696 or threonine 850 following three days hypoxia; both of these sites are known to be amenable to inhibitory phosphorylation by Rho kinase. We hence extrapolate that the downregulation of MLCP activity seen at this time point in our hypoxic PPHN model is unrelated to activity of this pathway. This was surprising, given the recognized role of the Rho kinase pathway in other hypertensive pathologies [183 296 177]. Rho kinase is multiply regulated in smooth muscle, particularly by cell strain, and less so by hypoxia. Activation of RhoA has been observed in hypertension independent of cause, suggesting it is a common point in downstream signaling and a critical component of hypertension [147]. Rho kinase is known to also phosphorylate CPI-17 in vitro [297] and in vivo [83], so one cannot conclude that the Rho kinase pathway is uninvolved in PPHN upon these data alone.

Considering the two modes of phosphatase regulation, MYPT phosphorylation by Rho kinase and pseudosubstrate interaction with CPI-17, in further detail (see Fig 18), it is reported that MYPT is relatively slow to dephosphorylate, implying a slow increase in myosin phosphatase activity when Rho kinase is inactivated [244]. Circulatory transition at birth requires a very rapid increase in pulmonary vascular relaxation capability, allowing pulmonary perfusion to match with early ventilatory efforts. After an initial drop in pulmonary vascular resistance in conjunction with pulmonary expansion [33], the majority of the fall in blood pressure is due to myocyte elongation and relaxation [287], which must hence occur at great speed. If MYPT dephosphorylation were required at this time, this would always be a rate-limiting step for circulatory transition. Therefore, it makes some sense that MYPT regulation via the Rho kinase pathway is not developmentally crucial for neonatal pulmonary blood flow. In this context, the lack of change we find in MYPT phosphorylation in early hypoxic PPHN supports this theory of the unsuitability of altered MYPT phosphorylation as a mechanism of perturbation of circulatory transition.

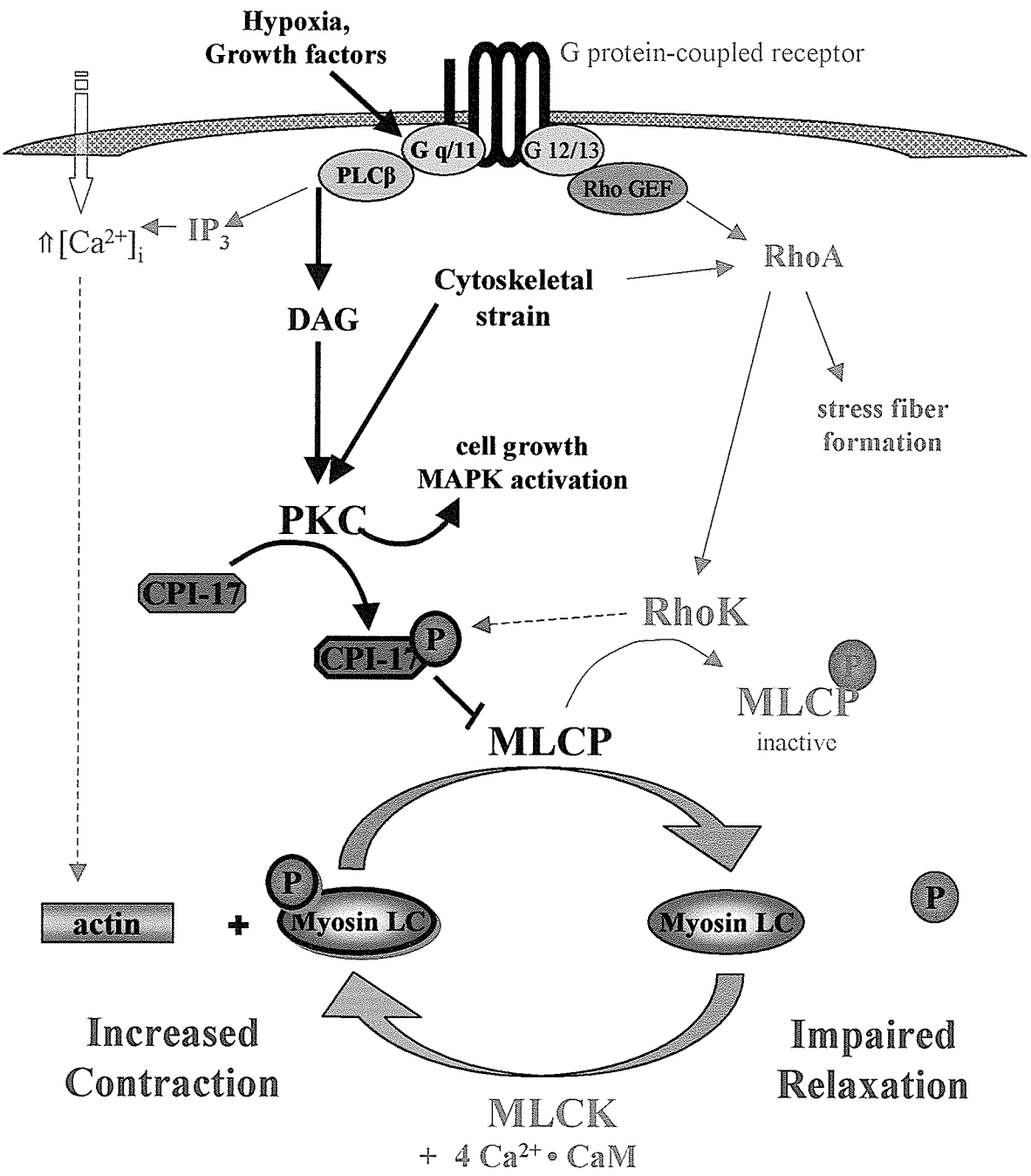
CPI-17 phosphorylation is key to regulation of relaxation in both tonic [237] and phasic smooth muscle [243]. It is known to be amenable to rapid dephosphorylation by cytosolic protein phosphatase type 2, allowing for immediate smooth muscle relaxation [244]. Tonic muscle is known to express several times the magnitude of CPI-17 found in phasic muscle, and the expression ratio of native CPI-17 to MLCP has been identified as an indicator of calcium sensitivity. Agonist stimulation of arterial smooth muscle with a high CPI-17 and low MLCP expression generated greater force and MLC phosphorylation than stimulation of visceral muscle with a relatively low CPI-17 and high MLCP content [84]. This implicates CPI-17 inhibition of MLCP as an important component in modulating vascular muscle tone in tissues having higher CPI-17 to MLCP expression ratio.

We found CPI-17 to be highly expressed in pulmonary vascular homogenates from newborn animals, with a postnatal decline in expression apparent by the third day of life. CPI-17 expression appears higher in hypoxic animals, although not to the extent present on the first day of life, and not achieving statistical significance. Phospho-CPI-17, the active moiety with an over 1000-fold increase in MLCP affinity [236], is higher in newborns than in normal three day olds. It is also markedly elevated in hypoxic animals compared to age-matched normoxic animals; pulmonary arterial phospho-CPI-17 content after three days hypoxia is comparable to the level seen in newborns. Ratio of phosphorylated to native CPI-17, as estimated by relative band density, is unchanged over this interval. In other words, the primary agent of relaxation failure found in our model of PPHN is an unalleviated increase in CPI-17 content, with a proportionate rise in CPI-17 phosphorylation. This implies importance of CPI-17 in maintenance of high fetal pulmonary vascular resistance, with regulation at the level of both CPI-17 protein expression and phosphorylation in the normal course of perinatal development, and derangement of this regulation by short course hypoxia. Examination of CPI-17 to MYPT ratio, by relative band density of paired samples after the fashion of Woodsome and colleagues [84], due to a high within-group variation, reveals no change that might be suggestive of greater intrinsic calcium sensitivity in the hypoxic tissue. The implication is of an inducible calcium sensitization, which may be closely linked to agonist stimulation and CPI-17 expression or activity modulation.

To date, regulation of vascular CPI-17 expression is not elaborated in the literature; this may be of much importance in the neonate. Phosphorylation of CPI-17 is known to occur by several agents, including PKC and also Rho kinase. Given that the other target of Rho kinase, MYPT, is not differentially phosphorylated in this model, we consider it more likely that the route of phosphatase downregulation in early hypoxic

PPHN induced in our model may involve processes that control CPI-17 protein expression. Increased CPI-17 phosphorylation, perhaps through activation of the PKC pathway, may also be contributory (pathway detailed in Fig 18); the degree to which this mode of regulation is important in short course hypoxia could be further elucidated by examination of absolute phosphorylation ratio of CPI-17 through immunoprecipitation, which may have a smaller margin of error than ratiometric comparison of band densities.

Fig. 18 : Postulated pathway of MLCP regulation in hypoxic PPHN



Activation of CPI-17 is associated with decreased MLCP activity, which results in impairment of vascular relaxation. The most probable pathway for this regulation is through PKC pathway activation; although the RhoK pathway also interacts with CPI-17, it is unlikely to do so in absence of measurable MLCP phosphorylation.

Clinical Significance

Until birth, phosphatase activity in the pulmonary arterial media is tightly regulated, in part by an abundance of regulatory protein CPI-17, maintaining high tone in the pulmonary circuit. In normoxic newborns, pulmonary arterial myosin light chain phosphatase activity (but not content) increases in the first 3 days of life, in conjunction with the natural decrease in pulmonary resistance. Hypoxic pulmonary hypertension is associated with loss of this developmentally regulated increase in phosphatase activity; attenuated phosphatase activity may be an early etiology of pulmonary arterial calcium sensitization and relaxation failure, independent of endothelial function.

Downregulation of pulmonary arterial MLCP activity in hypoxia may be related to inhibition by CPI-17, as increase in phospho-CPI-17 is observed within this timeframe. MYPT regulation by Rho kinase-mediated phosphorylation does not appear to be a factor in this process. If the observed increase in phosphorylation of CPI-17 in early PPHN is associated with upstream activation of the PKC pathway in hypoxia, this might suggest that smooth muscle proliferative activation may be initiated earlier in the natural history of PPHN than previously thought. The crux of the pathophysiology of PPHN development lies in the timing of the onset of smooth muscle proliferation; is PPHN a two-stage phenomenon, in which impairment of relaxation occurs as a result of an initial stimulus with later vascular medial thickening occurring as a result of subsequent stimuli, or a single-hit disease marked by the progressive manifestation of smooth muscle responses to the initiating phenomenon of hypoxia? PKC activation and smooth muscle mitosis were not examined in this study. The pathways of upstream regulation of calcium sensitization in PPHN, which interconnect with pathways regulating protein synthesis and mitosis, may be important in determining the timing of onset of more ominous changes of smooth muscle proliferative activation, which are treatment limiting and rarely reversible.

Early therapeutic use of antiproliferative agents, such as nitric oxide [298], may be useful in upregulating myosin light chain phosphatase as well as in delaying smooth muscle proliferative activation. Nitric oxide has been shown to decrease CPI-17 phosphorylation, in association with decreased myosin light chain phosphorylation and tissue force generation, in intact carotid rings [299]; while the relationship of nitric oxide to CPI-17 activation in hypoxia is not known, this may be of importance in the clinical efficacy of nitric oxide as a pulmonary arterial relaxant in hypoxic PPHN. Given that nitric oxide sensitivity may also be impaired in hypertension and vascular hyperplasia [300], research into other downregulators of CPI-17 activation state would be of clinical importance. In addition, if a causal link is established between smooth muscle calcium sensitization in hypoxia, CPI-17 content or activation, and upstream PKC pathway activity, this may necessitate early consideration of pulmonary vascular smooth muscle antiproliferative therapies, in order to prevent inexorable activation of medial proliferation in refractory cases of PPHN.

Limitations of This Study

Pulmonary hypertension is known to be a common end point for a number of pathologies, with many effectors and many active signalling pathways, perhaps with multiple defects occurring in the same patient. Focussing on any one defect entails the risk of missing the forest for the trees. It is also impossible, therefore, to discuss the entirety of this multifactorial disease as stemming from any individual derangement. Contemplation of clinical significance of the findings of this study must bear this caveat.

Much effort was expended initially to attempt mechanical analysis of neonatal pulmonary arterial smooth muscle in hypoxia, both at the level of the isolated dispersed single cell, and employing tissue strips attached to a computerized lever system. Due to fragility of small resistance vessels obtained from piglet lungs, as well as membrane disruptions engendered by enzymatic dispersion, neither of these approaches has been fruitful. Impairment of vascular relaxation in hypoxic pulmonary hypertension has been well established by other groups [85] [68] [1]; therefore, that is not considered a limitation of this investigation of cellular mechanisms of relaxation failure.

All animals used in this study were of the same species of swine and obtained from the same farm; however, controls were not littermates, but were comparable size and age-matched. Hypoxic animals were raised in our institution, while controls of appropriate ages were farm raised and obtained on day of use. Other researchers using the hypoxic neonatal swine model have validated this approach, as no differences have been detected in weight, vigour or measurable pulmonary arterial characteristics between institutionally fed and farm raised piglets [51].

In the current study, PPHN was induced in a whole animal model by the application of a single agent of pathogenesis, in this case moderate normobaric hypoxia.

This does maintain the complex in vivo regulation of hemodynamic, hormonal, and cellular environments found in the clinical condition better than would be the case with hypoxic exposure in a cell culture dish. However, there are limitations associated with the interpretation of protein abundance in whole tissue homogenates in a heterogeneous tissue such as the arterial wall. Firstly, cells other than smooth muscle are included in the homogenate, both because of small vessel size precluding proper cleaning of endothelial and adventitial layers, and because of the need for very rapid dissection. The contribution of these remnants of other tissue layers to the protein content as a whole is felt to be small, given the predominance of smooth muscle protein. Secondly, protein alterations in one subpopulation of smooth muscle cells are generalized to the whole homogenate, so smaller variations lose significance; while this could be teased out using immunohistochemical techniques, the case can be made that any alteration of sufficient magnitude to be apparent in a tissue homogenate is likely to be of functional significance to that tissue. Using this threshold argument, we conclude that the differences in protein abundance or enzyme activity between treatment groups seen in this study are unlikely to be spurious, and therefore may be applied to clinically observed functional alterations.

Wide within-group variation in protein expression is more often a problem encountered in tissue-based investigation, as opposed to cell culture, as tissues are heterogeneously composed, and individual animals may vary in their disease development or physiological responses. This was exacerbated to some extent in this study by the fact that pulmonary arteries were pooled to create a single tissue sample from each animal, in view of the small quantities obtained. There is increased risk of both type I and type II error under such circumstances. This will be addressed by cultured cell experimentation in more controlled conditions, detailed subsequently.

Time is required for the dissection and preservation of tissues under study, during which period it is possible to encounter changes in protein phosphorylation and enzyme regulation. We ensured that all pulmonary arterial specimens were obtained in less than one hour post mortem, and immediately placed in dry ice-cold acetone to freeze further cellular processes. Phosphatase activity assay of tissue samples obtained at the beginning and end of the allotted hour were no different (data not shown), indicating that this dissection time was reasonable for the preservation of accurately measurable enzyme activity. Some alterations in protein phosphorylation may occur, however, between animal death and the time of tissue freezing, and during mechanical tissue homogenization despite the presence of protein kinase and phosphatase inhibitors. While this was felt to be unavoidable beyond maintenance of scrupulous technique and timing, it was a limitation equally applicable to tissue studied in all treatment groups, and hence should not unduly influence interpretation of results.

Closer examination of regulatory pathways responsible for altered phosphatase activity requires the use of agonists and antagonists applied to the cells under study, usually in advance of the studied stimulus. This degree of control is not possible in an in vivo system, where up- or down-regulation of crucial pathways may be lethal, and where counterregulatory pathways may be activated by any number of additional stimuli. Elucidation of the specific contribution of PKC activation to CPI-17 phosphorylation in hypoxic smooth muscle would necessitate further experimentation in an isolated cell culture system.

Future Research Directions

We have commenced primary neonatal pulmonary arterial smooth muscle cell culture, and have found that even in the presence of serum, these cells maintain markers of contractile smooth muscle phenotype. Neonatal myocytes are also notably fast growing compared to adult cells, which suggests smooth muscle cells from the newborn age group may simultaneously retain contractile machinery and capabilities of proliferation. Functional competence of primary cultured vascular smooth muscle has been established by measurement of calcium mobilization. This makes it a viable system for the examination of signalling pathways for vascular myogenic response.

Two major local factors can be identified as etiologic in PPHN: hypoxia as a consequence of ventilation/perfusion mismatch, and wall strain due to overload of the contracted pulmonary circuit. Both are known to cause mitotic pathway activation, but the individual contributions of hypoxia and strain to the initiation of relaxation failure, and later to medial fibrosis, are not known. This may be important in the sequential planning of clinical interventions, in order to extend the therapeutic window for vasodilators. As a future direction for research in this area, a more detailed examination of the mechanism of smooth muscle calcium sensitization and proliferative activation in the context of wall strain (cyclic stretch) and hypoxia in cultured vascular myocytes would involve use of known agonists and antagonists of the individual signal transduction pathways, PKC and Rho kinase, investigated in the current study. Some of these experiments are presently underway.

It should also be possible to investigate specific isoforms of the PKC family using selective inhibitors or by myocyte transfection with dominant negative mutants where available, in order to delineate which kinase is responsible for CPI-17 activation, and

hence myosin phosphatase downregulation. We would like to pursue an analysis of the timeframe of this activation, in the context of the natural history of neonatal pulmonary hypertension. Another potential avenue of investigation is the transcriptional control of CPI-17 in the neonatal period, as this may be of developmental significance.

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