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**MATURATIONAL FEATURES
OF THE NEONATAL
PULMONARY CIRCULATION**

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**MATURATIONAL FEATURES
OF THE NEONATAL
PULMONARY CIRCULATION**

Rijpingskenmerken van de neonatale longcirculatie

Proefschrift

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam,
op gezag van de Rector Magnificus
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en volgens besluit van het College voor Promoties

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PREFACE

The studies described in this thesis were performed in the Cardiovascular Pulmonary and Developmental Biology Laboratories, University of Colorado Health Sciences Center, Denver, Colorado, Department of Pharmacology, University of South Alabama, College of Medicine, Mobile, Alabama, USA and in the Departments of Cell Biology and Pharmacology, Erasmus Medical Center Rotterdam, Rotterdam, The Netherlands. The work was performed under the supervision of K.R.Stenmark M.D., Professor of Pediatrics, T.Stevens Ph.D., Professor of Pharmacology, and D.Tibboel Ph.D., M.D., Professor of Experimental Pediatric Surgery.

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1

INTRODUCTION

1.1 Clinical background

It is generally assumed that pulmonary vascular resistance in utero is high with resulting low blood flow through the lung. Near the end of fetal life new vessel growth and remodeling coincides with a progressive fall in pulmonary vascular resistance, thus allowing the lung to assume its postnatal role in gas exchange.^{2,3,7,8,21} When the pulmonary vasculature fails to adapt to postnatal life, right to left extra-pulmonary shunting and severe hypoxemia are bound to occur.¹³ As a consequence the clinical syndrome of Persistent Pulmonary Hypertension of the Newborn (PPHN) may arise. PPHN is observed following perinatal asphyxia, either in combination with developmental abnormalities of the lungs (lung hypoplasia with or without congenital diaphragmatic hernia) and/or heart, or idiopathically. PPHN presents approximately in one in 1000 newborns. Despite new treatment modalities, such as nitric oxide (NO) inhalation, intra-tracheally administered prostacyclin (PGI₂),²⁰ high frequency oscillation and extracorporeal membrane oxygenation (ECMO),¹ which have decreased its morbidity and mortality, 20-50% of infants diagnosed with PPHN are still expected to die.¹⁷ Those infants who survive often have continuing respiratory morbidity as a result of volutrauma from intermittent inflation/deflation and high inspiratory oxygen levels. Thus, PPHN continues to be a major problem in Neonatal and Pediatric Surgical Intensive Care Units worldwide.

Infants dying with pulmonary hypertension exhibit structural abnormalities of the pulmonary vessels, including thickening of media and adventitia and extension of smooth muscle into normally non-muscularized vessels.¹⁵ Although it is hard to understand the direct relationship between morphological abnormalities and the deviant vascular reactivity so often observed in these patients, the changes in smooth muscle cells (SMCs) appear particularly striking when compared with vascular wall alterations in adult human forms of pulmonary hypertension.⁵ Observations in animal models suggest that the proliferation and matrix-producing^{4,9} abilities of SMCs in the neonatal pulmonary circulation in response to injury also exceed those seen in adults.^{24,25,27} Relevant intracellular signaling mechanisms that account for the enhanced growth responsiveness are incompletely

understood.^{6,11,16,23,28} One of the most important intracellular messengers is adenosine 3', 5'-cyclic monophosphate (cyclic AMP), which is synthesized from adenine triphosphate (ATP) by the enzyme adenylyl cyclase.^{10,14,26} Cyclic AMP (cAMP) has been associated with proliferation in various cell types amongst which SMCs are the most important.^{18,19,22}

Therefore, for clinical practice, improving our knowledge of the cellular mechanisms that drive growth and differentiation of smooth muscle cells residing in the pulmonary vascular wall is essential:

1. To better understand normal and abnormal pulmonary vascular development, and
2. To develop therapeutic modalities which may affect the excessive pulmonary vascular remodeling in response to lung injury seen in some critically ill newborns.

1.2 Definition of research objectives

The objectives of this thesis are threefold:

1. To investigate morphological, functional, and cell-biological characteristics of the developing pulmonary vasculature;
2. To explore mechanisms of developmental regulation of proliferation and growth in pulmonary vascular cells;
3. To determine cell-cell interactions within the vascular wall.

These objectives are addressed in the following chapters. Chapter 2 comprises an initial discussion of morphological and functional aspects of the developing normal pulmonary vasculature and the pulmonary circulation in (models of) chronic hypoxic pulmonary hypertension and congenital diaphragmatic hernia in the newborn.

Control of the pulmonary circulation by adenylyl cyclase (AC) has been well documented in clinical and experimental settings, though these studies were

largely performed before the consideration of isoform-specific AC responses. Recently, antibodies suitable to distinguish between AC isoforms became available. In chapter 3, a study into pulmonary vascular distribution of AC isoforms (types II-IX) is given.

The vascular wall alterations in a calf model of chronic hypoxic pulmonary hypertension resemble those seen in human PPHN. Chapter 4 deals with the unique growth properties of SMCs derived from the bovine pulmonary artery (PA) and with signaling mechanisms that may underlie the particular growth patterns at various developmental stages. A role for the second messenger cAMP in mediating neonatal PA SMC proliferation is implicated.

The cAMP response element binding protein (CREB) is a transcription factor that has been linked to growth and differentiation. CREB is stimulated by elevated levels of cAMP. An extension of our observations on cAMP mediated growth of neonatal PA SMC is presented in chapter 5.

One of the most striking initial features observed during artificial ventilation in newborns is development of edema. Endothelial cell disruption is known to play a pivotal role in the initiation of this process.¹² Chapter 6 addresses the relationship between cAMP and adenylyl cyclase on the one hand and pulmonary microvascular permeability on the other hand.

Finally, significant research results are discussed and summarized in chapters 7 and 8 respectively.

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2

THE NORMAL AND ABNORMAL DEVELOPING PULMONARY VASCULATURE

Partly based on:

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2.1 Introduction

From the extensive literature mechanisms governing lung morphogenesis^{28,74} and maturation^{33,37,57,75} it appears that development and maintenance of normal pulmonary vascular structure clearly plays a critical role in lung function. As, several major questions about the regulation of this process in the normal and abnormal developed lung still remain unanswered.

This chapter addresses the morphological and functional aspects of normal and abnormal pulmonary vascular development. The latter concerns primarily the developing pulmonary circulation in (animal models of) chronic hypoxic pulmonary hypertension⁶⁶ and congenital diaphragmatic hernia.⁶⁸

2.2 Morphological aspects of normal pulmonary vascular development

In the embryonic lung, endothelial precursor cells (angioblasts) initially form a primary vascular plexus within the tissue, which eventually links up to the main circulation coming from the heart.^{3,15} Primitive pulmonary arteries become incorporated into the sixth aortic arch and inter-segmental arteries involute by the end of the 5th week of gestation. Connections with systemic arteries may persist in abnormal situations.

Reid, after having studied pulmonary vascular development, formulated three laws. According to the third law, pre-acinar vessels (both arteries and veins) develop at the same time as airways, so after the 16th week all pre-acinar artery branches are present.⁵⁵ The relationship of the blood vessels to the airways and air spaces permits useful land marking or timing of critical events in the development and function of the pulmonary circulation.^{9,13,14,15}

The structure of pulmonary arteries varies with vessel size and the developmental stage of the lung. The muscular coat of an artery develops during the canalicular stage. Axial arteries from hilum to the 7th generation are elastic; more peripheral arteries are muscular, partially muscular or, at the level of intra-acinar arteries, predominantly non-muscular. By definition, an elastic artery has more than five elastic laminae in its media, a muscular

between two and five. A partially muscular artery has smooth muscle cell (SMC) tissue in only one part of its circumference; at this level the continuous muscular coat is replaced by a spiral of SMCs. A non-muscular artery is similar in structure to an alveolar capillary, except for its (larger) diameter.^{54,57} Smaller muscular, and probably also partially muscular, arteries represent the so-called resistance arteries. This muscularization decreases towards the periphery. A newborn has one artery for every 20 alveoli. Due to formation of new alveoli postnatally, this ratio is reduced to 1:8 in an adult human being.^{9,31,54,57}

Two types of pulmonary arteries can be distinguished: conventional arteries, which accompany airways branching from the axial airway, and additional or supernumerary arteries which are lateral branches that arise between conventional arteries and run a short course to supply the capillary bed of alveoli immediately adjacent to the pulmonary artery at the peribronchial parenchyma.¹⁴ The latter are considerably more numerous and contribute in a significant way to the cross-section of the totally recruited vascular bed. Branching is more frequent towards the periphery. Supernumerary arteries constitute approximately 25% of the cross-sectional area total at the preacinar level, whereas at the intra-acinar level they comprise approximately 33%. In the normal lung, according to Hislop and Reid, 23 generations of conventional arteries exist along the posterior basal artery and 64 supernumerary branches. It has also been suggested that supernumerary arteries facilitate blood oxygenation by allowing passage of venous blood to the more remote alveoli adjacent to large arteries, veins and airways.^{13,31,32} The intra-acinar arteries represent the resistance arteries in the pulmonary vascular bed. The external diameter of pre-acinar arteries is usually $>200\ \mu\text{m}$, while arteries running with respiratory bronchioli represent intra-acinar arteries with a diameter of $50\text{-}200\ \mu\text{m}$.^{23,24} These intra-acinar arteries, together with supernumerary arteries, rapidly increase in number and dilate near term, thus accommodating postnatal demands of the pulmonary circulation.⁷⁰⁻⁷³

Following the description of morphological changes in the developing pulmonary vasculature, the role(s) of growth factors, especially the isoforms of fibroblast growth factor (FGF),⁴⁰ transforming growth factor β (TGF- β)³⁰ and isoforms of platelet-derived growth factor (PDGF)⁶ were investigated. Especially a member of the PDGF-family, the vascular endothelial growth factor (VEGF) likely plays a role in pulmonary vessel formation. Unlike the FGFs, VEGF appears to be an endothelial-specific mitogen. Fms-like tyrosine kinase (flt-1) and the fetal liver kinase (flk-1) are receptors for VEGF and are known to be expressed during early vascular development in the mouse embryo.^{20,39} Heterozygous VEGF-deficient mice demonstrate abnormal vessel formation, embryos lacking both VEGF alleles die at mid-gestation.⁷ In addition to these findings, it has been demonstrated that over-expression of VEGF in pulmonary epithelium results in an increase in vascularization surrounding the airways combined with an abnormal pulmonary morphogenesis.^{17,21,81} VEGF has been reported to be a target gene of hypoxia inducible factor-1(HIF-1),⁶² a well known mediator of physiological and pathophysiological responses to hypoxia.⁶⁰ Loss of HIF-1 impairs vascular function, consistent with the recent observation that VEGF mRNA in human fetal lung explants was found to increase (in response to cAMP) in the presence of 20%, but not 2% O₂.¹

Several vasoactive mediators produced by the vascular endothelium may also play a role in vascular development. As blood flow to the lung is limited during fetal life, regulation by locally produced vasoconstrictors is important. These same vasoconstrictors may exert growth promoting effects on vascular SMCs, as has been described for thromboxane A₂, Angiotensin II, 5-hydroxytryptamine and Endothelin I.^{12,18,22,46,65,80} The differences in animal models, as well as the variety of techniques and culture systems used in these experiments, make the definitive role of growth factors and their target genes hard to define.^{6,25,30,40,52,59}

2.3 Abnormal pulmonary vascular development

In abnormal pulmonary vascular development it is especially important to compare the possible differences in pulmonary artery structures in a standardized way.

Four main features need to be assessed in this respect in order to determine postconceptional age:

1. branching pattern;
2. number or density of arteries;
3. wall structure; and
4. arterial size.¹³

Hislop and Davies described a way to process lung tissue into histological slides resulting in barium-gelatin filled arteries and dark stained elastin layers easy to distinguish from the surrounding, but empty lung veins.^{9,31} In these slides, external diameter, wall thickness, and wall structure regarding the muscle coat (muscular, partially muscular, or nonmuscular) are registered for each artery, as is the type of the accompanying airway. The percentage wall thickness ($2 \times \text{wall thickness} / \text{external diameter}$) is calculated (Picture 1 on page 141) In addition, the use of a radiopaque injection medium allows rapid assessment of the pulmonary circulation through arteriograms.^{56,69}

Based on this technique, Geggel and Reid distinguished the abnormal pulmonary vasculature as a result of maladaptation, maldevelopment or underdevelopment.²⁴

Maladaptation is represented by a structurally normal lung at birth, in which no increase in compliance of small resistance arteries is present. Therefore, the pulmonary vascular bed is highly reactive and a vicious circle of acidosis, hypoxia, hypercarbia, pulmonary vasoconstriction and pulmonary hypertension may develop. Maldevelopment indicates new and precocious muscularization as seen in idiopathic persistent pulmonary hypertension of the newborn (PPHN). Excessive muscularization is seen in hypoplastic left heart syndrome, chronic intra-uterine hypoxia, and also following meconium

aspiration syndrome. Underdevelopment represents the reduced size of arteries seen in congenital anomalies associated with pulmonary hypoplasia, such as CDH, renal agenesis, or dysplasia.

2.4 Cellular and molecular mechanisms of vascular growth in chronic hypoxic pulmonary hypertension model in the newborn

Proliferation of endothelial cells, as well as SMCs and fibroblasts, is necessary for continued pulmonary vascular growth.⁹ Although the exact mechanisms that control vascular cell proliferation during lung development *in vivo* are not known, there is circumstantial evidence from *in vitro* studies of pulmonary vascular cells suggesting that the local production and/or action of second messenger pathways may underlie particular growth patterns in various developmental stages.^{63,65}

The most important contribution in the understanding of the different cell populations which are present in the pulmonary vessels is made by the research group of Stenmark and Reeves. They developed a model using neonatal calves at high altitude to study both the pathophysiology of severe pulmonary hypertension as well as the cell biology of normal and abnormal pulmonary vessels.⁶⁶ Structural changes known as vascular remodeling^{27,29} are observed in these animals. Furthermore, factors which have been identified to play a role in the pathogenesis of muscular hyperplasia⁴ observed in pulmonary hypertension, are also identified in this model.

Evidence is accumulating to suggest that SMCs and fibroblasts in the vascular wall can respond directly to changes in local environment conditions i.e with increased production of growth and differentiation factors (e.g. PDGF) capable of acting in autocrine or paracrine fashions on vascular wall cells.⁷⁷ Chronic hypoxia and mechanical stress are two stimuli important for the development of pulmonary hypertension.¹⁰ These impulses may stimulate growth directly or they could promote cell proliferation indirectly by enhancing responsiveness to other mitogens.⁶³ Dempsey has reported that

hypoxia stimulates protein kinase C (PKC) to a small extent in PA SMC, which does not by itself lead to cell proliferation but may stimulate growth factors, such as Insulin-like Growth Factor-I (IGF-I).¹⁶

Recent observations demonstrated that SMCs in the pulmonary artery are a diverse population of cells capable of expressing multiple phenotypes.⁷⁶ This heterogeneity in vascular SMCs may be reflected in differences in intracellular signaling which in order may contribute to e.g. mitogenic responses in the pulmonary circulation during development and disease. Thus, it is not only important to know which cells secrete the different forms of cytokines, but also which cells are expressing the receptors that will recognize the cytokines. Alterations in receptor expression, signal transduction (including PKC and cyclic AMP),^{44,58} proto-oncogene expression (including c-fos and c-myc) and growth factor production (PDGF), have been demonstrated during periods of rapid cell growth and differentiation and are consistent with the rapid proliferative response of the neonate to growth promoting stimuli.¹¹ It has been demonstrated that PDGF activates mitogen-activated protein kinase (MAPK), which in turn plays a crucial role in regulating the entry of cells into the growth cycle. In a number of cells it has been proposed that the kinetics of MAPK activation may dictate the relative efficacies of both growth factor and G-protein-coupled receptor agonists as mitogens.^{44,64}

Earlier work demonstrated that matrix protein producing genes for elastin and collagen show their maximal activity in late foetal and early neonatal life. Tropoelastin mRNA expression for example is nearly completely suppressed in the adult. Therefore, it has been suggested that the capacity of the SMCs to respond to matrix producing stimuli depends not only on local hormonal and cell-cell interactions, but also on the developmental state of the cell. In the model of the hypoxic neonatal calf, in situ hybridization revealed radial gradients and a heterogeneous pattern of elastin expression in the medial layers of the intralobar pulmonary arteries.¹⁹

Furthermore, previous studies showed decreased arterial wall prostaglandin production in neonatal hypertensive calves.² In this study the endothelial cell

was the main source for PGI₂ and PGE₂ production. No detectable levels of the prostaglandines were found in SMCs.

The earliest and most striking proliferative changes in the more peripheral vessels occur in the adventitial layer rather than in the medial layer.^{53,66} Stenmark et al⁶⁴ demonstrated that in isolated fibroblasts, hypoxia can specifically activate select members of the PKC isozyme family, as well as members of the MAPK family of proteins. A better understanding of why these cells react differently to hypoxia than e.g. SMCs derived from the pulmonary artery, will certainly contribute to our understanding of normal and abnormal pulmonary vascular development.

The morphological findings in the model of chronic hypoxic pulmonary hypertension in the calf⁶⁶ resemble the pathological findings in diseased human newborns. However, the preceding courses of development are not completely similar, as the calf is born after a normal pregnancy and then placed at 4300 m simulated altitude for 2 weeks. The elegance of this model is beyond doubt, however a contrast to the human situation exists when a persistent foetal circulation maintains hypoxemia and vice versa. Thus, the question remains whether pre-existing vascular abnormalities in the human can precede the pathophysiological processes seen and which are not always reversible both at the functional and at the morphological level.

2.5 Vascular morphology in human CDH

The main pathological findings in CDH lungs are hypoplasia and vascular abnormalities. The latter consist of:

1. Reduced total pulmonary vascular bed and fewer vessels per volume unit of lung;
2. Medial hyperplasia of pulmonary arteries together with peripheral extension of muscle layer into small arterioles,
3. Adventitial thickening.^{67,78}

Intra-acinar arteries in healthy newborns are virtually all non-muscular; however, in PPHN most of these arteries are completely muscularized. Geggel et al gave a detailed morphometric analysis of the lungs in a series of 7 infants with CDH.²³ They divided these patients into two groups: 4 infants who were never able to be adequately ventilated (the no-honeymoon group), and 3 who did well initially following repair of their diaphragmatic hernia, but then developed increased pulmonary vascular resistance and died (honeymoon group). No-honeymoon patients have smaller lungs, increased muscularization of intra-acinar arteries, and decreased luminal area of preacinar and intra-acinar arteries. Persistent hypoxemia in the no-honeymoon group is determined by severity of pulmonary hypoplasia and structural remodeling of pulmonary arteries (Picture 2 on page 141).

Studying lungs of CDH patients, Kitagawa et al.³⁸ observed abnormalities in both number and muscularization of arterial branches. The number of conventional branches was reduced to 14 in the right lung and to 12 in the left lung. They found only 17 supernumerary branches in the right lung, but still 36 in the left lung. Finally, smaller-diameter arteries (external diameter <300 μm) appeared to have thicker muscular walls. Other investigators reported reduction in the cross-sectional area of the pulmonary vascular bed and uniform thickening of the pulmonary artery muscle mass in CDH lungs.^{42,47,48} In contrast to the functional studies in the neonatal model of the calf at high altitude, hardly any studies on the functional and or biochemical changes of the abnormal pulmonary vasculature in CDH are available.

2.6 Studies in rats

We used the nitrofen rat model in order to unravel the pathogenesis of CDH.⁶⁸ In this model, CDH rats show respiratory insufficiency directly after birth. Newborn rats were killed by an intraperitoneal injection of sodium pentothal (200 mg/kg of body weight). We studied the pulmonary arterial bed in a control and a nitrofen treated group. The latter was

subdivided on the basis of presence and absence of CDH. The chest wall was removed and the pulmonary arterial bed was injected and perfused through a cannula in the right ventricle, with a barium-gelatine mixture at 60°C and constant pressure. Perfusion was stopped when this solution reached the visceral pleura in all segments, resulting in so-called "snow flocks". Subsequently tracheal cannulation and lung fixation with Davidson solution (40 vol % ethanol 100%; 5 vol % acetic acid 96%; 10 vol % formaldehyde 37%; 45 vol % saline; pH 7.3) was performed; fixation was maintained under constant pressure of 20 cm water. After fixation, the lungs were dissected out of the thoracic cavity. Then, the number of animals with a diaphragmatic defect, the position (right-or left-sided) and size of the defect, as well as the contents of the thorax (liver, bowel, stomach) were determined. An arteriogram was taken of each pair of lungs. Dissected lungs were processed for routine histology resulting in paraffin embedding of total lungs. Six μm frontal sections showing both lungs were made and stained with hematoxylin-eosin and Lawson, combined with van Gieson staining. Each section was analysed in a blind fashion by means of an ocular micrometer (1 unit is 1.48 μm , Zeiss Optical Industry) and standard magnification (10 x 63). A total of at least 35 arteries of each animal (15 from each section) were examined. For each artery, external diameter, wall thickness, wall structure (muscular, partially muscular, or nonmuscular) and the accompanying airway were noted. Wall thickness was defined as the distance between luminal surface and adventitia. External diameter was defined as the distance between the outer edges of the adventitia. The percentage wall thickness ($2 \times \text{wall thickness} / \text{external diameter} \times 100\%$) was calculated. The mean value for each group was calculated with respect to the accompanying structure. No correction was made for processing and shrinkage factors. A frequency tabulation of the artery structure was made with respect to the accompanying airway structure.

At the level of the respiratory bronchioles, significant differences in the vessels were found. Decreased external diameter and increased wall thickness were seen in CDH, but not in control lungs. Abnormal muscularization of the peripheral branches of the CDH pulmonary arteries was also found.

2.7 Functional studies

The question remains whether the morphological features seen in the pulmonary vasculature in CDH can directly be correlated to responses of the pulmonary vasculature during the perinatal period.⁶⁸ In the clinical situation reactions to various vasoactive agents, including inhaled nitric oxide (NO), appeared to be highly unpredictable.^{36,51}

The mechanisms that control normal and abnormal tone in the neonatal pulmonary circulation are not exactly known. SMCs and connective tissue in the vessel wall may be highly important and influenced by various mediators (eg, bradykinin, ANG II, ET-I, epinephrine, thromboxane B₂, and metabolites of arachidonic acid) in a complex and yet not completely understood mechanism.

Although high levels of circulating immunoreactive ET-1 have been reported in human neonates with PPHN and CDH,⁴¹ pulmonary expression of ET-1 and the exact mechanisms through which ET-1 and its receptors interact to regulate pulmonary vascular tone are not fully known.⁷⁸

We hypothesized that in CDH, altered pulmonary vascular reactivity might be related to differential expression of ET-1 and its receptors. We, therefore, examined the pulmonary expression of ET-1, ET_A, and ET_B receptor mRNAs in a rat model of CDH and compared the expression pattern with those of age matched controls.⁴³ Significantly ($p < 0.05$) enhanced levels of ET-1 mRNA were observed in CDH rat lungs as compared to controls. No significant difference in expression of ET-1 mRNA between right and left lung (the most hypoplastic) in CDH rats was observed. A 3.0 ± 0.9 -fold increase in ET_A mRNA was observed in CDH as compared to controls, whereas ET_B mRNA levels remained unchanged in both CDH and control rats.

Gosche revealed in the same model, that responses to potassium-induced depolarization, phenylephrine, ANG II, serotonin, and U46619, were not different in pulmonary arterioles derived from control and CDH rats (personal communication). These data suggest that structural alterations of the pulmonary vasculature observed in infants with CDH may not necessarily

be responsible for exaggerated vasoconstrictive responses to normal stimuli. Karamanoukian et al.³⁶ detected both decreased NOS expression and activity in CDH rat lungs using a [14 C]-L-arginine to [14C]-L-citrulline conversion assay and Western blots, suggestive of an absence of primary NO deficiency in CDH.

In other studies, levels of several eicosanoids in lung homogenates and in broncho-alveolar lavage fluid of controls and rats with CDH were measured.^{34,49} In controls, concentrations of a stable metabolite of prostacyclin, thromboxane A₂, PGE₂, and leukotriene B₄ decreased after birth. CDH lungs demonstrated higher levels of 6-keto-PGF_{1α} compared to controls. We concluded that in CDH, abnormal lung eicosanoid levels are present perinatally.^{5,22} Elevated levels of 6-keto-PGF_{1α} in CDH may reflect a compensatory mechanism for increased resistance in the pulmonary vascular bed.^{26,35}

2.8 Discussion

Animal models have provided greater understanding of the pulmonary vascular remodeling observed in pulmonary hypertension in general and congenital diaphragmatic hernia in particular. In humans, despite well defined morphological and functional features of normal and abnormal pulmonary development, the actual situation continues to raise concern; e.g. whether vascular abnormalities result from maturational arrest or coincide with abnormal development of lung parenchyma. In CDH, it is assumed that normal remodeling of the pulmonary vasculature towards the end of fetal life does not occur, potentially because genes responsible for this process are not switched off. Thus, the genes responsible for these processes need to be established in order to explain when/how the system can be expected to go awry.

Although more detailed information on morphology and function of the pulmonary vasculature has become available, clinicians still deal with unpredictable pulmonary reactivity in PPHN and the variable responses to inhaled NO^{8,50} and ECMO treatment. Clinical experience in many centers

worldwide has provided well-documented cases of patients who were responders, and others who continued to be non-responders, to known therapies. Shehata et al.⁶¹ demonstrated decreased adventitial thickening of pulmonary arteries after ECMO treatment; however the specimen they investigated were derived from autopsy cases only. This thinning of the vascular wall may result from the ECMO treatment itself and/or reflect delayed remodeling after birth. Whether this phenomenon can be correlated to distinct morphological changes or differences in expression of a variety of growth factors requires to be clarified.

It will probably not be easy to directly correlate the morphological findings to an abnormal pulmonary vascular response as observed in many of our patients. The same holds true for the wide spectrum of reactivity (no versus high response to vasodilating agents), that may be observed with identical morphology. To this end, we need again a better understanding of the biochemical processes involved in growth and proliferation of the SMCs within the vascular wall.

2.9 References

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PULMONARY VASCULAR DISTRIBUTION
OF ADENYLYL CYCLASE ISOFORMS:
HETEROGENEITY BETWEEN AND
AMONG CELL PHENOTYPES

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3.1 Abstract

Adenylyl cyclases (AC) catalyze the conversion of ATP to cAMP. In recent years a substantial diversity of enzyme isoforms has been described, each with different sensitivities to G_s and G_i proteins and unique regulatory properties. Such diversity of enzymes suggests cells govern function by regulating the complex of ACs they express. It is broadly recognized that tissue distribution of AC isoforms differ, however the cell-specific expression of AC isoforms has not been clearly defined in any organ. The diverse functions required of blood vessels include regulation of permeability and vascular tone. We therefore sought to examine cell-specific expression of ACs in bovine lung vasculature. Pulmonary artery and vein endothelial cells expressed AC types II-IX, though the protein kinase C-stimulated (type II) enzyme exhibited the greatest immunoreactivity. This expression pattern was not conserved in capillaries, where alveolar endothelial cells did not express AC II or VI but rather most abundantly expressed the calcium (type VIII)- and calcineurin (type IX)-stimulated isoforms. The medial layer of pulmonary arteries demonstrated diffuse expression of AC II, III, and V/VI and focal expression of types IV, VII, VIII and IX, whereas the adventitial layer expressed only AC IV and VII. Heterogeneity of AC expression decreased in the media of smaller vessels, suggesting homogeneity of smooth muscle cell phenotypes exists in more distal vessel segments. Thus, the pattern of AC expression is different between and among cell types in the pulmonary circulation; this pattern of expression may fundamentally regulate the cell's response to its environment.

3.2 Introduction

Cyclic AMP is a ubiquitous second messenger molecule that regulates diverse cell functions including growth and differentiation,³⁶ motility,^{13,15,24,29,31,37,38,50} shape,^{19,27,28,32,35} and contraction.^{17,18,25,30,39,54} Because of its broad-ranging significance, cellular cAMP is controlled

within a narrow concentration range by multiple regulatory pathways. Central to this control is the transmembrane protein adenylyl cyclase (AC) which catalyzes conversion of ATP to cAMP. Understanding of this enzyme system has increased substantially over the last decade in accordance with cloning and expression of IX related isoforms^{8,21,45,47} (Table 1). Activity of all isoforms characterized so far is increased by G_s proteins whereas activity of some ACs is decreased by G_i proteins. For example, pertussis toxin sensitive G_i proteins selectively target AC VI over AC II or IV.⁴⁸ Gβγ subunits appear to bind ACs near the G_sα binding domain and conditionally activate some, but not all, isoforms.^{5,12,33} Independent of G protein activity nearly all transmembrane ACs are directly stimulated by the hydrophobic dipterene, forskolin.²¹ AC IX is the exception to this rule; it possesses a Ser → Ala and a Leu → Tyr substitution in the forskolin binding pocket absolutely conserved in AC I-VIII. Indeed, reversal of these substitutions in AC IX by site-directed mutagenesis restores forskolin sensitivity.⁵⁵

Table 1. Partial list of regulatory properties of AC isoforms.

| Adenylyl Cyclase Isoform | G _s α | G _i α | Gβγ | For | Ca ²⁺ | PKC |
|--------------------------|------------------|------------------|-----|-----|------------------|-----|
| I | + | | - | + | + | |
| II | + | | + | + | | + |
| III | + | | | + | ? | |
| IV | + | | | + | | |
| V | + | - | | + | - | +* |
| VI | + | - | | + | - | - |
| VII | + | | | + | | + |
| VIII | + | | | + | + | |
| IX | + | | | | | |

For = forskolin; *PKC* = protein kinase C. * denotes conditional regulation.

Each of the distinct AC isoforms possess unique regulatory properties used to functionally classify their activity. Several isoforms are regulated by calcium. Whereas AC V and VI are inhibited by sub- μM calcium concentrations independent of calmodulin,^{22,26} AC I and VIII are stimulated by calcium-calmodulin.^{11,46} AC III may be inhibited by calcium in the intact cell,⁵² though initial reports suggested this enzyme was conditionally activated by calcium in the presence of $G_s\alpha$.⁷ Calcium may also regulate enzyme function indirectly since AC IX is activated by the calcium stimulated phosphatase calcineurin.^{1,2} Similarly, AC II and VII are directly stimulated by protein kinase C (PKC),⁵¹ while AC V is stimulated by PKC when activated by $G_s\alpha$.²³ Taken together, global AC activity and thus cAMP responses are highly susceptible to complex regulation of AC isoforms expressed within a cell, providing means for a specific cell type to uniquely sense and respond to its environmental demands.

Control of the pulmonary circulation by AC activity is well documented in clinical and experimental settings, though these studies were largely performed before the consideration of isoform-specific AC responses. Prostacyclin activates smooth muscle AC to promote low pulmonary vascular tone. This physiological response has been adapted pharmacologically; epoprostenol stimulation of AC is utilized clinically to reduce the pulmonary vascular resistance and vascular remodeling apparent in various forms of pulmonary hypertension.^{4,26} Stimulation of AC in endothelial cells enhances barrier function associated with both decreased macromolecular flux and leukocyte transmigration.²⁸ Such anti-inflammatory properties of AC activation have been documented in response to diverse lung injury models. Perhaps the best example of the anti-inflammatory properties of AC activators however is in asthma, where β -adrenergic agonists produce bronchodilation and decrease the accompanying vascular permeability.³ Thus, AC represents an important physiological and clinical target in control of pulmonary vascular function. Cell-specific AC isoform expression has not been evaluated in lung or in any other tissue to date, however, largely due to the lack of available antibodies suitable for distinguishing between AC isoforms. A growing

knowledge of the molecular diversity of AC isotypes along with their unique regulatory properties demonstrate the importance of understanding cell-specific expression of AC isoforms as a means to determining cell-specific function. Current studies therefore utilized recently developed antibodies suitable to discriminate between AC II-IX to examine their pulmonary vascular distribution.

3.3 Methods

Tissue Processing.

Lung tissue samples 1 x 2 x 0.5 cm were taken immediately after the sacrifice of one year old healthy heifers and placed into 70% ethanol for 48 hours. Samples were routinely processed in paraffin and 5 μ m thick sections mounted on poly-L-lysine coated slides.

Antibodies.

Peptide antibodies for AC II-VIII were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against AC subtypes II, III, IV, V/VI were rabbit polyclonal IgG, and the subtype VII and VIII were goat polyclonal IgG. Each of the antibodies targeted the C-terminal domain of the respective AC isoform (Fig. 1). There is minimal sequence overlap between AC species within the C-terminal domains providing a suitable target to distinguish the respective isoforms, with exception of the antibody to AC V/VI which does not distinguish AC V from AC VI. Both AC V and AC VI are highly conserved, calcium inhibited enzymes with similar structure and function. Rabbit polyclonal antibodies against AC subtype IX were a generous gift from Dr. Richard T. Premont, Duke University, Durham, NC. This antibody was generated as a fusion peptide to the C-terminal 110 amino acids of AC IX, which shares limited homology to any of the other AC isoforms.³⁴

Histochemical Staining.

The slides were deparaffinized in xylene and placed for 5 min in ethanol with 0.5% H₂O₂ to extinguish endogenous peroxidase activity. After 5 minutes rehydration in phosphate buffered saline (PBS) with 0.5% bovine serum albumin and 0.05% Tween (PBS), the primary antibody was applied at 1:50 dilution and slides were placed into a moist chamber and incubated for 2 h. A peroxidase-labeled anti-rabbit IgG (DAKO) was used as secondary antibody with the exception of anti-AC VII and VIII antibodies which were raised in goat. In this case a mouse anti-goat IgG was applied as the intermediate incubation step for 1 h. The 1 h long incubation with secondary antibody was followed by the development of color reaction with aminoethyl carbazole for 5-7 min. Slides were washed in tap water, counter-stained with hematoxylin and mounted in glycerol-gelatin (DAKO). Each step was followed by 2 washes in PBS for 5 min and all incubations were at 37 °C.

Slides incubated with PBS instead of the primary antibody served as negative controls and co-incubation of the primary antibodies with equal protein concentration of the appropriate antigen were used as controls for reaction specificity. Positive immunoreactivity was evaluated semi-quantitatively, where ± represented inconsistent or weak expression and + to +++ represented increased staining intensity. All data presented are representative of multiple sections from 3-5 different lungs.

3.4 Results

Endothelial Cell AC Expression.

Staining was positive for all AC isoforms tested in endothelial cells of the large, intra-lobar pulmonary artery (Picture 3 on page 142; Table 2). Highest staining intensity was observed for AC II and IV. AC VII was the only isoform that displayed a low or inconsistent staining pattern. Endothelial cells of smaller arterioles similarly expressed all isoforms tested, though a switch in staining intensity was observed (Picture 4 on page 143; Table 2). In arterioles the most intense immunoreactivity was

found with AC VIII and IX, supporting the idea that endothelial cells in more distal vascular segments undergo a change in expression of prominent AC isoforms. Consistent with this idea staining for AC VIII and IX was intensely positive, whereas staining for AC II, III, and V/VI was not observed or was inconsistently observed in capillary endothelial cells (Picture 5 on page 144; Table 2). This decrease in endothelial cell immunoreactivity for AC II, III, and V/VI occurred in vessels $\leq 15 \mu\text{m}$ in diameter. Staining for AC IV and VII was similar in intensity to endothelial cells of larger arterioles.

Venule endothelial cells possessed a unique staining pattern compared with cells from other vascular segments. Overall staining intensity was considerably lower in venules than in comparably sized arterioles (Picture 5 on page 144; Table 2). Whereas staining was detected for all isoforms tested, only AC II and VII exhibited abundant immunoreactivity. Staining for other isoforms was of low magnitude and, in the case of AC VIII and IX, absent or inconsistent.

ADENYLYL CYCLASE

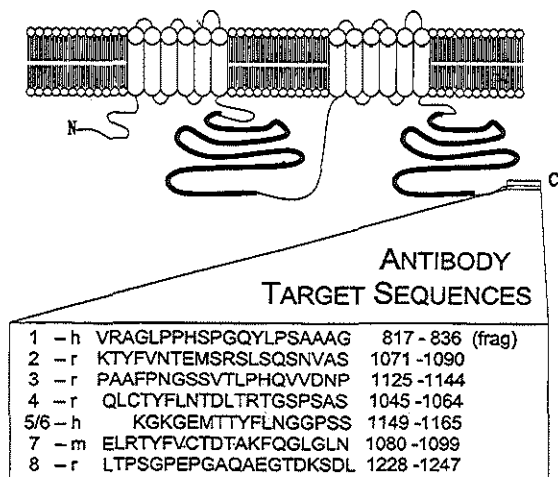


Figure 1. Summary of target sequences for AC antibodies 2-8. In each case the distal-terminus was targeted by polyclonal antibodies, though limited overlap between AC species is observed in this region.

AC Expression In The Pulmonary Vascular Media.

AC isoforms II and III exhibited diffuse, strong positive staining throughout the media of large (Picture 3 on page 142; Table 3) and small (Picture 4 on page 143; Table 3) pulmonary arteries. AC V/VI exhibited a similar diffuse staining pattern in the large pulmonary artery media, though staining was considerably reduced in intensity and was entirely absent in small pulmonary arteries. In contrast, AC IV, VII, VIII, and IX expression in the large pulmonary artery was not diffuse but rather characterized by focal staining of small cell groups. These cell groups were similarly distributed throughout the media for isoforms IV and IX and VII and VIII, respectively. A consistent staining pattern was observed for AC IV and IX in small arteries, though expression of AC VII and VIII was not observed in the 100 μm vessels. Thus, with the exception of AC VII and VIII medial expression of AC isoforms in the large pulmonary artery and arteriole was similar.

Table 2. Histochemical detection of different subtypes of adenylyl cyclase in pulmonary vascular media and adventitia.

| Adenylyl Cyclase Subtype | Lobar PA | | PA(100 μm) | | PV(100 μm) |
|--------------------------|--------------------|------------|------------------------|------------|------------------------|
| | Media | Adventitia | Media | Adventitia | |
| II | +++ ^d | - | ++ ^d | - | ++ ^d |
| III | ++ ^d | - | +++ ^d | - | ++ ^d |
| IV | ++ ^f | +++ | + ^f | +++ | ++ ^f |
| V/VI | \pm ^d | - | - | - | + ^d |
| VII | \pm ^f | +++ | - | - | \pm ^f |
| VIII | + ^f | - | \pm ^f | - | - |
| IX | ++ ^f | - | ++ ^f | - | + ^f |

PA = pulmonary artery; PV = pulmonary vein; ^d = diffuse distribution of positivity; ^f = focal positivity of individual or groups of cells.

The staining pattern for AC isoforms in pulmonary veins (100 μm) generally resembled comparably sized arterioles, though staining intensity was reduced (Picture 6 on page 145; Table 3). AC II, III, and V/VI exhibited a diffuse staining pattern whereas AC IV and IX exhibited focal positivity. AC VII and VIII were not readily distinguishable.

AC Expression In The Adventitia.

Intense staining for AC IV and VII was observed at the medial-adventitial border of large pulmonary arterial segments (Picture 3 on page 142; Table 3). Although a similarly intense staining was observed for AC IV in smaller arterioles, staining for AC VII was not apparent in the adventitia of smaller vessels (Picture 4 on page 143; Table 2). Expression of other isoforms, including AC II, III, V/VI, VIII, and IX was not detected in this vessel layer.

Table 3. Histochemical detection of different subtypes of adenylyl cyclase in endothelial cells of the pulmonary vascular bed.

| Adenylyl Cyclase Subtype | PA | PA 100 μm | PV 100 μm | Capillary |
|--------------------------|-----|-------------------------|-------------------------|-----------|
| II | +++ | ++ | ++ | - |
| III | ++ | + | + | - |
| IV | +++ | ++ | + | ++ |
| V/VI | ++ | ++ | + | - |
| VII | + | ++ | ++ | ++ |
| VIII | ++ | +++ | \pm | +++ |
| IX | ++ | +++ | \pm | +++ |

PA = pulmonary artery; PV = pulmonary vein

3.5 Discussion

AC activity is dynamically coordinated by multiple circulating mediators, neurotransmitters, and intracellular regulatory molecules.^{8,21,45,47} In the lung AC activity plays a central role in maintaining the normally low vascular resistance and has been utilized as a pharmacological target to reduce pulmonary artery pressure in hypertension.^{4,26} Endothelial cell AC activity contributes to constitutive barrier properties. Inhibition of AC activity increases endothelial permeability and stimulation of AC activity reduces endothelial permeability.²⁸ Thus, this enzyme system is critically linked to pulmonary vascular function. Recent advances in our understanding of the molecular diversity of AC isoforms, however, has not been paralleled by an improved understanding of how the isoforms regulate cell-specific lung function. As a first step toward this end, our current studies utilized recently available peptide antibodies to distinguish pulmonary vascular expression of AC isoforms II-IX.

Prior work in this field has been hampered by the lack of antibodies suitable to distinguish between AC isoforms. However, as indicated in Fig. 1 none of the sequences targeted by currently utilized antibodies possess shared homology, limiting the chance of cross-reactivity. Also, our current data indicate considerable heterogeneity of AC expression between cells within different segments of the vasculature. It is therefore likely the antibodies adequately discriminate between their target and other AC isoforms. Finally, the lack of apparent non-selective binding in all cross-sections, complete inhibition of immunoreactivity by blocking peptides, and the complete loss of staining in negative controls indicate antibody specificity is suitable for the purposes of our study.

Initial studies focused on endothelial cell AC expression. Endothelial cells of the large intra-lobar pulmonary artery expressed all isoforms tested, though types II and IV were most prominent. The intense staining of AC II is consistent with a prior report that activation of PKC increases cAMP in pulmonary artery endothelial cells.⁴³ Expression of AC V/VI is also

consistent with previous functional studies demonstrating that calcium decreases cAMP in conduit lung endothelial cells, due to inhibition of AC VI.^{42,43} In the initial functional studies expression of AC VI activity was detected in purified membrane preparations from pulmonary artery endothelial cells,⁴⁴ where sub- μM calcium inhibited global AC activity $\approx 35\%$. This calcium inhibition of cAMP promoted disruption of endothelial cell barrier function, suggesting decreased cAMP represents a functionally important response to inflammatory calcium agonists.

A switch in endothelial cell AC expression was detected when large arteries were compared with arterioles and capillaries. Unlike the large pulmonary artery, endothelial cell expression of AC VIII and IX was most prominent in pulmonary arterial segments $\leq 100 \mu\text{m}$. This isoform switch was particularly noteworthy in capillary endothelial cells where AC II, III, and V/VI immunoreactivity was not consistently detected. Staining for these isoforms was effectively lost in vessels $\leq 20 \mu\text{m}$ in diameter. It is not presently clear whether loss of immunoreactivity is due to the relatively low abundance of these isoforms, or their absence. Inconsistent staining of AC V/VI in the bovine microcirculation differs from our prior studies in rat lung tissue and rat and human cultured pulmonary microvascular endothelial cells (PMVECs). AC VI expression was detected in rat^{42,43} and human PMVECs⁴⁹ and, under certain experimental conditions, calcium conditionally inhibited cAMP accumulation in PMVECs.⁵² An additional difference between present and prior observations is that cultured rat PMVECs do not express AC VIII; AC VIII activity was not detected biochemically and its mRNA could not be verified by RT-PCR (Stevens, unpublished). Though speculative, it is possible the disparate detection of microvascular endothelial cell AC VI and AC VIII in bovine lung (immunological) versus rat/human lung (immunological and biochemical) represents a species variation; we have previously found species variations in lung expression of AC III.⁶ Nonetheless, either a decrease in AC VI or an increase in AC VIII expression would improve endothelial cell barrier function, consistent with reports that PMVECs form a more restrictive barrier than do macrovascular endothelial cells.²⁸

We next examined medial and adventitial expression of AC isoforms in intra-lobar large pulmonary arteries. AC II was most prominently expressed in this vascular segment; its staining pattern was diffuse suggesting the majority of medial SMCs expressed this enzyme, consistent with previous findings in bovine lung tissue.²⁰ AC II is a PKC stimulated isoform that coordinates the inositol 1,4,5-trisphosphate signaling pathway with cAMP.⁵⁷ Recent studies from our laboratory indicated AC II expression in cultured neonatal PASMCs linked ET-I and ANG-II-dependent increases in PKC activity with elevated cAMP necessary to promote proliferation.²⁰ Thus, identification of this isozyme as the prominent smooth muscle isoform of the main pulmonary artery media supports and extends prior functional data in this area.

In contrast to the expression pattern of AC II, AC IV, VII, VIII and IX exhibited a focal distribution in the large pulmonary artery media. Types IV and IX and VII and VIII exhibited similar distribution patterns. This punctate expression pattern of AC IV is striking because it was originally recognized as a ubiquitously expressed isozyme based on Northern analysis of RNA from tissue homogenates.¹⁶ Our data therefore provide evidence that AC IV expression is cell-type specific. Similar distribution of AC VII and VIII is also of significance since these enzymes are stimulated by PKC and calcium-calmodulin, respectively. Media-derived SMCs undergo calcium and PKC dependent proliferation,¹⁰ suggesting AC VII and VIII may coordinate the PKC and cAMP signaling pathways – with AC II - in control of vascular remodeling.²⁰ Overall, the unique staining pattern to different AC isoforms identifies phenotypically distinct cell groups within the pulmonary artery media.

That the large pulmonary artery media possesses multiple phenotypically distinct cell populations has already been well characterized. Work from Stenmark and colleagues^{14,41} identified bovine main pulmonary artery media possesses multiple layers of SMCs, characterized as L1-L3 layers where L1 is near the lumen and L3 is near the medial-adventitial border. Cells in these respective layers exhibit vast differences in expression patterns of SMC markers. To date these markers are mostly limited to

cytoskeletal or cytoskeletal-associated proteins. Nonetheless, discrete cell populations have previously been identified in medial smooth muscle layers using immunohistochemical techniques. Supportive evidence for multiple cell phenotypes was also provided from electrophysiological recordings of K^+ conductances.⁵³ Altered oxygen sensitivity of various smooth muscle phenotypes was detected which, consistent with the present findings, determined that phenotypically unique responses could be observed in signal transduction properties of these diverse cell groups. Future studies will be required to assess the important cell-specific responses conferred by discrete expression of AC isoforms.

Adventitia stained positive for AC IV and VII. AC IV is not recognized as a highly regulated enzyme, though AC VII is stimulated by PKC. Fibroblasts inherent to this vascular segment undergo significant activation and proliferation in various forms of pulmonary hypertension, at least partly dependent upon PKC activity.^{9,40} Thus, in future studies it will be important to identify the extent to which PKC crosstalk with cAMP coordinates fibroblast activation.

In summary, current studies demonstrated cell-specific expression of diverse AC isoforms within the pulmonary vasculature. These observations, when considered with currently available functional data, revise our interpretation of site-specific pulmonary function regarding vascular remodeling of pulmonary media and adventitia during the course of hypertension, and site-specific permeability responses to inflammatory calcium agonists. Present studies also raise questions regarding cell-specific expression of AC isoforms during the course of disease, and increase awareness of the potential complications in some patients unresponsive to pharmacological therapies targeting AC. For example, it is unclear whether agonists like prostacyclin similarly activate all AC isoforms. Future studies will be required to more rigorously assess cell-specific AC expression in the remodeled pulmonary circulation, and the response to AC stimulation in pulmonary vascular diseases.

3.6 Acknowledgements

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4

MECHANISMS REGULATING CYCLIC AMP-MEDIATED GROWTH OF BOVINE NEONATAL PULMONARY ARTERY SMOOTH MUSCLE CELLS

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4.1 Abstract

Neonatal pulmonary artery smooth muscle cells (PASMCs) exhibit enhanced growth capacity and increased growth responses to mitogenic stimuli compared with adult PASMCs. Because intracellular signals mediating enhanced growth responses in neonatal PASMCs are incompletely understood, we questioned whether:

1. G_q agonists increase cAMP content and
2. Increased cAMP is pro-proliferative.

Endothelin-1 (ET-I) and angiotensin II (ANG II) increased both cAMP content and proliferation in neonatal but not in adult PASMCs. Inhibition of protein kinase C (PKC) and protein kinase A (PKA) activity nearly eliminated the ET-I- and ANG II-induced growth of neonatal PASMCs. Moreover, cAMP increased proliferation in neonatal but not in adult cells. PKC-stimulated adenylyl cyclase (AC) was expressed in both cell types, suggesting that insensitivity to stimulation of cAMP in adult cells was not due to decreased enzyme expression. Our data collectively indicate that PKC stimulation of cAMP is a critical signal mediating proliferation of neonatal PASMCs that is absent in adult PASMCs and therefore may contribute to the unique pro-proliferative phenotype of these neonatal cells.

4.2 Introduction

Adaptation of the pulmonary circulation to postnatal life is a process that requires both growth and differentiation of vascular wall cells. In SMCs, there is a transition from a fetal to a more adultlike phenotype.²⁷ Several studies^{15,26} have demonstrated that when the normal transition to postnatal life is interrupted by hypoxia or increased pulmonary blood flow, marked proliferative changes in PASMCs are observed that exceed those observed when adult animals are exposed to these stimuli. Similarly, SMCs derived from neonatal pulmonary arteries are less differentiated and exhibit enhanced growth responses to mitogenic stimuli compared with the

relatively differentiated and quiescent SMCs derived from the adult pulmonary artery.^{6,30} Thus the increased growth capacity of neonatal PASMCs likely contributes to both normal pulmonary vascular development and the predisposition to develop exorbitant pulmonary vascular remodeling in response to injury in the neonatal period.

Although it is generally accepted that neonatal PASMCs possess increased growth responses to mitogenic stimuli, the unique intracellular signaling mechanism(s) that account for the enhanced growth responsiveness are incompletely understood. Ligands such as IGF-I and PDGF are coupled to tyrosine kinase signal transduction pathways that activate extracellular signal-regulated kinases (ERKs) and potentially increase PASMC growth.² Dempsey and colleagues^{6,8} demonstrated that receptor tyrosine kinase-dependent agonists induced fourfold greater increases in neonatal than in adult PASMC growth, suggesting that ERK-dependent proliferation is developmentally controlled. It has additionally been shown that constitutive and phorbol 12-myristate 13-acetate-sensitive PKC activity is increased in neonatal compared with adult PASMCs and that increased PKC activity promotes neonatal PASMC growth and also synergistically promotes ERK-dependent PASMC proliferation.^{6,7} However, how PKC synergistically interacts with ERK to enhance neonatal PASMC growth and what accounts for enhanced PKC-dependent proliferation in neonatal compared with adult SMCs is not clear at the present time.

Emerging data indicate that in some cell systems PKC may synergistically promote ERK-dependent proliferation by elevating cAMP. Faure and colleagues^{11,12} demonstrated that either G_q - or $G_q\alpha$ - activation of PKC or direct activation of PKC with phorbol esters stimulated ERK activity, and, similarly, activation of G_s or direct activation of AC elevated cAMP and stimulated ERK activity. Although these data implicate either G_q - or G_s -coupled mechanisms in regulation of ERK activity, they do not clearly demonstrate how G_q -coupled agonists may elevate cAMP. However, recent elucidation of the molecular complexity of ACs revealed type II AC is activated by PKC.³² These data suggest the possibility that a linkage between PKC and ERK activation is PKC stimulation of type II AC and

elevation of cAMP. Thus, as suggested by Faure and colleagues,^{11,12} G_q-coupled signal transduction pathways activate PKC, which may promote AC II synthesis of cAMP that, in turn, regulates ERK. It is equally clear that cAMP can have opposite effects on ERK activity in other cell systems.¹¹ Thus second messenger regulation of ERK and proliferation may be unique in phenotypically distinct cell types.

As neonatal SMCs demonstrate unique PKC-associated growth properties, it is possible that PKC regulation of cAMP may play an important role in the increased growth responses in neonatal compared with adult PSMCs. Therefore, the goal of the present study was to test the hypothesis that G_q-coupled agonists promote PKC-dependent stimulation of cAMP in neonatal PSMCs and that such elevation of cAMP would be pro-proliferative. To test our hypotheses, we utilized two endogenous polypeptides, ET-1 and ANG II, widely recognized as G_q-coupled PKC agonists that control SMC growth and differentiation.^{1,9,10,13,14,16,24,25,27} Both cAMP responses and indexes of proliferation were measured in neonatal and adult PSMCs.

4.3 Methods

Isolation and Culture of Neonatal and Adult PSMCs.

SMCs were obtained from the main PAs of neonatal (14-days-old) calves and adult (2-yr-old) cows. Neonatal and adult PSMCs were considered matched because they were derived from the middle media at the same vascular site with previously described techniques.⁶⁻⁸ Briefly, main PAs were dissected from calves and cows immediately after death and transported to the laboratory immersed in MEM (pH 7.4) containing 200 U/ml of penicillin, 0.2 mg/ml of streptomycin, and 5 mg/ml of amphotericin B at 25°C. The PAs were opened, and the endothelium was scraped off. Explants of smooth muscle tissue (2 x 3 mm) were dissected from the middle media of PA strips. They were plated in petri dishes containing MEM with 10% serum, 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin. The PSMCs at confluence exhibited characteristic "hill-and-valley" (adult) and "swirllike" (neonatal) morphologies by phase-contrast microscopy and stained in a homogeneous fibrillar pattern with smooth

muscle-specific monoclonal anti- α -smooth muscle actin antibody (Sigma, St. Louis, MO).⁶⁻⁸ Cell cultures were maintained in MEM (pH 7.4) containing 1% L-glutamine, 200 U/ml of penicillin, 0.2 mg/ml of streptomycin and 0.5% MEM-nonessential amino acid solution (all from Sigma) with 10% bovine calf serum (BCS; Hyclone Laboratories, Logan, UT) and incubated in a humidified atmosphere with 5% CO₂ at 37°C. The medium was changed biweekly. To ensure that any differences seen between the cell populations were due to intrinsic differences and not induced *in vitro* in, we controlled identical sites of harvest, time in culture, passage number, and growth conditions of neonatal and adult PSMCs. The cells were studied between primary culture and third passage. Cells were grown to confluence in T 75 flasks in the presence of 10% CBS, removed from the tissue culture flasks by trypsinization (0.2 g/l of trypsin-0.5 g/l of EDTA; Sigma), and then seeded at equal density into 24-well tissue culture plates (50 x 10³ cells/ well). Cells were grown to confluence in the presence of 10% serum in 2-3 days and incubated for 72 h in serum-deprived medium (0.1% BCS) to achieve a quiescent state.

Measurement of [³H]thymidine Incorporation into DNA.

DNA synthesis was measured as previously described.⁶⁻⁸ For these experiments, neonatal and adult PSMCs were grown to confluence, and a quiescent state was achieved after 72 h in 0.1% CBS-MEM. [³H]thymidine (0.5 μ Ci/well, ICN Biochemicals, Irvine, CA.) was added together with ET-1, ANG II, forskolin, or 8-bromo-cAMP (Sigma) for 24 h. In studies of [³H]thymidine incorporation during PKC or cAMP blockade, the cells were pretreated with the specific PKC inhibitors cherythrine chloride and RO 31-8220 or the cAMP antagonist *R_p* diastereomer of cAMP (*R_p*-cAMP; all Alexis, San Diego, CA) for 15 min, followed by application of [³H]thymidine (0.5 μ Ci/well) together with ET-1, ANG II, forskolin or 8-bromo-cAMP for 24 h. Cell counts were obtained at the end of the incubation with a hemacytometer. After the cells were washed with phosphate-buffered saline (PBS) and 0.2 M perchloric acid (0.5 ml/well) was added for 2-3 min, the cells were again washed with PBS (1 ml/well) and then 1.0% SDS-0.01 N NaOH (0.3 ml/well) was added. The contents of each well were added to 4 ml of Ecoscint H scintillation cocktail

(National Diagnostics, Atlanta, GA), and radioactivity was measured with a Beckman LS 7500 beta-scintillation counter (Irvine, CA). Incorporation of [^3H]thymidine into DNA is expressed as counts per minute (cpm) per cell.

Measurement of Change in Cell Number.

Cells were trypsinized for 10 min, gently triturated four times after the addition of an equal volume of MEM-10% serum, and counted with a standard hemacytometer.

Measurement of cAMP Accumulation.

cAMP measurements were made with confluent, quiescent neonatal and adult PASCs grown to confluence in 24-well plates (plated at 50×10^3 cells/well) with a standard radio-immunoassay (Biomedical Technologies, Stoughton, MA). Studies were conducted with MEM at pH 7.35-7.45. In studies of cAMP accumulation, either vehicle control, ET-1, or ANG II was added to the cells, and the cells were incubated at 37°C for 90 min. In selected experiments, the cAMP signal was amplified with the α -adrenergic agonist isoproterenol (Sigma) along with vehicle control, ET-1, or ANG II. In all studies, the solutions contained the phosphodiesterase inhibitor 3-isobutyl-1 methylxanthine (IBMX; Sigma) to inhibit cAMP breakdown. In studies of cAMP accumulation during PKC blockade, the cells were pretreated with the specific PKC inhibitors cherythrine chloride or RO 31-8220 for 15 min, followed by application of vehicle control, ET-1, or ANG II. After incubation, the cells were washed with PBS, and the reactions were stopped with 1 M NaOH and then neutralized to pH \approx 7.0 with 1 M HCl. The solutions were acetylated, the tubes were centrifuged, and the supernatant was decanted. Radioactivity of the precipitate was counted, and sample cAMP was calculated from a standard curve. cAMP was standardized to cell counts obtained from untreated wells at the end of the incubation.

RT-PCR detection of adenylyl cyclase.

Expression of PKC-stimulated AC (type II) was tested generally as described.⁴ Briefly, total RNA was extracted from cell monolayers with Qiagen RNeasy (Qiagen, Santa Clarita, CA) and subjected to first-strand

synthesis with reverse transcriptase (GIBCO BRL) and oligo (dT) primer after treatment of RNA with DNase I (GIBCO BRL). Degenerate primers directed to the highly conserved C_{2A} region of AC were used for PCR amplification.²⁸ PCR products were ligated into a cloning vector (TOPO TA cloning kit, Invitrogen Inc., San Diego) and transformed into competent cells. After PCR screening of clones for proper inserts, bacterial cultures were made and grown for 16-18 h. Plasmids were purified by QIAprep Spin Plasmid kit (Qiagen). Sequencing was performed by automated fluorescence sequencing (ABI370A DNA Sequencer).

Lung Fixation.

Neonatal calves were killed by intravenous overdose of barbiturate and exsanguinated before removal of the lungs. The superior lobe of the left lung was fixed in Formalin and cut, and lung blocks were embedded in paraffin. Sections of paraffin-embedded tissue were cut using a microtome at 4 μ m. Before the slides were stained, the paraffin was removed from the slides with xylene, and the tissue was rehydrated in a graded alcohol series.

Immunohistochemistry.

Immunostaining_s was generally as previously described.^{4,17} To test for AC expression, the slides were incubated in 0.3% H₂O₂ in methanol for 30 min to decrease endogenous peroxidase activity. The slides were incubated with blocking solution (1% BSA and 0.05% Triton X-100 in PBS) to reduce nonspecific binding of antibodies. Primary antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for type II AC were diluted 1:250 in PBS with 0.1% BSA and 0.05% triton X-100 and incubated overnight at room temperature. After the slides were washed, biotinylated goat anti-rabbit IgG antibody diluted 1:250 in PBS with 0.05% Tween was added for 2 h. The slides were washed and incubated in avidin-biotin-horseradish peroxidase complex (Vectastain ABC kit, Vector Laboratories Inc., Burlingame, CA) diluted 1:250 in PBS with 0.05% Tween for 1.5 h. The slides were again washed and developed in 5 mg of diaminobenzidine, 10 ml of 50 mM Tris, pH 7.4, and 10 μ l of 30% H₂O₂ for 1 min, rinsed with tap water, and counterstained briefly with hematoxylin before dehydration and mounting. In control experiments, blocking peptide (Santa Cruz

Biotechnology) was diluted 1:250 and coincubated with the type II AC polyclonal antibody.

The antibody for type II AC was generated against the sequence KTYFVNTEMSRSLSQSNVAS of the type II enzyme. Specificity of the antibody for type II AC has been confirmed by Santa Cruz Biotechnology with Western blotting and immunocytochemistry.

Statistical Analysis.

Data are reported as means \pm SEM. One-way ANOVA with multiple comparisons was used to compare means between groups. A $p < 0.05$ was used to indicate significance.

4.4 Results

Stimulation of cAMP

To address whether ET-1 and ANG II could increase proliferation in neonatal PSMCs through a cAMP-mediated pathway, we first measured cAMP levels in PSMCs in the presence of the phosphodiesterase inhibitor IBMX (500 μ M) and in response to ET-1 and ANG II. Baseline cAMP was higher in neonatal PSMCs than in adult cells (Fig. 1A). Both ET-1 (10 nM) and ANG II (10 nM) stimulated cAMP synthesis (\approx 12- and 4-fold respectively; $p < 0.05$; $n=6$.) in neonatal PSMCs over 90 min but did not change cAMP levels in adult cells (Fig 1A). Similar results were observed in response to ET-1 and ANG II in the presence of isoproterenol (25 μ M) and IBMX over a 5-min time course (data not shown).

We investigated the possibility that ET-1 and ANG II increased cAMP in neonatal PSMCs by PKC-mediated stimulation of AC. Pretreatment of these cells with the PKC inhibitor cherythrine (1 μ M) reduced baseline cAMP (30%) and eliminated ET-1 and ANG II stimulation of cAMP (Fig 1B) Similar results were obtained with the specific PKC blocker RO 31-8220 (5 μ M; data not shown). These data are consistent with the idea that basal cAMP levels and elevation of cAMP after application of ET-1 and ANG II are regulated through PKC stimulation of AC.

Expression of type II (PKC-stimulated) adenylyl cyclase in vitro and in vivo.

The activity of type II AC is stimulated by PKC.³² We next sought to identify whether the type II enzyme is expressed in neonatal PSMCs using RT-PCR cloning. Sequence analysis of a 261-nucleotide product revealed 94 and 90% homology between the bovine product and respective human and rat species at the nucleotide level. Deduced amino acid alignments demonstrated 97 and 95% homology between the presently cloned bovine and respective human and rat sequences (Table 1).

Although type II ACs is expressed in cells in culture, isolation and culture per se may induce phenotypic changes in the population of PSMCs. Thus we determined whether PKC-stimulated AC was evident in neonatal and adult PSMCs in vivo using established immuno-histochemical techniques. A recently developed AC type II-specific polyclonal antibody was utilized. We found expression of type II AC throughout the pulmonary vasculature. Especially important is detection of the type II PKC-stimulated AC in the medial layer at the site where excessive proliferation of PSMCs in response to mitogenic stimuli occurs (Picture 7 on page 146). Immunoreactivity was eliminated by coincubation of the PKC-stimulated AC type II antibody with a blocking peptide, suggesting antibody specificity to type II AC.

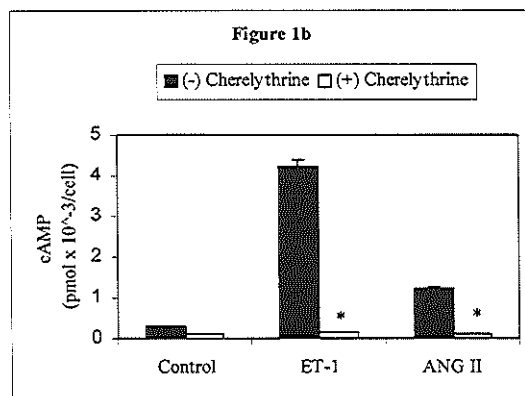
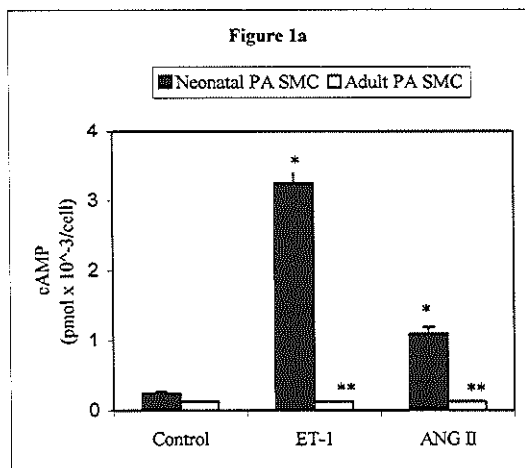


Figure 1. Endothelin 1 ET-1 and Angiotensin II (ANG II) activate protein kinase C and increase cAMP in neonatal pulmonary artery (PA) smooth muscle cells (SMC). Cells were plated at 50,000 cells/well and studied in a confluent and quiescent state. After application of vehicle or treatment conditions, cAMP was measured using radio-immunoassay and was standardized to cell counts. Values are means \pm SE. A: baseline cAMP in the presence of IBMX was higher in neonatal than in adult PASMCS. ET-1 (10 nM) and ANG II (10 nM) increased baseline cAMP over a 90- min time course in neonatal but not adult cells. (n=6/group). Significantly different ($p < 0.05$) from *control PASMCS: **neonatal PASMCS

B: pretreatment with (+) cherythrine (1 μ M) reduced baseline cAMP and eliminated ET-1 and ANG II-induced rise in cAMP in neonatal PASMCS (n=6/group).-, Without. *Significantly different from without cherythrine, $p < 0.05$.

Table 1. Comparison of rat, human and bovine amino acid sequences of type II adenylyl cyclase.

| Type II Isoform | Sequence |
|-----------------|---------------------|
| | 1 10 |
| Rat | KTIGSTYMAA |
| Human | KTIGSTYMAA |
| Bovine | KTIGCTYMAA |
| | 11 20 |
| Rat | TGLSAIPSQE |
| Human | TGLSAVPSQE |
| Bovine | TGLSAVPSQE |
| | 21 30 |
| Rat | HSQEPERQYM |
| Human | HSQEPERQYM |
| Bovine | HAQEPERQYM |
| | 31 40 |
| Rat | HIGTMVEFAY |
| Human | HIGTMVEFAF |
| Bovine | HIGTMVEFAF |
| | 41 50 |
| Rat | ALVGKLDAIN |
| Human | ALVGKLDAIN |
| Bovine | ALVGKLDAIN |
| | 51 60 |
| Rat | KHSFNDFKLR |
| Human | KHSFNDFKLR |
| Bovine | KHSFNDFKLR |
| | 61 70 |
| Rat | VGINHGPVIA |
| Human | VGINHGPVIA |
| Bovine | VGINHGPVIA |
| | 71 80 |
| Rat | GVIGAQKPQY |
| Human | GVIGAQKPQY |
| Bovine | GVIGA H KPQY |
| | 81 87 |
| Rat | DIWGNTV |
| Human | DIWGNTV |
| Bovine | DIWGNTV |

Sequences are within the C_{2A} region of the 2nd cytosolic loop of adenylyl cyclase. Nos. on top, amino acid no. Boldface type, nonhomologous amino acids. Bovine sequence is 97% and 95 % similar to human and rat sequences, respectively, within this region.

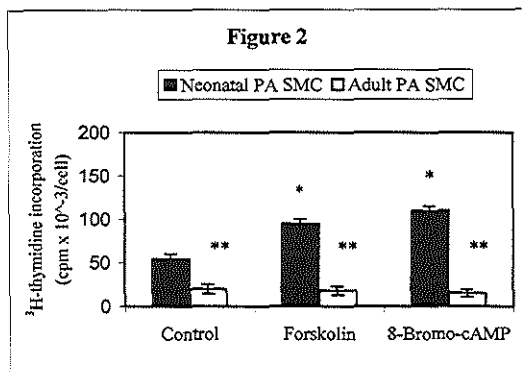


Figure 2. *Elevated cAMP promotes growth in neonatal PASM Cs. Cells were plated at 50,000 cells/well and studied in a confluent and quiescent state. After application of treatment conditions, incorporation of [³H]thymidine into DNA was measured. cpm, counts/min. Values are means \pm SE; n=4 cells/group. Basal [³H]thymidine incorporation in neonatal PASM C was threefold higher than in adult cells. Forskolin (10 μ M) and 8-bromo-cAMP (1 μ M) increased [³H]thymidine incorporation after 24 h in neonatal but not adult cells. Significantly different (p < 0.05) from: *control PASM Cs; **neonatal PASM Cs.*

SMC Proliferation

To access the contribution of cAMP to growth in neonatal and adult PASM Cs, we measured the effects of the direct AC agonist forskolin (10 μ M) and the cAMP analog 8-bromo-cAMP (1 μ M) on [³H]thymidine incorporation. Basal [³H]thymidine incorporation was threefold higher in neonatal than in adult cells. Forskolin (10 μ M) and 8-bromo-cAMP (1 μ M) induced a twofold increase in [³H]thymidine incorporation in neonatal PASM Cs but did not effect [³H]thymidine incorporation in adult PASM Cs (p < 0.05; n=4 cells; Fig. 2). Cell counts after forskolin and 8-bromo-cAMP application were higher in neonatal but not in adult PASM Cs (data not shown), consistent with a pro-proliferative effect of cAMP in these neonatal cells.³⁰

Our next studies investigated whether neonatal PASM Cs exhibit greater growth responses than adult cells to the G_q α agonists ET-1 (10 nM) and ANG II (10 nM). ET-1 and ANG II increased [³H]thymidine incorporation three- and twofold, respectively, in neonatal PASM Cs but did not increase

[³H]thymidine incorporation in adult PASMCS ($p < 0.05$; $n = 4$ cells; Fig. 3A). Cell counts after ET-1 and ANG II application were also higher in neonatal but not in adult PASMCS (data not shown).

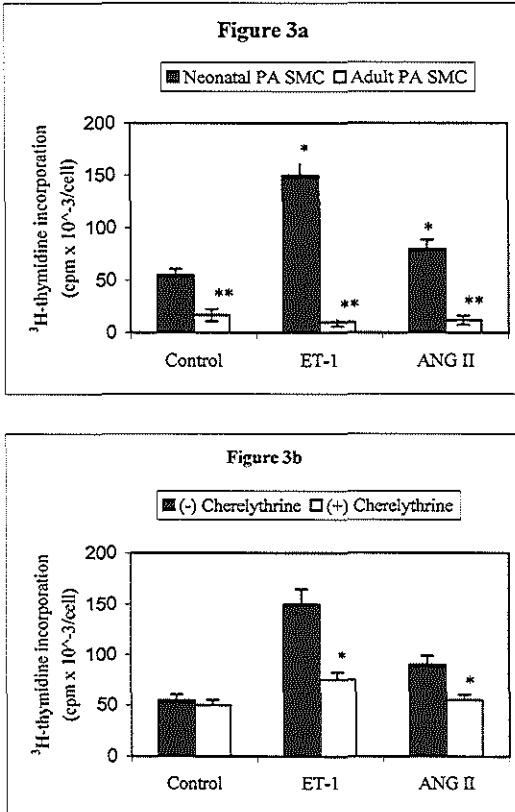
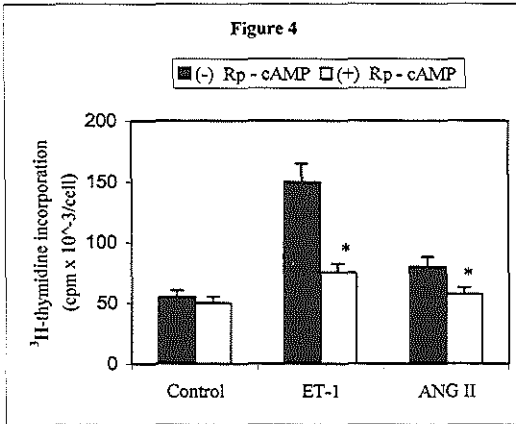


Figure 3. ET-1 and ANG II activate PKC and stimulate growth of neonatal PASMCS. Cells were plated at 50,000 cells/well and studied in a confluent and quiescent state. After application of treatment conditions, incorporation of [³H]thymidine into DNA was measured. Values are means \pm SE; $n = 4$ cells/group. A: ET-1 (10 nM) and ANG II (10 nM) increased [³H]thymidine incorporation after 24 h in neonatal but not in adult cells. Significantly different ($p < 0.05$) from: *control PASMCS; **neonatal PASMCS. B: pretreatment with chereylythrine (1 μ M) inhibited ET-1 and ANG II-stimulated neonatal PASC proliferation. *Significantly different from without chereylythrine, $p < 0.05$.



*Figure 4 Inhibition of PKA prevents ET-1 and ANG II stimulation of neonatal PASM C growth. After pretreatment with Rp diastereomer of adenosine 3',5'-cyclic monophosphate (Rp-cAMPS; 1mM) and application of ET-1 or ANG II, incorporation of [³H]thymidine into DNA was measured. Values are means \pm SE; n=4 cell/group. PKA blockade attenuated basal and the ET-1 and ANG II-induced increase in [³H]thymidine incorporation in neonatal PASM Cs. *significantly different from neonatal PASM C, p<0.05.*

We investigated the role of PKC in the proliferative response to ET-1 and ANG II in neonatal PASM Cs using cherylythrine. Figure 3B shows that PKC inhibition attenuated the basal and ET-1 and ANG II-mediated increase in [³H]thymidine incorporation (6, 75 and 75% respectively; p<0.05; n=4 cells). Identical results were achieved with the PKC blocker RO 31-8220 (5 μ M; data not shown). A previous report from our laboratory³⁰ has shown that PKC inhibitors at concentrations presently reported do not cause significant cell death, confirming that inhibition of PKC decreased proliferation rather than induced apoptosis or necrosis. We next tested the role of AC and cAMP in increased proliferation by blocking cAMP dependent protein kinase activity with Rp-cAMPS (1 mM). Rp-cAMPS attenuated basal and ET-1 and ANG II-mediated increases in [³H]thymidine incorporation (6, 85 and 78%, respectively; p<0.05; n=4 cells; Fig. 4), confirming a pro-proliferative action of cAMP in neonatal PASM Cs. Altogether, these data suggest that stimulation of proliferation in

quiescent neonatal PASMCs is at least partly regulated through PKC stimulation of AC and cAMP.

4.5 Discussion

Vascular SMCs derived from neonatal PAs exhibit enhanced growth capacities to growth-promoting stimuli compared with SMCs derived from adult pulmonary arteries.^{6,30} The reason for this unique phenomenon is unclear, although enhanced growth capacity may contribute to normal adaptive mechanisms after birth as well as the need for continued pulmonary vascular growth. Recent evidence^{2,6-8} indicated that compared with adult PASMCs, neonatal PASMCs exhibit enhanced growth responses to activation of ERK and PKC. Moreover, stimulation of ERK occurred after elevation of cAMP, suggesting that PKC stimulation of cAMP may be a critical link to ERK-dependent proliferation.^{2,6,8,11,12,22} However, it was unclear whether activation of PKC influences PASMC cAMP content and whether cAMP is pro-proliferative in PASMCs. Novel findings from our study are that:

1. G_q agonists ET-1 and ANG II elevate neonatal but not adult PASMC cAMP,
2. Both neonatal and adult PASMCs express a PKC-stimulated AC, and
3. ET-1, ANG II, and direct elevation of cAMP is pro-proliferative in neonatal but not in adult PASMCs.

These data suggest that PKC stimulation of cAMP is a critical signal mediating proliferation of neonatal PASMCs that is absent in adult PASMCs and therefore may contribute to the unique pro-proliferative phenotype of neonatal PASMCs.

Our initial studies sought to determine whether G_q agonists ET-1 and ANG II promote cAMP synthesis. Activation of PKC in diverse cell types, including bronchial SMCs, increases cAMP content.²² Both ET-1 and ANG

II increased cAMP content in PASMCs over a 90-time course, and inhibition of PKC prevented the ET-I and ANG II-induced rise in cAMP. Interestingly, neither G_q agonist tested elevated cAMP content in adult PASMCs. Thus these data are the first to demonstrate that PKC stimulation of cAMP is developmentally controlled.

Recent elucidation of multiple AC species revealed that certain isoforms (e.g. type II) are stimulated by PKC,³² providing a putative mechanism through which PKC may increase cAMP content. Our next studies therefore determined whether PKC-stimulated AC was selectively expressed in neonatal PASMCs. We tested expression of the type II isoform by RT-PCR cloning using sequence-specific oligonucleotide primers. Sequence analysis revealed that type II AC is expressed in both neonatal and adult PASMCs. To confirm that expression of this enzyme was not an artifact of cell culture per se, immunostaining was performed on sections from intact neonatal and adult bovine lungs. Positive staining was observed in the medial layers of large and small vessels from animals of both developmental stages. Thus these data indicate that the expression of type II AC is not developmentally controlled and does not account for the distinct ET-1 and ANG II responses in neonatal versus adult PASMCs.

Although PKC-stimulated AC is expressed in both neonatal and adult cells, our data indicated that PKC only stimulated the type II enzyme in neonatal PASMCs, supporting the idea that mechanisms controlling AC are developmentally regulated. Dempsey et al.⁶ previously demonstrated that relative to adult cells, neonatal PASMCs exhibit increased PKC activity under basal conditions and increased sensitivity to the direct PKC activator phorbol 12-myristate 13-acetate. It is therefore reasonable that increased PKC activity in neonatal PASMCs stimulates type II AC, whereas lower PKC activity in adult cells does not stimulate type II AC. Multiple isoforms of PKC are present in neonatal PASMCs, but the α -isozyme has been implicated in increased growth responses.³⁰ Interestingly, the α -isozyme of PKC activates type II AC in Sf9 cells.³⁴ Future studies will be required to directly test the nature of PKC stimulation of AC activity in neonatal

PASMCs, e.g. which PKC isoforms account for increased whole cellular PKC activity and activation of type II AC.

Our next studies were designed to address whether a link exists between PKC stimulation of cAMP and neonatal PASMC proliferation by determining whether:

1. Elevated cAMP is pro-proliferative,
2. PKC activation is pro-proliferative, and
3. PKC stimulation of proliferation depends on cAMP

The role of cAMP on SMC proliferation is controversial. Previous reports suggested that cAMP may have either a negative or positive influence on proliferation,²³ with the effect of cAMP depending on cell type,²³ state of cell differentiation,³ and stage of cell cycle.²¹ Neonatal and adult PASMCs were "growth arrested" to mimic the *in vivo* environment. Although previous studies^{6,30} showed that neonatal PASMCs exhibit enhanced growth capabilities, our present studies demonstrated that these cells also exhibit higher basal cAMP levels, consistent with the possibility that cAMP may function as a positive stimulus for proliferation. We found that two agents that increase cAMP (8-bromo-cAMP and forskolin) also stimulate [³H]thymidine incorporation and cell proliferation in neonatal but not in adult PASMCs. Furthermore, direct inhibition of the cAMP dependent PK lowered basal [³H]thymidine incorporation in neonatal PASMCs. These data suggest that cAMP is pro-proliferative in neonatal PASMCs and that the action of cAMP-induced growth is developmentally regulated as recently suggested in Schwann cells.³¹

We next evaluated the influence of PKC on growth in neonatal PASMCs. PKC activity is increased in neonatal versus adult PASMCs, and ET-I and ANG II activate PKC. Furthermore, activation of PKC is generally found to stimulate proliferation.^{6,16,20,25,34} In our present studies, inhibition of PKC with chelerythrine and Ro 31-8220 decreased basal and ET-I- and ANG II-stimulated [³H]thymidine incorporation, suggesting that increased growth in neonatal PASMCs depends at least partly on PKC activity.

However, PKC inhibitors did not influence proliferation in adult PSMCs. Both ET-1 and ANG II are generally believed to stimulate growth in adult SMCs derived from the systemic circulation.^{1,5,10,27} Indirect evidence for the involvement of ET-1 and ANG II in medial thickening of pulmonary arteries has also been shown in adult rats,^{18,33} but direct effects of the polypeptides on PASMCM proliferation are less clear. For example, ET-1 was previously reported¹⁶ to increase growth in adult swine PSMCs in the presence of 0.5% serum, whereas in the present study, ET-1 and ANG II were not pro-proliferative in adult bovine PSMCs in the presence of 0.1% serum. The reason for this discrepancy is unclear, although it is possible that these agents act as comitogenic stimuli, requiring other growth factors to stimulate proliferation. Independent support for this idea comes from work of Morrell and Stenmark,¹⁹ who observed that ANG II stimulated proliferation of adult rat PSMCs only when the cells were primed by preincubation with 10% serum but not under serum-deprived conditions (0.1%). Thus our data are consistent with the idea that ET-1 and ANG II alone are insufficient to promote proliferation in adult PSMCs and suggest that PSMCs possess a developmentally regulated sensitivity to these vasoconstrictors.²⁹

Our final series of experiments tested whether inhibition of PKA blocks ET-I and ANG-II-stimulated increase in neonatal PSMCM proliferation. Indeed, the PKA inhibitor *RP*-cAMPS prevented G_q activation from stimulating neonatal PSMCM proliferation but did not affect proliferation of adult PSMCs. Our data therefore demonstrate that ET-I and ANG-II stimulate PKC-dependent production of cAMP that is pro-proliferative in neonatal PSMCs; inhibitors of either PKC or PKA prevent this stimulation of proliferation.

In summary, ET-I and ANG-II activation of G_q activates PKC, which increases cAMP and promotes proliferation of neonatal PSMCs. In contrast, ET-I and ANG II activation of G_q neither increases cAMP nor promotes proliferation of adult PSMCs. The explanation for this apparent developmental distinction is not yet fully determined, however it is not due to altered expression of PKC-stimulated (type II) AC. Based on earlier

work from our laboratory,^{6,8} a likely explanation is that increased constitutive PKC activity and enhanced PKC responsiveness to activation accounts for PKC stimulation of cAMP in neonatal versus adult PSMCs. Now that a key link between PKC and cAMP production has been established in neonatal PSMCs, future studies may address the regulation of ERK-dependent proliferation by cAMP.

4.6 Acknowledgements

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5

ROLE OF CYCLIC AMP RESPONSE ELEMENT BINDING PROTEIN IN THE DEVELOPING PULMONARY VASCULATURE

5.1 Abstract

The second messenger cyclic AMP (cAMP) mediates exorbitant proliferation of neonatal bovine pulmonary artery smooth muscle cells (PASMC) in response to G_q coupled agonists through protein kinase C (PKC) stimulation of type II adenylyl cyclase. Elevated levels of cAMP activate protein kinase A (PKA), which influences gene transcription through phosphorylation of cAMP response element binding protein (CREB). We found PKA activity was present in neonatal but not in adult PASMC. Serum stimulated growth of both neonatal and adult PASMC resulted in phosphorylation of CREB. In contrast to detectable phospho-CREB in serum-deprived neonatal PASMC, no phospho-CREB was detected in quiescent adult cells (Western blot). Using immunohistochemistry we located (phospho-) CREB throughout the pulmonary vasculature within endothelial and medial SMC. Furthermore, neonatal lungs showed enhanced expression of (phospho-) CREB, compared with adult lungs. While immunolocalisation for CREB was mainly in the cytosol, staining for phospho-CREB appeared nuclear, suggesting translocation. We concluded that cAMP mediated activation of CREB might well contribute to enhanced growth of neonatal PASMC.

5.2 Introduction

The morphologic changes that occur in the pulmonary vasculature during the transition to extra-uterine life have been described.¹² However, the mechanisms that lead to these changes are not completely understood. Distinct mechanisms controlling growth during pre-and perinatal development and disease in particular (e.g. Pulmonary Hypertension of the Newborn) have been suggested. Differences in the activation of growth factors, such as insulin-like growth factors and fibroblast growth factor and second messenger pathways, such as PKC may underlie particular growth patterns in various developmental stages.^{9,15} Our previous data implicate a role for the second messenger cAMP, through PKC stimulation of type II

adenylyl cyclase, in mediating exorbitant proliferation of neonatal pulmonary artery smooth muscle cells (PASMC) in response to G_q coupled agonists (Angiotensin II, Endothelin I).¹¹

The principal intracellular target for cAMP in mammalian cells is cAMP-dependent protein kinase (PKA).²³ Elevated levels of cAMP activate PKA which influences gene transcription through phosphorylation of cAMP response element binding protein (CREB).⁷

CREB is a transcription factor that is constitutively expressed in most cells and tissues.⁴ It was originally identified as a regulator of gene transcription in response to elevated intracellular cAMP levels.¹³ In the last decade, numerous studies have contributed to our understanding of CREB structure, function and CREB-mediated transcription.^{3,4,5,6,7} In this capacity, CREB binds to a specific target sequence or cAMP-response element (CRE) in the promotor regions of cAMP-regulated genes, such as c-fos, c-jun.²² Under conditions which increase cAMP levels, CREB is phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (PKA) at Ser 133. Phosphorylation of CREB increases its association with transcriptional adapter proteins like CREB binding protein (CBP) or P300 which also interact with the core transcriptional machinery and thereby increase the rate of transcription.^{2,10,19,20}

While CREB clearly regulates numerous functions in fully differentiated cells,¹⁶ multiple lines of evidence also support the hypothesis that CREB participates in regulating the growth and differentiation of SMCs.^{13,14} However, how activation of CREB synergistically interacts with growth factors to enhance neonatal PASMC growth and what accounts for enhanced PKC-dependent proliferation in neonatal compared with adult PASMC is not clear at this moment. A possible mechanism by which CREB modulates neonatal PASMC development is through its ability to regulate the transcription of target genes.^{17,22}

We hypothesized that activation of CREB in neonatal PASMC due to stimulated cAMP production, may account for the increased growth of neonatal PASMC. We first found that PKA activity was present in neonatal but not in adult PASMC. Furthermore, we found that serum stimulated growth of both neonatal and adult PASMC results in phosphorylation of

CREB, but that in contrast to detectable phospho-CREB in serum deprived neonatal PASMC, no (phospho-)CREB was noticed in quiescent adult PASMC. We then sought to identify expression and distribution of CREB and phospho-CREB in neonatal and adult lung *in vivo*. Using immunohistochemistry we located CREB throughout the pulmonary vasculature within endothelial cells and medial SMCs. Furthermore, neonatal lungs showed enhanced expression of (phospho-) CREB, compared with adult lungs. While immunolocalisation for CREB was mainly in the cytosol, staining for phospho-CREB appeared nuclear, suggesting translocation. We concluded that activation of CREB might well contribute to the enhanced growth potential of neonatal PASMC.

5.3 Methods and materials

Cell culture

We used smooth muscle cells which were obtained from the main pulmonary arteries (PASMC) of neonatal (14 days old) calves and adult (2 year old) cows (kind gift from Dr. K.R. Stenmark, Denver, CO, USA). The PASMC at confluence exhibited characteristic "swirl-like" (neonatal) and "hill and valley" (adult) morphologies by phase-contrast microscopy and expressed smooth muscle actin protein in a homogeneous manner as detected with a monoclonal antibody against smooth muscle actin (Neomarkers, Union City, CA, USA)(data not shown).²⁴ Cell cultures were maintained in modified eagle's medium (Gibco BRL, Paisley, Scotland) with 10% bovine calf serum (BCS; Dako, Glostrup, Denmark) and grown to confluence in T75 flasks and then seeded at equal density into 24 well tissue culture plates (10×10^3 cells/well). Cells were grown to confluence in the presence of 10 % serum in 2-3 days and incubated for 72 h in serum-deprived medium (0.1% BCS) to achieve a quiescent state.

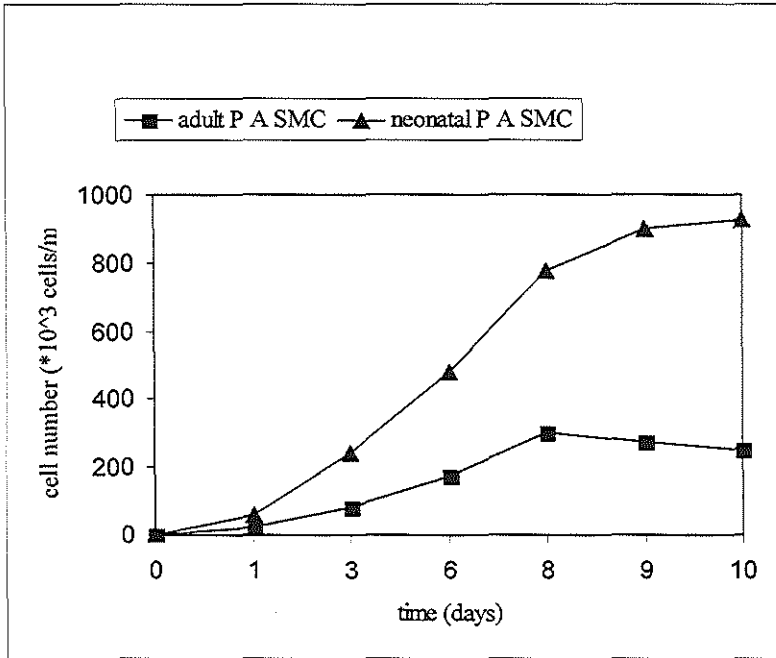


Figure 1 PASM from neonatal calves proliferate faster and achieve higher plateau densities than adult cells. Cells seeded at 10×10^3 cells/well in 10% serum for 10 days. Similar results were reproduced with 2 other groups of matched cell populations.

Measurement of change in cell number

Cells were trypsinized for 10 min, gently triturated four times after addition of an equal volume of 10% serum, and counted with a standard hemocytometer. PASM derived from neonatal calves proliferate faster and achieve higher plateau densities than adult control cells, when exposed to MEM-10% serum (figure 1).⁸

Protein Kinase A activity measurements

PKA activity measurements were made with confluent, quiescent neonatal and adult PASM using a peptag non-radioactive assay (Promega, Madison, WI, USA). In studies of PKA accumulation, either vehicle

control or ET-1 [10nM], VEGF [11.4 pM (0,5 ng/ml)], 8-Bromo-cAMP [1 μ M] (Sigma-Aldrich, Steinheim, Germany) or 10% serum respectively were added to the quiescent cells for 10 min. Then medium was removed, cells were washed and cells were kept on ice to stop any reaction; cell extraction buffer was added, cells were scraped off the T75's into the buffer and stored on ice until homogenized ultrasonically and spinned down at 13.000 rpm during 15 min at 4°C; cytosol was collected and kept at -80 °C until measurements were taken. Cell number measurements were taken as described before,²⁴ protein measurements were done to correct for cell amount. Two μ g of protein/ml was incubated for 30 minutes at room temperature as described in the standard PKA assay protocol. The samples were separated at a 1% agarose gel at 100V for 15 minutes. The gel was photographed on a transilluminator.

Western blot analysis

Cell cultures were maintained in MEM with 10% BCS and grown to confluence in T75 flasks and then either serum deprived for 72 hours or medium was removed from the cells and cells were directly lysed with 500 μ l SDS loading buffer. After correcting for protein concentrations, 50 μ g portions of the lysates were resolved on 10% polyacrylamide-SDS gels and transferred to a nitrocellulose membrane. Duplicate blots were probed with monoclonal antibodies that recognize CREB phosphorylated at Ser 133 (P-CREB) or antibodies that recognize both unphosphorylated and phosphorylated CREB. These antibodies are produced by immunizing rabbits with a synthetic peptide derived from the sequence of human CREB (New England Biolabs., Beverly, MA, USA). The blots were washed and subsequently treated with goat anti-rabbit IgG conjugated with alkaline phosphatase. Following washing of the blots, specific immune complexes were visualized with bromochloroindoyl phosphate and nitro blue tetrazolium.

Immunohistochemistry

The superior lobes of neonatal and adult cows were fixed in formalin and cut, and lung blocks were embedded in paraffin. Sections of paraffin-

embedded tissue were cut with a microtome at 4 μm . The slides were deparaffinized in xylene, and placed in 0.3% H_2O_2 in methanol for 30 min to decrease endogenous peroxidase activity. Immunostaining was performed as previously described.¹⁸ In short, to test for (P-)CREB expression, primary antibodies for (P-)CREB were diluted 1:100 in PBS with 0.1% BSA and 0.05% Triton X-100 and applied. Slides were placed into a moist chamber and incubated overnight at 4°C. After washing, biotinylated goat anti-rabbit immunoglobulin G antibody diluted with PBS at 1:250 was added for 2h. Following washes, expression was visualized using diaminobenzidine. After washing, slides were counter-stained with hematoxylin.

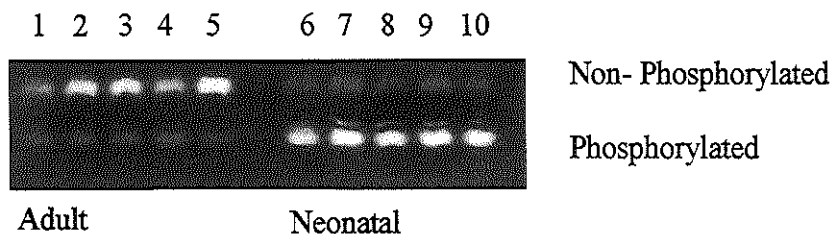


Figure 2 *Protein Kinase A activity is present in the cytosol of neonatal but not adult pulmonary artery smooth muscle cells (PASM). Cells were plated at 10,000 cells/well and studied in a confluent and quiescent state. After application of vehicle control (lane 1+6), 10% bovine calf serum (lane 2+7), ET-1 (100 nM, lane 3+8), VEGF (0.5 ng/ml, lane 4+9), or 8-Bromo-cAMP (1 μM , lane 5+10) respectively, PKA activity was measured using a peptag non-radioactive assay and standardized to protein amount. Under each conditions PKA activity was qualitatively higher in neonatal than in adult cells PASM*

5.4 Results

Stimulation of Protein Kinase A

The principle intracellular target for cAMP in mammalian cells is the cAMP-dependent protein kinase (PKA). We sought to investigate the

possibility that agonist-increased cAMP may increase proliferation through activation of PKA. First PKA activity in neonatal and adult PASMNC were evaluated. In studies of PKA accumulation, either vehicle control or ET-1, VEGF, 8-Bromo-cAMP or 10% serum respectively were added to the quiescent cells. Under each conditions PKA activity was present in neonatal but not adult cells (Figure 2).

Expression of total and phosphorylated CREB in vitro

Neonatal PASMNC exhibit increased basal and stimulated cAMP content when compared to adult cells; increased cAMP induces proliferation in neonatal but not adult PASMNC. Elevated levels of cAMP activate cAMP-dependent PKA which influences gene transcription through phosphorylation of CREB (P-CREB). First we sought to identify whether neonatal PASMNC possess higher constitutive P-CREB than adult cells using Western blotting. Serum stimulated growth of both neonatal and adult PASMNC resulted in phosphorylation of CREB, but in contrast to detectable P-CREB in serum deprived neonatal PASMNC, quiescent adult PASMNC did not express (P-)CREB (Figure 3). These data suggest that activation of ¹³³CREB through phosphorylation at Ser 133 may contribute to the uniquely different growth pattern of neonatal PASMNC, compared to adult PASMNC.

Expression of CREB in vivo

Although CREB is expressed in cells in culture, isolation and culture *per se* may induce phenotypic changes in the population of PASMNC's. Thus we determined whether CREB was evident in neonatal and adult PASMNC's *in vivo* using established immunohistochemical techniques. Recently generated polyclonal antibodies for total and phosphorylated CREB were utilized. Staining for total CREB was observed throughout the pulmonary vasculature including arteries, capillaries and veins. Expression in intimal and medial layers of the vessels appeared most prominent, although notably not all cells within the media stained positive suggesting heterogeneous SMC populations (Picture 8, on page 147). Staining in the adult lung was observed throughout the pulmonary vasculature in a similar but less pronounced pattern (not shown). While immunolocalization for CREB was mainly in

the cytosol, staining for P-CREB appeared nuclear, suggesting translocation (Picture 8 on page 147). Our data indicate cell specific expression of CREB throughout the bovine lung, and importantly suggest that activation of CREB may contribute to increased growth of neonatal PASMC.

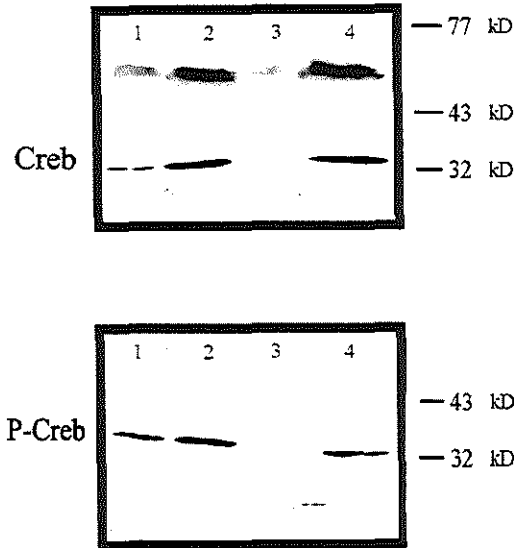


Figure 3 (P-)CREB is expressed in both growing and quiescent neonatal (lanes 1 and 2) and growing adult (lanes 3 and 4) pulmonary artery smooth muscle cells. Cells were either serum deprived for 72 hours (lanes 1 and 3) or directly lysed (lanes 2 and 4). After correcting for protein concentrations, 50 μ g portions of the lysates were resolved on 10% polyacrylamide-SDS gels and transferred to a nitrocellulose membrane. Duplicate blots were probed with monoclonal antibodies (dil.1:1000) that recognize P-CREB or antibodies that recognize both unphosphorylated and phosphorylated CREB. Immune complexes were visualized with bromochloroindyl phosphate and nitro blue tetrazolium. Results are representative of 4 independent experiments.

5.5 Discussion

The pulmonary vasculature has to adapt to postnatal life in order to allow the lung to establish gas exchange. Major changes take place in especially the SMCs residing in the pulmonary vascular wall. Previous studies showed enhanced growth capacities of neonatal compared to adult SMCs in the pulmonary artery.^{8,24} The mechanisms through which these occur are largely unknown. Our recent observations established a relationship between cAMP and the increased growth potential of neonatal PASMCs.¹¹ Endothelin-1 and Angiotensin II increased cAMP levels and promoted growth in neonatal but not in adult PASMC. Present studies were undertaken to further determine second messenger induced pathways in these cells. Elevated levels of cAMP are known to influence gene transcription through phosphorylation of CREB.^{10,17} Recently it has been demonstrated that the cAMP dependent protein kinase gene is developmentally regulated in fetal lung. Acaregui et al.¹ demonstrated that mRNA levels for PKA subunits C and R1 alpha were highest in 16-day old rat lung, decreased at newborn and were lowest in the adult rat lung. However, it was unknown whether elevated levels of cAMP activate PKA in PASMC and furthermore whether an apparent developmental distinction as seen for PKC,⁸ is present in these cells.

Our study demonstrated that:

1. PKA activity was present in neonatal but not in adult PASMC
2. Serum-stimulated growth of both neonatal and adult PASMC resulted in phosphorylation of CREB, however, no detectable (P-)CREB was noticed in quiescent adult cells
3. (P)-CREB is located throughout the pulmonary vasculature, but neonatal lungs showed enhanced expression of (P)-CREB
4. Immunolocalisation for CREB was mainly in the cytosol, but P-CREB appeared nuclear.

These data collectively suggest that cAMP-mediated activation of CREB may contribute to increased growth of neonatal PASMC.

It is important to recognize that protein kinases, such as PKA, do not function in isolation within the cell. To the contrary, protein phosphorylation is rapidly reversed by the action of protein phosphatases. These serve to terminate the responses initiated by receptor activation of protein kinases. The level of phosphorylation of PKA substrates like CREB are thus determined by a balance between the intracellular activities of PKA and protein phosphatases. We did not investigate this aspect, but recent studies suggested that protein phosphorylation may be a mechanism by which alveolarization is regulated in developing lungs.²¹

Our first studies sought to determine whether stimulation of cAMP would activate PKA and whether this was developmentally regulated. We found that PKA activity was present in neonatal but not in adult cells. These data are consistent with the idea that cAMP levels regulate PKA in neonatal PASMCM and that activation of PKA may contribute to proliferation of neonatal PASMCM. Previous experiments demonstrated that PKA inhibition did not affect adult PASMCM growth.¹¹ This suggests that this pathway may be developmentally controlled, as recently seen in newborn rat lungs.²¹

After establishing that elevated levels of cAMP activate cAMP-dependent PKA in neonatal PASMCM, we tested whether gene transcription of CREB was also developmentally regulated. We then sought to identify whether neonatal PASMCMs possess higher constitutive (P-)CREB than adult cells. Western blotting revealed that neonatal PASMCM express CREB. However, P-CREB was seen in both quiescent and growing neonatal cells, but not in quiescent adult cells. This indicates that activation of CREB may be involved in the excessive growth potential of neonatal cells. This is supported by the observation that transfected CREB^{-/-} neonatal PASMCM do not proliferate at all (DJ Klemm, personal communication).

Although (P-)CREB is expressed in neonatal PASMCM in culture, isolation and culture *per se*, may induce phenotypic changes in the cells. We therefore also tested expression of CREB *in vivo*. Immunostaining indicated cell specific expression of CREB throughout the bovine lung, and

importantly demonstrated that phenotypically distinct cell populations within the vascular media differentially express CREB. Upon activation of CREB by phosphorylation at Ser 133, immunolocalisation moved from cytosol to nucleus, suggesting translocation.

Coupling of CREB to physiologic function *in vivo* awaits future studies, but our data suggest a potential role for cAMP and CREB in the proliferative response of neonatal PASMC.

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6

Ca²⁺-INHIBITABLE ADENYLYL CYCLASE AND PULMONARY MICROVASCULAR PERMEABILITY

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6.1 Abstract

Intracellular mechanisms responsible for endothelial cell disruption are unknown, although either elevated cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$), or decreased adenosine 3', 5'-cyclic monophosphate (cAMP) promotes permeability. Recent identification that Ca^{2+} -inhibitable adenylyl cyclase (AC) establishes an inverse relationship between $[\text{Ca}^{2+}]_i$ and cAMP in macrovascular endothelial cells provided a possible mechanism of development of permeability. However, these data utilized an *in vitro* model; lacking was evidence supporting:

1. expression of Ca^{2+} -inhibitable AC in pulmonary microvascular endothelium and
2. Ca^{2+} -inhibition of AC and cAMP content as a paradigm for inflammatory mediator-induced permeability in the intact circulation.

We therefore addressed these issues in microvascular endothelial cells derived from rat lung and in an isolated perfused rat lung preparation. Results demonstrate expression of a Ca^{2+} -inhibitable AC in microvascular endothelial cells. Furthermore, data suggest that Ca^{2+} -inhibition of AC is necessary for development of microvascular permeability in the intact circulation. We conclude Ca^{2+} -inhibition of cAMP represents a critical step in genesis of microvascular permeability in the intact pulmonary circulation.

6.2 Introduction

Maintenance of an extremely thin blood-gas barrier promotes efficient gas exchange in the lung. Microvascular endothelial cell disruption resulting in increased fluid accumulation, and loss of the thin blood-gas barrier is a hallmark feature of non-cardiogenic pulmonary edema(s).¹⁰ Thus discovery of mechanisms responsible for initiating and sustaining endothelial cell

disruption are critical to development of improved therapies for treatment of non-cardiogenic pulmonary edema(s).

Multiple neuro-humoral inflammatory/vasoactive factors and ischemia-reperfusion- and/or white cell-induced oxygen radicals promote endothelial cell disruption, likely through elevation of intracellular free Ca²⁺ ([Ca²⁺]_i).^{16,22} Elevated [Ca²⁺]_i induces endothelial cell permeability by promoting contraction,^{16-18,26,36,49,50} reorganizing cytoskeleton,^{16,18,22,35} and disrupting focal adhesions,^{18,27} which collectively cause development of intercellular gaps that form a paracellular pathway for the transport of macromolecules.^{4,9,21,32,34,39,44} Recent evidence, however, suggests a rise in [Ca²⁺]_i alone is insufficient to induce permeability.^{3,19} The observation that endothelia express an AC inhibited by Ca²⁺ suggests a paradigm wherein physiological elevations in [Ca²⁺]_i lower cAMP content.⁴² Prior experimental and clinical studies demonstrated that an elevation of endothelial cAMP content prevented or reversed pre-established edema.^{1,2,5,14,20,24,37,38,40} It now appears that decreases in cAMP content accompany increases in [Ca²⁺]_i as part of the cell's normal response to multiple inflammatory agonists.

Although evidence for a role of elevated [Ca²⁺]_i and decreased cAMP in mediating development of endothelial permeability is becoming clear, transfer of these data to the intact circulation is problematic. It has become apparent that endothelia exhibit remarkable phenotypic differences based upon their vascular origin;^{33,41} as an example, [Ca²⁺]_i and cAMP responses differ between pulmonary artery and microvascular endothelial cells in culture.⁴¹ Similarly, most data regarding mechanisms of endothelial permeability have been obtained from macrovascular and not microvascular endothelial cells. The current studies were therefore designed to address two key issues regarding mechanism(s) that regulate microvascular permeability:

1. whether a Ca^{2+} -inhibitable AC is expressed in pulmonary microvascular endothelium both *in vitro* and *in vivo*, and
2. Ca^{2+} -inhibition of AC and cAMP content represents a paradigm for inflammatory mediator-induced permeability in the intact circulation.

Our data clearly demonstrate that Ca^{2+} -inhibitable AC is expressed in microvascular endothelial cells and, most importantly, that decreased cAMP likely accompanies elevated $[\text{Ca}^{2+}]_i$ in order for inflammatory mediators to induce permeability.

6.3 Methods

Microvascular endothelial cell isolation and culture.

Rat pulmonary microvascular endothelial cells were obtained and cultured as previously described, with minor modification.¹¹ In brief, rat lungs were perfused without recirculation with 300 ml of Krebs-Henseleit buffer containing 8% bovine serum albumine (BSA). Perfusate was changed to serum-free Aim V medium (Life Technologies, Gaithersburg, MD) containing 8% BSA, 50 mg hyaluronidase I (Sigma Chemical, St. Louis, MO), and collagen IV coated 50 μM microspheres (DuPont, Wilmington, DE), and the lung was perfused with 100 ml.

Flow ($0.03 \text{ ml.g body wt}^{-1}\text{min}^{-1}$) was alternated in forward and reverse directions and the perfusate was collected. The suspension of perfusate, microspheres, and cells was centrifuged at 200 g (4°C) and was washed three times with RPMI containing 25% fetal calf serum (Intergen). The pellet was resuspended in 2 ml of media containing one part endothelial cell conditioned medium and two parts RPMI with 20% rat serum and 0.1% gentamicin. An additional 0.5 ml media was added after 1 wk in culture. Endothelial cells were evident in ~ 2 wk, at which time the media was changed and supplemented with 200 μg of heparin (Calbiochem, San Diego, CA), 300 μg endothelial cell growth supplement (Collaborative Biomedical Research), and 100 ng rat epidermal growth factor (Biomedical

Technologies, Stoughton, MA) to promote endothelial cell growth. The cells grew initially as capillary-like structures and assumed typical cobblestone morphology of endothelial cells at confluence. The cells stained with Factor VIII antibody and showed uptake of the acetylated low-density lipoprotein labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanide perchlorate. In addition, the cells demonstrated intense *Ulex europaeus* I, *Ricinus communis*, and *Arachis hypogaea* lectin binding criteria for microvascular endothelial cells. Electron micrographs were obtained to confirm endothelial cell identity and purity in culture.

Isolation of total RNA.

Total RNA was isolated from confluent monolayers of rat pulmonary microvascular endothelial cells as described (5' to 3', Boulder, CO.^{41,42}) Cells at passage 5 were utilized.

Amplification of adenylyl cyclases expressed in pulmonary microvascular endothelial cells and cDNA sequencing.

Amplification of AC was performed as described previously^{41,42} Briefly, total RNA was isolated, and reverse transcriptase-polymerase chain reaction (PCR) was performed using degenerate oligonucleotide primers to the C_{2A} region from the second cytosolic loop of AC. PCR products were excised from a 1% agarose gel and annealed to linearized pDIRECT cloning vectors (Clontech, Palo Alto, CA). pDIRECT cloning vectors containing inserts transformed into competent *Escherichia coli* (Invitrogen) were selected by PCR screen and bacterial cultures were grown for 14-18 h. Plasmid DNA was prepared for sequencing using standard mini-prep and alkaline denaturation techniques (Promega, Madison, WI). A silver stain sequencing technique was performed with modified Taq DNA polymerase (Silver Sequence DNA Sequencing System, Promega,⁴¹).

Lung fixation.

Rats were anesthetized with pentobarbital sodium (60 mg/ml, ip), sternotomy was performed, and lungs were ventilated and perfused as described (see *Lung isolation and perfusion* and ref 37). Lungs were ventilated and perfused for 20 min under full flow (e.g. 100% cardiac output) conditions. Ventilation was stopped at end inspiration so that airways and alveoli were distended. Lungs were then perfusion fixed with 1% paraformaldehyde, 0.1 M borate buffer, pH 9.5 for 30 min. After perfusion fixation, a lung section was placed in the paraformaldehyde solution for 4 h at 4° C. After this incubation period, the lung section was dissected into 2-cm² fragments suitable for sectioning with cryostat. These pieces were incubated overnight in filtered paraformaldehyde, 0.1 M borate, 0.45 M sucrose solution at pH 7.0. The next day, tissue was mounted with optimum cutting temperature embedding compound and was placed in frozen 2-methylbutane. Sections were cut using a cryostat at 4-6 µM, placed on a super-frost plus slide, air-dried for 10 min, and acetone fixed for 1 min.

Immunostaining.

Immunostaining was generally as described previously.²⁵ To test for AC expression, slides were incubated in 3% hydrogen peroxide in PBS for 15 min to decrease endogenous peroxidase activity. Slides were incubated with blocking serum (3% goat). Primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for type VI AC were diluted 1:250 in phosphate-buffered saline (PBS) with 0.1% BSA and 0.05% triton X-100 and were incubated overnight at room temperature. After washing, biotinylated goat anti-rabbit IgG antibody diluted with PBS at 1:250 was added for 2 h. Slides were washed and incubated in streptavidin-biotin-horseradish peroxidase at 1:250 in PBS 3% horse serum for 1.5 h. Slides were washed again and developed in diaminobenzidine (5 mg), 10 ml of 50 mM Tris pH 7.4, and 10 µl of 30% hydrogen peroxide. Slides were developed for 1-5 min, rinsed with tap water, and counterstained briefly with hematoxylin before dehydration and mounting. In control experiments, blocking peptide (Santa

Cruz) was diluted 1:250 and was co-incubated with the type VI AC polyclonal antibody.

The antibody for types V/VI AC is generated against amino acids 1149-1165 of the type VI enzyme, which is KGKGEMTTYFLNGGPSS. Specificity of the antibody for type V/VI AC has been confirmed by Santa Cruz Biotechnology using Western blotting and immunocytochemistry. Incubation of rat brain homogenates, enriched in AC I-VI, with the type V/VI antibody yielded a single 130-kDa band corresponding to type VI AC. Thus cross-reactivity of the antibody with other isoforms of AC is not apparent.

Lung isolation and perfusion.

Adult male Sprague-Dawley rats (250-350 g; SASCO, Omaha, NE) were anesthetized with pentobarbital (60 mg/kg i.p.), and a tracheotomy catheter was inserted. Lungs were ventilated with room air at inspiratory and expiratory pressures of 5.5 and 2.0 cmH₂O, respectively, until the heart was cannulated, after which time the lungs were ventilated with 21% O₂, 5% CO₂, and 74% N₂. After a median sternotomy, heparin (200 units) was administered via the right ventricle and allowed to circulate for 3 min. A pulmonary artery and double-lumen left ventricular catheter were inserted and secured with sutures. Lungs were perfused (Gilson Minipuls 3; Gilson, Middleton, WI) at constant flow of 0.04 ml.g body wt⁻¹.min⁻¹ with a bicarbonate buffered physiologic salt solution (Krebs-Henseleit, Sigma Chemical) that contained (in mM): 11.1 D-glucose, 1.2 MgSO₄, 1.2 KH₂PO₄, 4.7 KCl, 118.1 NaCl, 1.5 CaCl₂. The salt solution was osmotically stabilized with 4% bovine serum albumin (Sigma Chemical). Lungs and heart were removed en bloc, suspended from a force transducer (Grass FT03; Grass Instruments, Quincy MA) and placed in a chamber with 100% humidity at 37°C. Twenty milliliters of perfusate was used to flush the lung of resident blood cells. An additional 60 ml of perfusate were used for recirculation. Pulmonary artery (P_a) and venous (P_v) were continuously monitored (TSD 104, Biopac, Goelta CA) and were captured to a

Macintosh computer using an analog-to-digital converter (MP100A, Biopac). Zone 3 conditions were maintained in all experiments such that arterial > venous > alveolar pressures to ensure even distribution of perfusion. The protocol was approved by the University of Colorado Health Science Center Animal Care and Use Committee.

Assessment of permeability.

Capillary filtration coefficient (K_{fc}) is a measurement of hydraulic conductivity (permeability) and was estimated using a gravimetric method, modified from Drake et al.¹² In the isolated perfused lung, perfusion and colloid oncotic pressures are constant. Edema formation (net filtration) was estimated by lung weight gain and pulmonary capillary pressure (P_c) was estimated by double occlusion. When lung weight is stable (weight equilibrium) hydrostatic forces are equally opposed by oncotic forces. The weight equilibrium was disturbed by increasing P_v from 3.5 to 9.0 mm Hg for 15 min. P_c was estimated before and after P_v was increased. A rapid rise in weight due to vascular distention (recruitment and distention) was followed by gradual weight gain (net filtration). Over the period of measurement it was assumed that changes in interstitial pressure and oncotic forces were minimal. Because the rate of gradual weight gain between 5 and 15 min of pressure challenge increases in a linear fashion, it was analyzed by linear regression. The initial rate of weight change at time = 0 ($\Delta W/\Delta T_0$, where W is weight and T_0 is time 0) is estimated by the slope of the linear regression. K_{fc} was estimated by determining the ratio of $\Delta W/\Delta T_0$ to ΔP_c . K_{fc} measurements were normalized to left lung dry weight and were expressed as milliliter per minute per millimeter mercury per 10 g lung tissue.

Statistical methods.

Within-group comparisons were made using a test-retest design (repeated measures) whereas between-group assessments were made using a two-way analysis of variance with repeated measures. A Dunn Ferroni or Dunnett's

two-tailed post hoc test was applied, as appropriate. Differences were considered significant at $p < 0.05$.

6.4 Results

Adenylyl cyclase expressed in pulmonary microvascular endothelial cells.

Type VI Ca²⁺-inhibited AC is expressed in bovine pulmonary artery endothelial cells.^{41,42} Expression of a Ca²⁺-inhibited isoform, however, had not been demonstrated in endothelial cells derived from the rat pulmonary microvasculature. Reverse transcriptase-PCR was therefore performed using total RNA derived from confluent monolayers of rat pulmonary microvascular endothelial cells. The PCR products were subcloned and sequenced; seven clones were read. Cloned products corresponded to nucleotides 3208-3358 of the type VI (Ca²⁺-inhibited) isoform. Nucleotide similarity was 100% identical to previously reported rat sequences.³¹ Deduced amino acid alignment of the type VI isoform corresponded to amino acids 1069-1119 and was identical to that previously reported.³¹

Also detected was an isotype of AC described in olfactory epithelium (type III; see ref 7). This was an unexpected result, since we have not previously observed expression of this isoform in bovine or human macro- or microvascular endothelial cells. To validate expression of type III AC, isotype-specific primers were used in bovine macro- and microvascular endothelial cells, human microvascular endothelial cells, and rat microvascular endothelial cells. Reverse transcriptase-PCR products were only found in rat microvascular endothelial cells (Fig. 1), suggesting that rat cells do express the type III isozyme and bovine and human cells do not express the type III isozyme.

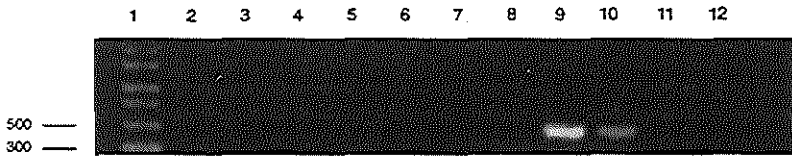


Figure 1. Rat microvascular endothelial cells express a type III AC. To confirm expression of type III AC in rat microvascular endothelial cells isoform-specific oligonucleotide primers were utilized in reverse transcriptase-polymerase chain reaction (PCR) experiments. Primer sequences were, sense: 5'-CATCGAGTGTCTACGCTTC-3'; antisense: 5'-TTATGACCTGTGTCTCTTCT-3'. Primers were applied to cDNA that was generated from poly(A)⁺ RNA from different populations of endothelial cells, with the exception of lane 9. In lane 9 primers were applied to DNA as a positive control. A product of expected size (≈ 420 bp) was detected only in lanes 9 and 10. Endothelial cell populations are described as follows:

Lane 1: PCR ladder;

Lane 2: Human pulmonary microvascular endothelial cells plus reverse transcriptase;

Lane 3: Human pulmonary microvascular endothelial cells minus reverse transcriptase;

Lane 4: Bovine microvascular endothelial cells plus reverse transcriptase;

Lane 5: Bovine microvascular endothelial cells minus reverse transcriptase;

Lane 6: Bovine microvascular endothelial cells plus reverse transcriptase;

Lane 7: Bovine microvascular endothelial cells minus reverse transcriptase;

Lane 8: Bovine microvascular endothelial cells plus reverse transcriptase;

Lane 9: Rat microvascular endothelial cell DNA;

Lane 10: Rat microvascular endothelial cells plus reverse transcriptase;

Lane 11: Rat microvascular endothelial cells minus reverse transcriptase;

Lane 12: Bovine pulmonary artery endothelial cells plus reverse transcriptase. Nos. on left are molecular weight.

Ca^{2+} -inhibitable AC expression *in vivo*.

Although Ca^{2+} -inhibitable AC is expressed in cells in culture, isolation and culture, *per se*, may induce phenotypic changes in the population of endothelia. Thus we determined whether Ca^{2+} -inhibitable AC was expressed in pulmonary endothelium *in vivo* using established immunohistochemical techniques.²⁵ A polyclonal antibody capable of discriminating Ca^{2+} -inhibited isoforms from other isoforms of AC was

utilized. Immunoreactivity was detected in endothelia throughout the pulmonary vasculature, including large arteries, arterioles, and capillaries (Picture 9 on page 148). Immunoreactivity was eliminated by co-incubation of the Ca²⁺-inhibitable AC antibody with a blocking peptide, demonstrating antibody specificity to type V/VI AC. Especially important is the detection of Ca²⁺-inhibited AC(s) in capillary endothelial cells, at the site where disruption contributes to development of permeability and/or edema in non-cardiogenic pulmonary edema(s). It is also apparent that immunoreactivity was not specific for endothelia in the lung. Type V/VI AC appears to be expressed in all lung vascular cells (Picture 9 on page 148).

Thapsigargin-induced microvascular dysfunction.

Ca²⁺ inhibition of AC and cAMP content promotes certain features of endothelial cell permeability *in vitro* models. However, evidence supporting a role for Ca²⁺ inhibition of cAMP in regulation of microvascular dysfunction in the intact circulation was lacking. We used an isolated perfused rat lung to evaluate the role of Ca²⁺ inhibition of cAMP content in microvascular dysfunction, where K_{fc} is used to estimate permeability.¹² Baseline K_{fc} was $0.303 \pm 0.054 \text{ ml} \cdot \text{min}^{-1} \cdot \text{mmHg}^{-1} \cdot 10 \text{ g lung tissue}^{-1}$ and did not change over the time course tested [$n=3$; p =not significant (NS); Fig. 2]. The plant alkaloid thapsigargin induces capacitative Ca²⁺ entry in cells *in vitro*, which is an efficient mechanism for inhibition of AC and cAMP content (see below). Thus, thapsigargin was applied to rat lungs over a range of doses, from 1 nM to 1 μ M. The threshold concentration for an effect of thapsigargin was above 1 nM (data not shown). However, 100 nM thapsigargin caused a reproducible increase in K_{fc} that at its peak was $\approx 400\%$ greater than baseline values ($n = 3$; $p < 0.05$; Fig. 2). This dramatic increase in permeability occurred with a small increase in capillary pressure ($p < 0.05$), suggesting that increased microvascular permeability and not hydrostatic edema was the cause. Hemodynamic data are presented in Table 1.

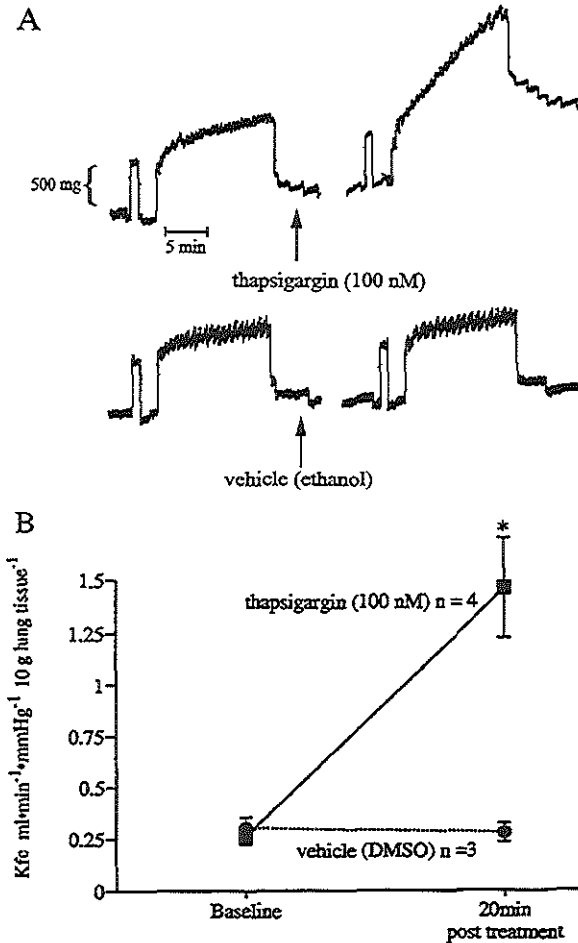


Figure 2. Thapsigargin induces microvascular permeability in an isolated perfused rat lung. Following medial sternotomy, lung isolation and establishment of a stable baseline, baseline capillary filtration coefficient (K_{fc}) was assessed by elevating venous pressure and measuring weight gain over a 15-min time course as described.¹² A: Whereas in vehicle control experiments repeated measurements of K_{fc} did not change over time, application of thapsigargin induced a large increase in K_{fc} as shown in this representative sample. B: Summary data show thapsigargin increased K_{fc} \approx 435% 20 min after application. DMSO, dimethyl sulfoxide *significantly different from the corresponding Control value at $p < 0.05$

Table 1. Effect of thapsigargin on hemodynamic variables in an isolated salt perfused rat lung.

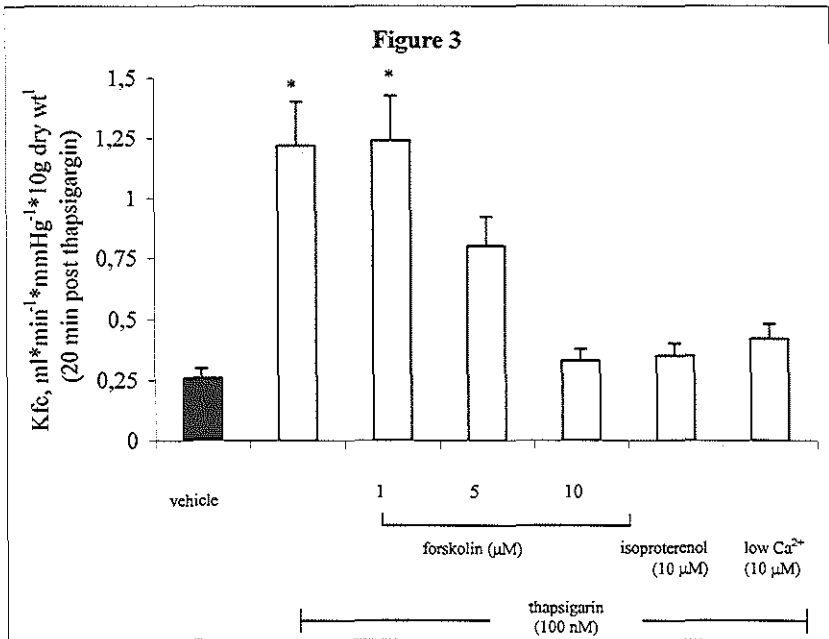
| | Control | | | Thapsigargin (100 nM) | | |
|----------|----------------|----------------|----------------|-----------------------|----------------|----------------|
| | P _a | P _v | P _c | P _a | P _v | P _c |
| Baseline | 7.9±0.09 | 3.6±0.07 | 4.5±0.14 | 7.7±0.36 | 3.6±0.06 | 4.3±0.26 |
| 20 min | 7.7±0.12 | 3.4±0.06 | 4.3±0.30 | 12.5±0.79* | 3.4±0.12 | 6.6±0.81* |

Values are means ± SE; n=3 lungs for control and thapsigargin studies. Rat lungs were isolated and perfused as described in Methods. Pulmonary artery (P_a) and venous (P_v) pressures were measured by a pulmonary artery and venous catheter coupled to independent force transducers. Pulmonary capillary (P_c) pressures were estimated by the double-occlusion technique. Thapsigargin increased P_a and slightly increased P_c pressure in the isolated rat lung. No change in P_v pressure was observed. * Significantly different from baseline value at p<0.05. cAMP and thapsigargin-induced microvascular dysfunction.

We next tested whether a change in endothelial cell cAMP was a prominent feature of the thapsigargin-induced microvascular dysfunction. To address this issue, inhibition of cAMP by thapsigargin was counterbalanced in three different ways. Previous reports suggested that activation of the cAMP pathway conferred protection against development of endothelial cell permeability¹. Thus, either the direct AC activator forskolin or the β-agonist isoproterenol were applied to lungs before application of thapsigargin; both treatments nearly eliminated the thapsigargin-induced increase in K_{fc} (n=3/group; p=NS vs. vehicle control; Fig. 3).

Reduced Ca²⁺ entry also prevents development of permeability in other experimental models, perhaps because Ca²⁺ inhibition of AC requires Ca²⁺ entry. We therefore tested whether reduction of perfusate Ca²⁺ would reduce the thapsigargin-induced increase in K_{fc}. However, these studies were complicated by the influence of low extracellular Ca²⁺ alone on permeability; as previously demonstrated,²⁸ lowering of extracellular free Ca²⁺ increases K_{fc} likely through disruption of intercellular junctional complexes, an observation we confirmed presently (data not shown). Yet, we found that reducing extracellular Ca²⁺ to 10 μM yielded a sufficiently high free concentration to preserve endothelial barrier function and

maintain a low K_{fc} over the time course tested ($n=3$; $p=NS$ over time). Indeed, with over 2 h of perfusion with 10 μM extracellular Ca^{2+} , K_{fc} changed from 0.343 ± 0.032 to 0.465 ± 0.019 $ml \cdot min^{-1} \cdot mmHg^{-1} \cdot 10$ g lung tissue $^{-1}$, whereas with over 2 h of perfusion with 2 mM extracellular Ca^{2+} , K_{fc} changed from 0.313 ± 0.078 to 0.363 ± 0.055 $ml \cdot min^{-1} \cdot mmHg^{-1} \cdot 10$ g lung tissue $^{-1}$. With 10 μM extracellular Ca^{2+} , the thapsigargin-induced increase in K_{fc} was greatly attenuated ($n=3$; $p=NS$ vs. time control in 10 μM extracellular Ca^{2+}).



*Figure 3. Thapsigargin-induced increases in K_{fc} require inhibition of cAMP. After baseline K_{fc} was assessed, interventions which prohibit a decrease in cAMP, e.g., the direct AC activator forskolin, β -agonist isoproterenol, and low extracellular Ca^{2+} - were applied to the perfusate, allowed to circulate for 20 min, and K_{fc} measured (data not shown). These treatments did not change baseline K_{fc} . Thapsigargin was then applied to the perfusate, and K_{fc} was measured a third time. Whereas thapsigargin alone increased K_{fc} , forskolin, isoproterenol and low extracellular (e) Ca^{2+} nearly prevented the response to thapsigargin. *significantly different from vehicle control; $p < 0.05$. $n = 3-4$ /group.*

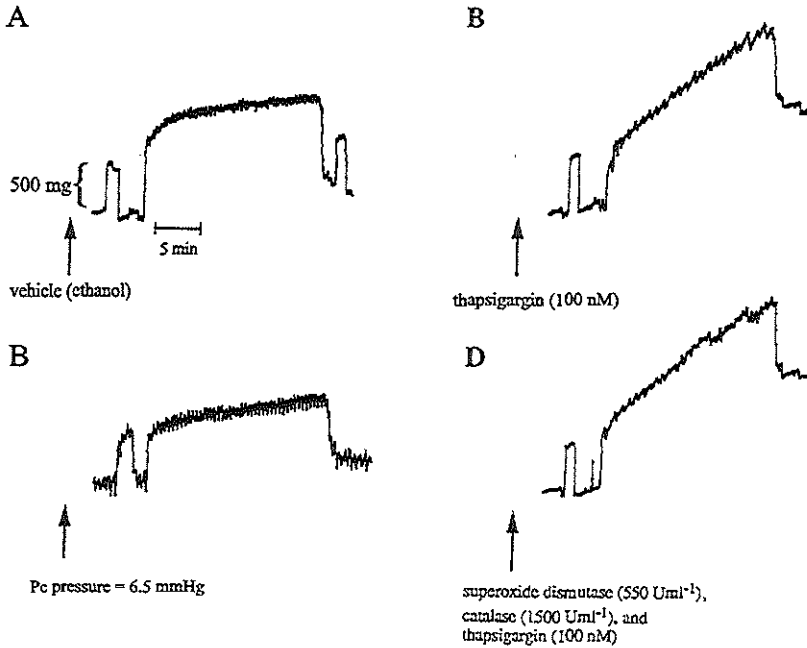


Figure 4. Thapsigargin-induced increases in K_{fc} were not due to increased capillary pressure (P_c) or oxidant production. To investigate alternative mechanisms of thapsigargin-induced increases in K_{fc} we considered whether increased P_c [C] or release of oxidants from resident neutrophils [D] was sufficient to increase K_{fc} . Representative tracings are shown. A: K_{fc} did not increase over time. B: Thapsigargin increased K_{fc} 20 min after application to the perfusate. C: Increases in P_c to ≈ 6.5 mm Hg to mimic the effect of thapsigargin were insufficient to increase K_{fc} . D: Superoxide dismutase and catalase did not prevent the thapsigargin-induced increase in K_{fc} .

P_c, oxidants, and thapsigargin-induced microvascular dysfunction.

Because thapsigargin increased P_a and P_c , we questioned whether a similar rise in hydrostatic pressure could account for the increase in K_{fc} observed after thapsigargin. To mimic the influence of thapsigargin, P_v was increased to achieve a $P_c \approx 6.5$ mm Hg. However, K_{fc} was not influenced by increased P_v ($n=1$), suggesting that increased P_c alone was not the cause of increased

K_{fc} in response to thapsigargin. The results of the experiment are shown in Fig. 4.

Thapsigargin likely does not solely influence endothelial cell function in the intact lung. Other resident lung cells which regulate endothelial barrier properties, e.g. neutrophils, may be influenced by thapsigargin.³⁷ For example, thapsigargin may induce resident neutrophils, which are present in buffer-perfused lungs, to release oxidants. Because oxidants could increase K_{fc} , we performed additional studies to determine whether the effect of thapsigargin on K_{fc} was due to increased oxidant production. Thus superoxide dismutase (550 U/ml) and catalase (1500 U/ml) were added to the perfusate before addition of thapsigargin. Inhibition of superoxide and hydroxyl radical production by superoxide dismutase and catalase did not influence the thapsigargin-induced increase in K_{fc} ($n=2$), suggesting increased oxidant production was not the cause of increased K_{fc} in response to thapsigargin. The results of a representative experiment are shown in Fig. 4.

6.5 Discussion

Development of non-cardiogenic pulmonary edema involves disruption of the microvascular endothelial cell barrier. Despite this, very little is known regarding mechanisms of microvascular endothelial cell disruption. Our recent demonstration that a Ca^{2+} -inhibitable AC establishes an inverse relationship between $[Ca^{2+}]_i$ and cAMP in bovine macrovascular endothelial cells provided a possible mechanism of development of permeability *in vivo*.⁴² However, these data utilized an *in vitro* model remote from the intact pulmonary circulation. The present studies therefore tested the hypothesis that Ca^{2+} -inhibitable AC establishes an inverse relationship between $[Ca^{2+}]_i$ and cAMP and promotes microvascular permeability in the intact pulmonary circulation.

The novel findings from the present studies are:

1. a Ca²⁺-inhibitable AC is expressed in microvascular endothelial cells *in vitro* and *in vivo*,
2. thapsigargin induces pulmonary microvascular permeability, and
3. strategies aimed at eliminating Ca²⁺ inhibition of cAMP, e.g. direct and indirect AC activation and low extracellular Ca²⁺, prevent thapsigargin-induced pulmonary microvascular permeability.

Our first series of experiments tested expression of type VI Ca²⁺-inhibitable AC in rat pulmonary microvascular endothelial cells²³ *in vitro*. We amplified RNA using degenerate oligonucleotide primers directed against the C_{2A} region of AC and sequenced the products. Sequence analysis confirmed expression of type VI (Ca²⁺-inhibited) AC.²³ The data suggest a role for Ca²⁺-inhibitable AC in regulating the balance between [Ca²⁺]_i and cAMP content [and permeability as our physiological data suggest (see below)] in endothelial cells derived from the rat pulmonary microvasculature.

In addition to a Ca²⁺-inhibitable isoform, we identified expression of type III AC.²³ This observation was unexpected, since in our previous studies using bovine,^{41,42} and human (unpublished observation) endothelial cells, we found no evidence for expression of this isoform. To validate our present observation, we performed additional reverse transcriptase-PCR experiments using type III isozyme-specific primers in different endothelial cell populations. Interestingly, expression of type III AC was only observed in rat microvascular endothelial cells and not in bovine or human macro- or microvascular endothelial cells. The reason for the difference in expression between cell types is unknown, although either different cell culture conditions or species variability are possible explanations.

It is presently unknown whether the type III enzyme contributes to Ca^{2+} -dependent regulation of AC in endothelia; however, previous reports have demonstrated Ca^{2+} sensitivity of the type III enzyme.^{7,13,46-48}

It was suggested first that type III AC was stimulated by Ca^{2+} in olfactory cells. These studies were performed using cell membrane fractions (not whole cells) though, and stimulation of AC by Ca^{2+} required concomitant activation of G_s .⁷ Subsequent reports have shown that, in intact cells, type III AC is inhibited by Ca^{2+} .⁽⁴⁶⁻⁴⁸⁾ Conflicting data by Fagan et al.¹³ have now shown that type III AC exhibits no Ca^{2+} sensitivity (physiologically relevant or otherwise) when transfected in HEK 293 cells. Thus the Ca^{2+} sensitivity of type III AC appears to vary in different cell preparations. Although we must consider the possibility that type III along with type VI AC contributed to Ca^{2+} inhibition of cAMP content in rat microvascular endothelial cells (see below), identification of the physiological role of the type III enzyme in endothelial cells awaits future studies.

Direct application of data from cell cultures to the intact circulation is not well justified, since cells may undergo phenotypic changes in the process of isolation and culture. Our second series of experiments therefore tested expression of Ca^{2+} -inhibitable AC *in vivo* by immuno-histochemistry. Immunostains of lung sections demonstrated expression of Ca^{2+} -inhibitable AC throughout the pulmonary vasculature. Prominent expression was observed in endothelial cells in pulmonary arteries, arterioles, and in capillaries. These data are consistent with our *in vitro* results showing expression in endothelial cells from large vessels and the microvasculature, and suggest in regard to expression of this enzyme that early passage endothelial cells retain their *in vivo* phenotype. Most importantly, these data clearly demonstrate expression of Ca^{2+} -inhibitable AC at the relevant site of edema formation *in vivo*.

Our third series of experiments therefore tested the role of Ca^{2+} -inhibitable AC in lung microvascular permeability. We utilized the plant alkaloid thapsigargin as a permeability-evoking agonist. This report demonstrates

that thapsigargin induces lung microvascular permeability, estimated by K_{fc} . Thapsigargin-induced permeability was not due to increased hydrostatic forces from venoconstriction because P_c pressures increased only slightly. Increased permeability was also not due to uneven perfusion, since the experiments were conducted under zone 3 conditions, where pressures are $P_a > P_v >$ alveolar. Furthermore, increased permeability was not due to gross injury, since the increase was only transient; by 50 min, K_{fc} had nearly returned to baseline levels (data not shown). Thapsigargin, therefore causes microvascular endothelial cell disruption that accounts for increased permeability.

Our fourth series of experiments evaluated the mechanism through which thapsigargin caused endothelial cell disruption that increased microvascular permeability. Thapsigargin increases $[Ca^{2+}]_i$ by inhibiting microsomal Ca^{2+} reuptake and inducing capacitative Ca^{2+} entry.⁴⁵ It seems likely that, since capacitative Ca^{2+} entry can prominently inhibit the type VI cyclase in other cell systems,⁸ thapsigargin induces large decreases ($\approx 50\%$) in endothelial cell cAMP content by a similar mechanism (unpublished observation). Both increased $[Ca^{2+}]_i$ and decreased cAMP promote permeability through interdependent and independent mechanisms. We counterbalanced the decrease in cAMP in three ways to investigate participation of the cAMP pathway in permeability induced by thapsigargin. We first applied isoproterenol, since β -adrenergic stimulation has previously been shown to prevent and reverse permeability induced by a variety of inflammatory stimuli in both experimental and clinical settings.^{1,2,5,14,20,24,37,38,40} Isoproterenol nearly eliminated the increase in permeability induced by thapsigargin. We next applied the direct AC activator forskolin over a range of doses. Forskolin also nearly eliminated the increase in permeability induced by thapsigargin. These data suggest a decrease in cAMP (and not just an increase in $[Ca^{2+}]_i$) is critical to development of permeability, since in these experiments the increase in $[Ca^{2+}]_i$ was not prevented.

Inhibition of AC by Ca^{2+} in intact cells requires Ca^{2+} entry.^{6,8} We also used low perfusate Ca^{2+} to limit Ca^{2+} entry and thus limit inhibition of cAMP by thapsigargin. This study was complicated, since nominally Ca^{2+} free perfusate itself increases K_{fc} by disruption of Ca^{2+} -dependent cadherins.²⁸ We therefore reduced extracellular Ca^{2+} to a free concentration of 10 μM which, without stimulation, was sufficiently high to preserve endothelial barrier function and maintain a low K_{fc} over the time course tested (data not shown). This suggests that 10 μM extracellular Ca^{2+} was sufficient to maintain cadherin function, while limiting Ca^{2+} entry; e.g. the inward Ca^{2+} gradient is usually $\approx 10,000$ -fold (1-2 mM extracellular vs. 100 nM intracellular), whereas with 10 μM extracellular Ca^{2+} the inward Ca^{2+} gradient is ≈ 100 -fold (10 μM extracellular vs. 100 nM intracellular). Thapsigargin did not induce microvascular permeability in the presence of 10 μM extracellular Ca^{2+} . We interpret these data as support for our hypothesis that elimination of decreased cAMP prevents development of permeability. However, the protective effects of 10 μM extracellular Ca^{2+} may also have been due to a decrease in endothelial cell $[\text{Ca}^{2+}]_i$.

In our final series of experiments, we considered alternative explanations for the mechanism of thapsigargin-induced microvascular permeability. Enhanced Ca^{2+} entry stimulates generation of neutrophil-derived oxidants that induce microvascular permeability. Resident neutrophils are present in the lung, even when perfused with a salt solution, and these resident neutrophils have been shown to be sufficient in number to induce oxidant-mediated lung injury.³⁷ We therefore tested whether thapsigargin-induced oxidants could account for increased permeability. In these experiments, superoxide dismutase and catalase were added to the perfusate before thapsigargin. However, scavenging of superoxide and hydroxyl radicals by superoxide dismutase did not influence permeability induced by thapsigargin. These data therefore suggest that thapsigargin-induced oxidants do not account for the observed increase in permeability, further supporting the notion that decreased endothelial cell cAMP is the critical mechanism.

An important consideration is whether treatments which prevented the thapsigargin-induced increase in K_{fc} (especially forskolin and isoproterenol) increased cAMP rather than eliminated a decrease in cAMP. Our data suggest that, when $[Ca^{2+}]_i$ is elevated, the influence of forskolin or isoproterenol on stimulation of AC would be minimized, due to Ca^{2+} inhibition of AC.³⁸⁻⁴⁰ Indeed, data in support of this hypothesis have been presented previously.⁴³ In these studies, application of hydrogen peroxide to endothelial cell cultures increased $[Ca^{2+}]_i$ and decreased cAMP by as much as 76%. Cholera toxin and forskolin each increased cAMP; however, in the presence of hydrogen peroxide the increased levels of cAMP dropped rapidly, consistent with the idea that increased $[Ca^{2+}]_i$ inhibited AC activity. The data suggest that, when AC activity is inhibited by Ca^{2+} , direct or receptor-mediated stimulation of AC is reduced. Taken together with our current data, Ca^{2+} inhibition of AC and cAMP represents an important step in development of pulmonary microvascular permeability.

Although the present studies implicate a role for decreased cAMP in mediating permeability induced by thapsigargin, precise molecular targets remain only speculative. Cyclic AMP likely influences permeability through regulation of protein kinase A, which in turn influence myosin light chain phosphorylation status, contraction, and cytoskeletal reorganization (^{36,40} and unpublished observation). Future studies will be required to determine the important cellular sites of action that mediate the cell's response to changes in cAMP content. It is also noteworthy that one model of endothelial cell permeability involves barrier disruption via mechanisms involving ADP-ribosylation.³⁰ Future studies will be needed to discriminate between relevant mechanisms of barrier disruption in different models of permeability.

Our current studies have demonstrated expression of Ca^{2+} -inhibitable AC in rat pulmonary microvascular endothelial cells *in vitro* and *in vivo*. We have demonstrated that thapsigargin induces microvascular permeability in the intact pulmonary circulation, and that increased permeability is partly due

to decreased cAMP. These data suggest that Ca^{2+} inhibition of AC and cAMP represents a critical step in the genesis of pulmonary edema. Direct linkage of this system to non-cardiogenic pulmonary edema is still missing. However, our data along with experimental and clinical reports that administration of cAMP agonists reduce permeability^{1,2,5,14,20,24,37,38,40} partly suggest a central paradigm in which edema results from decreased endothelial cell cAMP. Finally, these data suggest that therapies seeking to uncouple increases in endothelial cell $[\text{Ca}^{2+}]_i$ from decreases in cAMP may represent a novel strategy for prevention or treatment of non-cardiogenic pulmonary edema(s).

6.6 Acknowledgements

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7

GENERAL DISCUSSION

7.1 General discussion

Both morphology and function of the developing pulmonary vasculature have been widely researched.^{5,10} In the neonatal period, new vessels form while the existing vasculature undergoes adaptation.² A recent explosion in newly discovered vascular growth factors (e.g. VEGF) has coincided with increased knowledge of signal transduction pathways involved in pulmonary vascular development.²⁶ These new findings demand re-evaluation of therapeutic efforts in pathological settings, against the background of factors regulating blood vessel growth under normal circumstances. The clinical syndrome of persistent pulmonary hypertension of the newborn (PPHN) presents in approximately one in 1000 newborns.¹⁶ Although new treatment modalities, such as nitric oxide (NO) inhalation, intra-tracheally administered prostacyclin (PGI₂), high frequency oscillation and extracorporeal membrane oxygenation (ECMO), have decreased its morbidity and mortality, 20-50% of infants diagnosed with PPHN are still likely to die¹. PPHN thus continues to be a major problem in neonatal and pediatric surgical intensive care units worldwide. Infants dying with pulmonary hypertension exhibit structural abnormalities of the pulmonary vessels, including thickening of media and adventitia and extension of smooth muscle into normally non-muscularized vessels.¹²

Although it seems difficult to understand the direct relationship between morphological abnormalities and the deviant vascular reactivity so often observed in these patients, the changes in vascular wall architecture are particularly striking²³ when compared with vascular wall alterations in flow derived forms of pulmonary hypertension such as the plexogenic arteriopathies associated with congenital cardiac defects³ and adult forms of pulmonary hypertension.^{11,19} The studies described in this thesis were undertaken to improve our knowledge of normal and abnormal pulmonary vascular development. Once we understand the cellular mechanisms that drive growth and differentiation of cells residing in the pulmonary vascular wall, we could perhaps develop new treatment modalities acting specifically on the abnormally developed pulmonary vasculature.

It is hoped that these would also affect the excessive pulmonary vascular remodeling in response to lung injury seen in critically ill newborns, especially those born prematurely or those with underdeveloped lungs, as seen in congenital diaphragmatic hernia (CDH).

Research groups worldwide have greatly contributed to our knowledge of the pulmonary vascular wall,²⁴ but their *in vitro* approaches, although helpful in studying cellular events such as replication, changes in phenotype and growth responses, do no justice to the intimate relationship between medial smooth muscle cells and extracellular matrix (ECM) observed under normal circumstances. In this context, changes in expression of several proteins (e.g. tropoelastin)⁹ during normal and hypoxic conditions in initially normally developed pulmonary arteries were revealed with immunohistochemistry and *in situ* hybridization techniques. Cellular changes in animal models or human cases with abnormally developed pulmonary vascular bed prenatally have hardly been documented, except for VEGF expression described in the CDH rat (Nitrofen) model and in humans.²² Even basic topics, such as replication/apoptosis ratios, relating to processes of great importance in (ab)normal growth and remodeling, have not been studied in detail in abnormally developed pulmonary circulations.

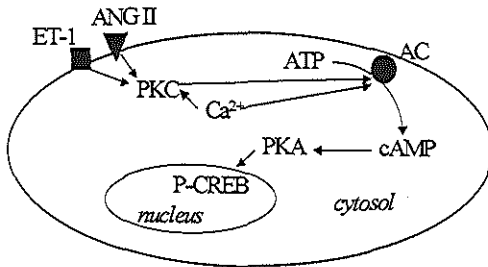


Figure 1. Signal transduction pathways in a smooth muscle cell. Arrow demonstrates activation of protein
 ET-1: Endothelin-I; ANG II: Angiotensin II; AC: Adenyl Cyclase;
 ATP: Adenosine Triphosphate; cAMP: cyclic AMP; PKC: Protein kinase C;
 PKA: Protein kinase A; P-CREB: Phosphorylated cyclic AMP Response Element Binding Protein; Ca²⁺: intracellular calcium

Researchers have realized that in order to increase understanding of abnormal vascular responses, they need to gain more knowledge of biochemical pathways within the cell that will definitely reveal the complex processes implicated in pulmonary vascular remodelling and growth.

The overall aims of the described studies were as follows:

1. To investigate morphological, functional, and cell-biological characteristics of both the normally and abnormally developing pulmonary vasculature; and more specifically:
2. To describe pulmonary vascular distribution of adenylyl cyclase isoforms;
3. To explore mechanisms of cyclic AMP mediated growth of pulmonary artery smooth muscle cells;
4. To investigate the role of the transcription factor CREB in the developing pulmonary vasculature; and
5. To evaluate pulmonary microvascular permeability.

Animal models of chronic hypoxic pulmonary hypertension and CDH in the newborn have been used to investigate the changes of the neonatal circulation in reaction to certain stimuli, such as hypoxia, hyperoxia, and artificial ventilation. However, although the morphological findings in these models resemble the findings in human newborns with PPHN and/or CDH, the models used show major limitations, which make it difficult to understand the exact mechanisms of growth and development in the "natural" environment. The mechanisms that control vascular cell proliferation during lung development *in vivo* are not precisely known, but *in vitro* studies of pulmonary vascular cells provide circumstantial evidence suggesting that activation of second messenger pathways may underlie particular growth patterns at various developmental stages.^{6,15}

Adenylyl cyclases (AC) catalyze the conversion of ATP to cyclic AMP (cAMP). Control of the pulmonary circulation by AC has been well documented in clinical and experimental settings.²⁵ In recent years a

substantial diversity of enzyme isoforms has been described, each with different sensitivities to G_s and G_i proteins and with unique regulatory properties.¹⁴ Such diversity of enzymes suggests that cells govern function by regulating the complex of AC they express. While it is broadly recognized that tissue distributions of AC isoforms differ, the cell-specific expression of AC isoforms has not been clearly defined in any organ. The diverse functions required of blood vessels include regulation of permeability and vascular tone. In this context, in chapter 3, cell-specific expression of AC in bovine lung vasculature was examined. Pulmonary artery and vein endothelial cells expressed AC types II-IX, though the protein kinase C (PKC)-stimulated (type II) enzyme exhibited the greatest immunoreactivity. This expression pattern was not conserved in capillaries, where alveolar endothelial cells did not express ACII or VI but rather most abundantly expressed the calcium (type VIII)- and calcineurin (type IX)-stimulated isoforms. The medial layer of pulmonary arteries demonstrated diffuse expression of AC II, III, and V/VI and focal expression of types IV, VII, VIII and IX, whereas the adventitial layer expressed only AC IV and VII. Heterogeneity of AC expression decreased in the media of smaller vessels, suggesting that more distal vessel segments display homogeneity of smooth muscle cell phenotypes.

We, therefore, concluded that:

1. The AC expression pattern differs between and among cell types in the pulmonary circulation; and
2. This expression pattern may fundamentally regulate the cell's response to its environment.

Expression of AC type II was predominantly seen in the main pulmonary artery. AC type II is a PKC-stimulated isoform. PKC has been demonstrated to be important in proliferation. Its precise role in developmental and hypoxia-induced changes in growth of pulmonary vascular cells has been subject of various research.^{4,7,8} Ca^{2+} influx has shown to be an important early signal transduction mechanism in SMC responses to hypoxia. Altered oxygen sensitivity within the vascular wall in

the fetal situation may, therefore, determine possible differences in expression of AC type VIII and IX in capillaries and veins.

Cyclic AMP is a second messenger that has been demonstrated to have a controversial role in proliferation.^{17,18,21} Because intracellular signals mediating enhanced growth responses are poorly understood, we investigated whether in neonatal pulmonary artery (PA) SMCs, cAMP is pro-proliferative and PKC activity, previously shown to be elevated in these cells, increases cAMP content via activation of AC. As described in chapter 4, our findings indicate that cAMP agonists increase proliferation in neonatal but not in adult PASMC. Furthermore, endogenous PKC activators Endothelin-1 (ET-1) and Angiotensin-II (ANG II) increased both cAMP content and proliferation in neonatal but not in adult PASMC. Inhibition of PKC and cAMP-dependent protein kinase nearly eliminated the ET-1 and ANG II induced growth of neonatal PASMC. A PKC stimulated isoform of AC (type II) was expressed *in vitro* and *in vivo*, providing a putative mechanism through which PKC activators may increase cAMP content. Thus, this study suggested that PASMC growth is developmentally regulated via PKC stimulated AC and increased cAMP (Figure 1).

Prostacyclin (PGI₂) exerts its vasodilating effect through increasing cAMP levels. Although often a successful therapy in older children and adults with primary pulmonary hypertension, its efficacy in newborns with PPHN is disappointing and unpredictable. Thus, it is suggestive that the effect of PGI₂ on targeting AC isoforms may be developmentally regulated.

The design of the study is also interesting, notably for its application of factors involved in vasoconstriction. Furthermore, it is relevant against the background of not yet fully revealed ET-I pathway and the repeated clinical observations of unpredictable responses to NO in CDH and PPHN. Data on eNOS/iNOS in experimental settings and after ECMO in humans are available, and findings on ET-1 in CDH and circulating ET-1 in newborns with PPHN, have shown enhanced levels.²⁰ On protein level no

data are available, but positive results of ET-A receptor blockade have been seen in PPHN.¹³ Our data are valuable in that they could serve as baseline data for the comparison of adult and neonatal responses. More experiments need to be done to answer questions on:

- Hypoxic conditions in normal cells;
- Cells derived from hypoxic animals with already different function and thus possible different responses to pathological stimuli.

Chapter 5 describes how we tested the hypothesis stating that elevated levels of cAMP will activate protein kinase A (PKA), which influences gene transcription through phosphorylation of cAMP response element binding protein (CREB). CREB has been identified as a key transcription factor in growth and differentiation of various cells. PKA activity was present in neonatal but not in adult PASMC. Furthermore, serum stimulated growth of both neonatal and adult PASMC resulted in phosphorylation of CREB. In contrast to detectable phospho-CREB (P-CREB) in serum-deprived neonatal PASMC, no (P-) CREB was noticed in quiescent adult cells. Using immunohistochemistry we located (P-)CREB throughout the pulmonary vasculature within endothelial and medial SMC. Furthermore, neonatal lungs showed enhanced expression of (phospho-) CREB, compared with adult lungs. While immunolocalisation for CREB was mainly in the cytosol, staining for phospho-CREB appeared nuclear, suggesting translocation (Figure 1). We concluded that cAMP mediated activation of CREB might well contribute to enhanced growth of neonatal PASMC.

Although we investigated differences in growth between neonatal and adult cells, the sensitive response of the neonatal pulmonary vascular bed cannot be interpreted only as a result of ongoing proliferation or a defective balance between cell replication and apoptosis. Morphology, as described earlier on, revealed increased medial SMC in cases of CDH and/or pulmonary hypertension. The question remains whether this is a result of abnormal proliferation or diminished apoptosis. Both are developmentally

regulated. This is especially intriguing, knowing that, the circumstances in CDH are far more complicated than those in PPHN in normally developed lungs. In CDH, the lungs are severely hypoplastic. This makes us wonder whether the degree of underdevelopment of the pulmonary vasculature compares with that of parenchymal underdevelopment or not. Questions like these can only be answered through comparative analysis of cells derived from normal pre-natally harvested lungs and from different forms of hypoplastic lungs, such as the ones presenting in oligohydramnios and CDH. To our knowledge, no data are available on SMC harvested under these conditions. This calls for further studies in these cells, combined with BrDU labelling and apoptosis markers in normoxia and hypoxia.

Pulmonary edema is one of the initial features observed during artificial ventilation. Endothelial cell disruption is known to play a central role in the onset of this process. The intracellular mechanisms responsible for endothelial cell disruption are unknown, although either elevated cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$), or decreased cAMP promotes permeability. The recent identification that Ca^{2+} -inhibitable AC establishes an inverse relationship between $[\text{Ca}^{2+}]_i$ and cAMP in macrovascular endothelial cells provided a possible mechanism of development of permeability.²⁵ However, this finding derived from an *in vitro* model, which gave no evidence supporting:

1. Expression of Ca^{2+} -inhibitable AC in pulmonary microvascular endothelium; and
2. Ca^{2+} inhibition of AC and cAMP content as a paradigm for inflammatory mediator-induced permeability in the intact circulation.

In chapter 6 these issues were addressed in microvascular endothelial cells from rat lungs and in an isolated perfused rat lung preparation. We demonstrated expression of a Ca^{2+} -inhibitable AC in microvascular endothelial cells. Furthermore, the results suggested that Ca^{2+} inhibition of AC is necessary for development of microvascular permeability in the intact circulation. We concluded therefore, that Ca^{2+} inhibition of cAMP

represents a critical step in the genesis of microvascular permeability in the intact pulmonary circulation.

This study refers to one of the earliest and most critical events involved in lung injury. Although extensive research has focused on the expression of numerous cytokines, endothelial cell disruption is still considered the prime event under these circumstances. However, biochemical processes underlying and preceding cellular changes have not been studied in great detail. In contrast to previously described experiments, an organ perfusion model was used in order to maintain tissue integrity. Once the pathway is established under these conditions, it is possible to study vascular dynamics enhancing or diminishing microvascular permeability. For obvious reasons, hypoxia is considered the crucial trigger in situations which resemble the clinical setting.

We consider this study a first step in our understanding of microvascular responses in hypoxic and hyperoxic settings, as observed in daily clinical practice in the intensive care unit. Here, the spectrum of respiratory injury is diverse: idiopathic respiratory distress syndrome in the prematurely born, respiratory insufficiency associated with different forms of pulmonary hypoplasia, and acquired/adult respiratory distress syndrome (ARDS) resulting from submersion or massive aspiration.

From a developmental point of view and against the background of our own findings in chapter 3, 4, and 5, we need more detailed studies extending our observations to clinical settings. Reviewing the different models used to study the (developing) pulmonary vasculature, a major discrepancy is observed between the amount of *in vitro* work using cell cultures to understand "cell behaviour" and the biochemical processes underlying enhanced or continued cell division.

A new field of investigation, i.e. the response of the abnormally developed pulmonary vasculature under hypoxic circumstances, lies evidently open for future investigation.

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8

SUMMARY / SAMENVATTING

8.1 Summary

The clinical syndrome of persistent pulmonary hypertension (PPHN) presents approximately in one in 1000 newborns. Despite new treatment modalities, such as nitric oxide (NO) inhalation, intra-tracheally administered prostacyclin (PGI₂), high frequency oscillation and extracorporeal membrane oxygenation (ECMO), which have decreased its morbidity and mortality, 20-50% of infants diagnosed with PPHN are still likely to die. PPHN thus continues to be a major problem in neonatal and pediatric surgical intensive care units worldwide.

Infants dying with pulmonary hypertension exhibit structural abnormalities of the pulmonary vessels, including thickening of media and adventitia and extension of smooth muscle into normally non-muscularized vessels. Although it seems difficult to understand the direct relationship between morphological abnormalities and the deviant vascular reactivity so often observed in these patients, the changes in vascular wall architecture are particularly striking when compared with vascular wall alterations in flow derived forms of pulmonary hypertension such as the plexogenic arteriopathies associated with congenital cardiac defects and adult forms of pulmonary hypertension.

The objectives of this thesis were:

1. To investigate morphological, functional, and cell-biological characteristics of the developing pulmonary vasculature;
2. To explore mechanisms of developmental regulation of proliferation and growth in pulmonary vascular cells; and
3. To determine cell-cell interactions within the vascular wall.

Chapter 2 comprises an initial discussion of (animal models of) chronic hypoxic pulmonary hypertension and congenital diaphragmatic hernia in the newborn. These models have been used to investigate the changes of the neonatal circulation in reaction to certain stimuli, such as hypoxia, hyperoxia and artificial ventilation. However, although the morphological findings resemble those seen in human newborns with PPHN and/or CDH, the models

used show major limitations, which make it difficult to understand the exact mechanisms of growth and development in the "natural" environment.

The mechanisms that control vascular cell proliferation during lung development *in vivo* are not precisely known, but *in vitro* studies of pulmonary vascular cells provide circumstantial evidence suggesting that activation of second messenger pathways may underlie particular growth patterns at various developmental stages.

Adenylyl cyclases (AC) catalyze the conversion of ATP to cAMP. Although it is broadly recognized that tissue distributions of the various AC isoforms differ, however the cell-specific expression of AC isoforms has not been clearly defined in any organ. The diverse functions required of blood vessels include regulation of permeability and vascular tone. In this context, in chapter 3 the cell-specific expression of AC in bovine lung vasculature was examined. Pulmonary artery and vein endothelial cells expressed AC types II-IX, though the protein kinase C-stimulated (type II) enzyme exhibited the greatest immunoreactivity. This expression pattern was not conserved in capillaries, where alveolar endothelial cells did not express AC II or VI but rather most abundantly expressed the calcium (type VIII)- and calcineurin (type IX)-stimulated isoforms. The medial layer of pulmonary arteries demonstrated diffuse expression of AC II, III, and V/VI and focal expression of types IV, VII, VIII and IX, whereas the adventitial layer expressed only AC IV and VII. Heterogeneity of AC expression decreased in the media of smaller vessels, suggesting that homogeneity of smooth muscle cell phenotypes exists in more distal vessel segments. It was therefore concluded that:

1. The pattern of AC expression differs between and among cell types in the pulmonary circulation; and
2. This pattern of expression may fundamentally regulate the cell's response to its environment.

The enhanced growth capacity and increased growth responses to mitogenic stimuli of neonatal vascular cells were investigated in chapter 4;

bovine neonatal pulmonary artery smooth muscle cells (PASMC) were compared with adult PASMC.

Cyclic AMP is a second messenger that has been demonstrated to have a controversial role in proliferation. Since intracellular signals mediating enhanced growth responses are poorly understood, we questioned whether in neonatal PASMCs, cAMP is pro-proliferative and whether protein kinase C (PKC) activity, previously shown to be elevated in these cells, increases cAMP content via activation of AC. The findings indicated that cAMP agonists increase proliferation in neonatal but not adult PASMC. Furthermore, the endogenous PKC activators endothelin-1 and angiotensin II increased both cAMP content and proliferation in neonatal but not in adult PASMC. Inhibition of PKC and protein kinase A (PKA) nearly eliminated the endothelin-1 and angiotensin-II induced growth of neonatal PASMC. A PKC stimulated isoform of AC (type II) was expressed *in vitro* and *in vivo*, providing a putative mechanism through which PKC activators may increase cAMP content. Thus, this study suggested that PASMC growth is developmentally regulated via PKC stimulated AC and increased cAMP.

Chapter 5 describes how we tested the hypothesis stating that elevated levels of cAMP will activate PKA, which influences gene transcription through phosphorylation of cAMP response element binding protein (CREB). CREB has been identified as a key transcription factor in growth and differentiation of various cells. PKA activity was present in neonatal but not in adult PASMC. Furthermore, serum stimulated growth of both neonatal and adult PASMC resulted in phosphorylation of CREB. In contrast to detectable phospho-CREB (P-CREB) in serum-deprived neonatal PASMC, no (P-) CREB was noticed in quiescent adult cells. Using immunohistochemistry we located (P-)CREB throughout the pulmonary vasculature within endothelial and medial SMC. Furthermore, neonatal lungs showed enhanced expression of (phospho-)CREB, compared with adult lungs. While immunolocalisation for CREB was mainly in the cytosol, staining for phospho-CREB appeared nuclear,

suggesting translocation. We concluded that cAMP mediated activation of CREB might well contribute to enhanced growth of neonatal PASMC.

Pulmonary edema is one of the initial features observed during artificial ventilation. Endothelial cell disruption is known to play a central role in the onset of this process. The intracellular mechanisms responsible for endothelial cell disruption are unknown, although either elevated cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$), or decreased cAMP promotes permeability. The recent identification that Ca^{2+} -inhibitable AC establishes an inverse relationship between $[\text{Ca}^{2+}]_i$ and cAMP in macrovascular endothelial cells provided a possible mechanism of development of permeability. However, this finding derived from an *in vitro* model; hence it gave no evidence supporting:

1. Expression of Ca^{2+} -inhibitable AC in pulmonary microvascular endothelium; and
2. Ca^{2+} -inhibition of AC and cAMP content as a paradigm for inflammatory mediator-induced permeability in the intact circulation.

In chapter 6 these issues were addressed in microvascular endothelial cells derived from rat lung and in an isolated perfused rat lung preparation. We demonstrated expression of a Ca^{2+} -inhibitable AC in microvascular endothelial cells. Furthermore, these results suggested that Ca^{2+} inhibition of AC is necessary for development of microvascular permeability in the intact circulation. We concluded, therefore, that Ca^{2+} inhibition of cAMP represents a critical step in the genesis of microvascular permeability in the intact pulmonary circulation.

The studies described in this thesis were undertaken to improve our knowledge of normal and abnormal pulmonary vascular development. Once we understand the cellular mechanisms that drive growth and differentiation of cells residing in the pulmonary vascular wall, we could perhaps develop new treatment modalities acting specifically on the excessive pulmonary vascular remodeling in response to lung injury seen in some critically ill newborns.

8.2 Samenvatting

Persisterende hypertensie bij de neonaat (PPHN) is een klinisch beeld dat bij ongeveer 1 op 1000 pasgeborenen voorkomt. Nieuwe therapievormen, zoals inhalatie van het gas stikstofoxide, intratracheaal toegediende prostacycline, hoogfrequente beademing en extracorporele membraanoxygenatie, hebben de morbiditeit en mortaliteit verminderd. Desondanks overlijden 20-50% van de kinderen bij wie PPHN is gediagnostiseerd. PPHN blijft dus wereldwijd een groot probleem op neonatale- en kinderchirurgische intensive care afdelingen.

Pasgeborenen overleden met een klinisch beeld van pulmonale hypertensie tonen structurele afwijkingen van de longvaten, bestaande uit een verdikte media en adventitia en de aanwezigheid van spierweefsel in de wand van kleine bloedvaten die normaliter geen spierweefsel bevatten. Het is moeilijk om de directe relatie tussen morfologische afwijkingen en de afwijkende reactiviteit van de bloedvaten, zoals vaak bij deze patiënten wordt waargenomen, te begrijpen. De afwijkingen in de vaatwandopbouw zijn echter zeer opvallend, zeker wanneer men die vergelijkt met veranderingen van de vaatwand bij andere vormen van pulmonale hypertensie, zoals de plexogene arteriopathie bij aangeboren hartafwijkingen en idiopathische pulmonale hypertensie bij volwassenen.

De doelstellingen in dit proefschrift zijn drieledig, namelijk:

1. Het onderzoeken van morfologische, functionele en celbiologische karakteristieken van de zich ontwikkelende longvaten;
2. Het verkennen van regulatiemechanismen van proliferatie en groei van de cellen die de bloedvaten van de long vormen;
3. Het typeren van cellulaire interacties in de vaatwand.

In hoofdstuk 2 worden diermodellen met chronisch hypoxische pulmonale hypertensie en congenitale hernia diafragmatica (CDH) besproken. Deze modellen worden gebruikt om veranderingen in de neonatale circulatie als reactie op verschillende stimuli, zoals hypoxie, hyperoxie en kunstmatige

beademing, te onderzoeken. Hoewel de morfologische bevindingen sterk lijken op de bevindingen bij pasgeborenen met PPHN en/of CDH, hebben deze diersmodellen grote beperkingen. Het is derhalve moeilijk om de exacte mechanismen van groei en ontwikkeling in de "natuurlijke" omgeving te begrijpen. De directe mechanismen, die de proliferatie van de cellen in de vaatwand van de long tijdens de ontwikkeling bepalen, zijn niet bekend. *In vitro* studies met deze cellen suggereren dat activatie van zogenaamde "second messenger-routes" aanleiding geeft tot specifieke groeipatronen tijdens de diverse ontwikkelingsstadia.

Het enzym adenylaatcyclase (AC) katalyseert de omzetting van ATP in de second messenger cyclisch AMP. In het algemeen wordt aangenomen dat de weefseldistributie van de iso-enzymen van AC onderling verschilt; de celspecifieke expressie van AC iso-enzymen in de verschillende organen is tot op heden niet in detail onderzocht. Bloedvaten hebben diverse functies, zoals regulatie van de doorlaatbaarheid en de vaattonus. In deze context wordt daarom in hoofdstuk 3 de celspecifieke expressie van AC in boviene longvaten beschreven. Endotheelcellen van de arteria- en vena pulmonalis laten expressie van het AC types II-IX zien. Type II, de door proteïne kinase C (PKC) gestimuleerde vorm, is het duidelijkst aanwezig. Dit expressiepatroon blijkt niet aanwezig in capillairen, want alveolaire endotheelcellen bevatten geen AC type II of VI, maar wel overduidelijk type VIII en type IX. Expressie van AC type II, III, V en VI werd in alle cellen van de media van de arteria pulmonalis waargenomen. Expressie van AC type IV, VII, VIII en IX werd in enkele cellen waargenomen; de adventitia daarentegen liet slechts expressie van type IV en VII zien. De heterogeniteit in adenylaatcyclase expressie was minder duidelijk in de media van de kleinere vaten, hetgeen suggereert dat in meer distaal gelegen vaten de gladde spiercellen grotere overeenkomst vertonen. Daaruit concludeerden wij:

1. Het patroon van adenylaatcyclase expressie verschilt in de diverse cellen van de longcirculatie, en
2. Dit expressiepatroon bepaalt hoe de cel op zijn omgeving reageert.

In hoofdstuk 4 worden de resultaten beschreven van studies naar het vermogen tot groei en de groei als reactie op mitogene stimuli van neonatale vaatwandcellen. Gladde spiercellen van de arteria pulmonalis (PASMC) van pasgeboren kalveren werden vergeleken met dezelfde cellen van volwassen dieren.

Cyclisch AMP (cAMP) heeft een controversiële rol met betrekking tot celgroei. De intracellulaire signalen die de groei bepalen zijn slechts gedeeltelijk bekend. Wij onderzochten of cAMP in gladde spiercellen van de arteria pulmonalis van pasgeboren kalveren groei bevordert en of de activiteit van PKC, welke hoger is in deze cellen, cAMP stimuleert na activatie van AC. Wij vonden dat cAMP-agonisten proliferatie stimuleren in de neonatale cellen, maar niet in de volwassen cellen. Endotheline I (ET-I) en Angiotensine II (ANG II) stimuleerden de cAMP concentratie in en groei van neonatale cellen, maar niet van volwassen cellen. Remming van PKC en proteïne kinase A (PKA) schakelde de door ET-I en ANG II geïnduceerde groei van neonatale PASMC nagenoeg uit. Een door PKC gestimuleerd iso-enzym van AC (type II) werd zowel *in vitro* als *in vivo* in PASMC aangetoond. Daarmee wordt aannemelijk dat activatie van PKC de hoeveelheid cAMP in de cel verhoogt. Deze studie suggereert dat de groei van gladde spiercellen in de arteria pulmonalis tijdens de ontwikkeling gereguleerd wordt door PKC gestimuleerde AC en door een hoge concentratie cAMP.

In hoofdstuk 5 wordt de hypothese getoetst dat hoge spiegels van cAMP PKA activeren en daardoor gen transcriptie door phosphorylering van het cAMP response element binding protein (CREB) beïnvloeden. CREB is bekend als een zogenaamde sleuteltranscriptiefactor betrokken bij groei en differentiatie van diverse cellen. PKA activiteit werd in gladde spiercellen van de arteria pulmonalis van neonatale kalveren, maar niet in die van volwassen dieren gevonden. De celgroei, welke was ontstaan na het toevoegen van serum, resulteerde bij beide celtypen in phosphorylering van CREB. In niet-delende neonatale cellen werden dezelfde bevindingen gedaan. Niet-delende cellen van volwassen dieren toonden echter geen P-CREB. Met behulp van immunohistochemische kleuringen werd

(P-) CREB in de longvaten aangetoond, met name in endotheel- en gladde spiercellen van de media. Op longen van pasgeboren kalveren waren de kleuringen duidelijker dan op de volwassen longen. CREB werd in het cytosol gelokaliseerd, P-CREB werd ook in de celkern gezien, hetgeen translocatie suggereert. De conclusie van deze studie is, dat de activatie van CREB door cAMP mogelijk bijdraagt aan de toegenomen groeimogelijkheden van gladde spiercellen uit de arteria pulmonalis van neonatale kalveren.

In tegenstelling tot eerder beschreven studies, wordt in de laatste studie gebruik gemaakt van een proefopstelling waarmee het ontstaan van weefseloedeem kan worden onderzocht. De ontwikkeling van longoedeem is één van de eerste pathologische verschijnselen die tijdens kunstmatige beademing optreedt. Het verlies van functie van endotheelcellen speelt een centrale rol in de beginfase van dit proces. Het is niet precies bekend welke intracellulaire mechanismen verantwoordelijk zijn voor dit verlies van functie van endotheelcellen. Wel is bekend dat een hoog calciumgehalte of een lage cAMP concentratie in het cytosol de doorlaatbaarheid van de vaatwand bevordert. Adenylaacyclase, dat geremd wordt door calcium, veroorzaakt een omgekeerde evenredigheid van het intracellulaire calciumgehalte en de cAMP concentratie in endotheelcellen van grote bloedvaten. Dit mechanisme draagt mogelijk bij aan het ontwikkelen van een verhoogde doorlaatbaarheid van de vaatwand. Echter, deze bevinding is gedaan in een *in vitro* model en als zodanig geen bewijs voor:

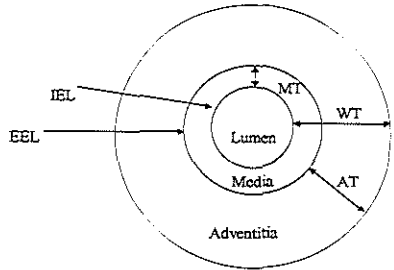
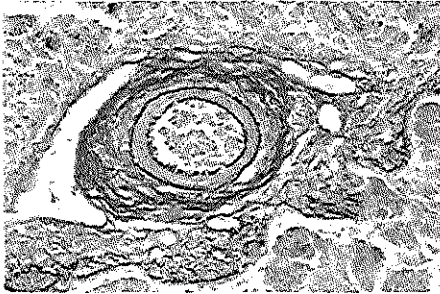
1. Expressie van een AC welke door calcium geremd wordt in het endotheel van kleinere longvaten en
2. Remming door calcium van AC en cAMP concentratie zoals wordt waargenomen als uiting van verhoogde doorlaatbaarheid van bloedvaten tijdens een infectie.

In hoofdstuk 6 werden deze processen nagebootst met behulp van geïsoleerde endotheelcellen uit kleinere bloedvaten en een

rattenlongperfusiemodel. Wij toonden aan dat het door calcium geremde AC in endotheelcellen van kleinere bloedvaten tot expressie komt. Verder suggereerden wij, dat remming van AC door calcium nodig is voor het ontstaan van verhoogde doorlaatbaarheid van deze vaten. De conclusie van deze studies is, dat remming van cAMP door calcium een kritische stap is in de ontwikkeling van verhoogde doorlaatbaarheid van de kleinere vaten van de long.

De studies, zoals beschreven in dit proefschrift, werden gedaan om onze kennis aangaande groei en differentiatie van cellen in de longvaatwand te vergroten. Aanvullende studies zijn nodig om effectieve nieuwe therapieën te ontwikkelen, die specifiek gericht zijn op de excessieve remodelering van de bloedvaten van de long bij pasgeborenen met pulmonale hypertensie.

PICTURES



Picture 1: chapter 2: page 13

A. Histology pulmonary arteriole in PPHN

Black: elastin fibers

20 x

B. Cross section of pulmonary artery

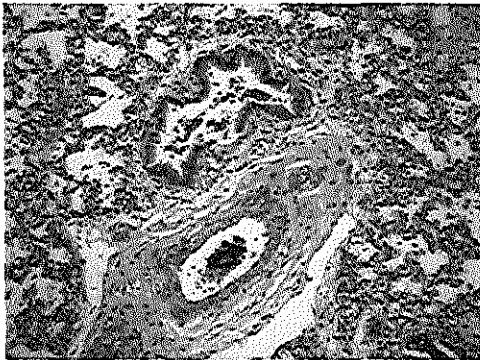
EEL= external elastic lamina

IEL= internal elastic lamina

WT= wall thickness

AT= adventitial thickness

MT=medial thickness

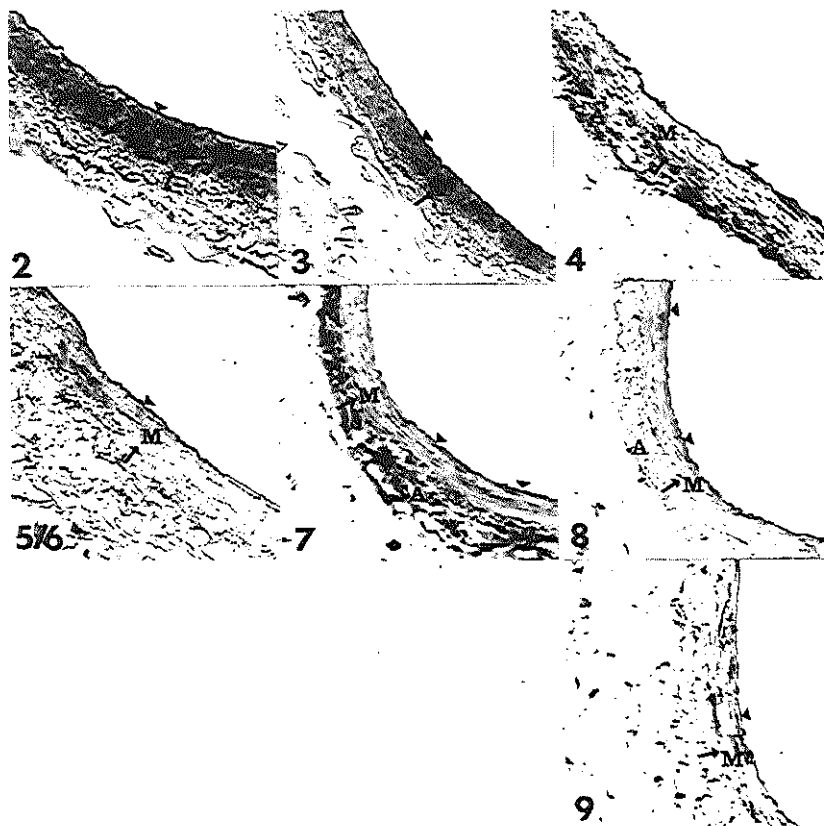


Picture 2: chapter 2: page 17

Histology human congenital diaphragmatic hernia lung

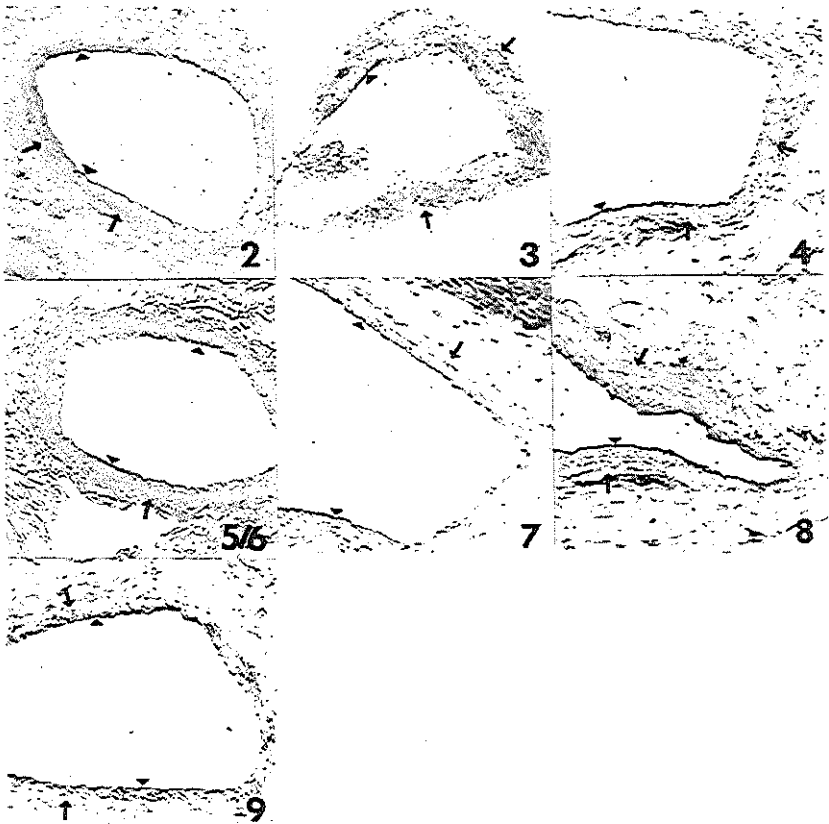
Arteriole (arrow) with increased medial and adventitial thickening, accompanying bronchiole.

Hematoxylin-eosin; 20 x



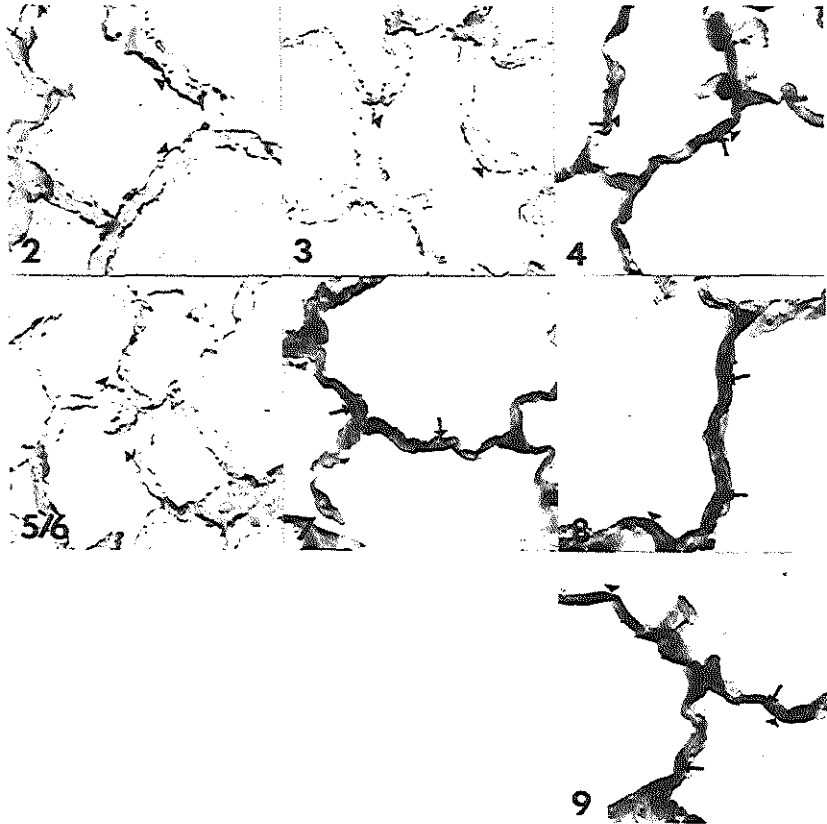
Picture 3: chapter 3: pages 34, 36, 37

Adenylyl Cyclase (subtypes denoted by number in left lower corner) expression in bovine main pulmonary artery. Endothelial cell positivity (arrowhead) was the strongest for AC II and IV. Smooth muscle cells in the media (M) showed diffuse positivity for AC II and III, while other AC subtypes exhibited a focal pattern of expression. Cells in adventitia (A) expressed AC IV and VII. Arrow identifies the medial-adventitial border. Immunoperoxidase-aminoethyl carbazole (AEC); counterstained with hematoxylin; 240 x.



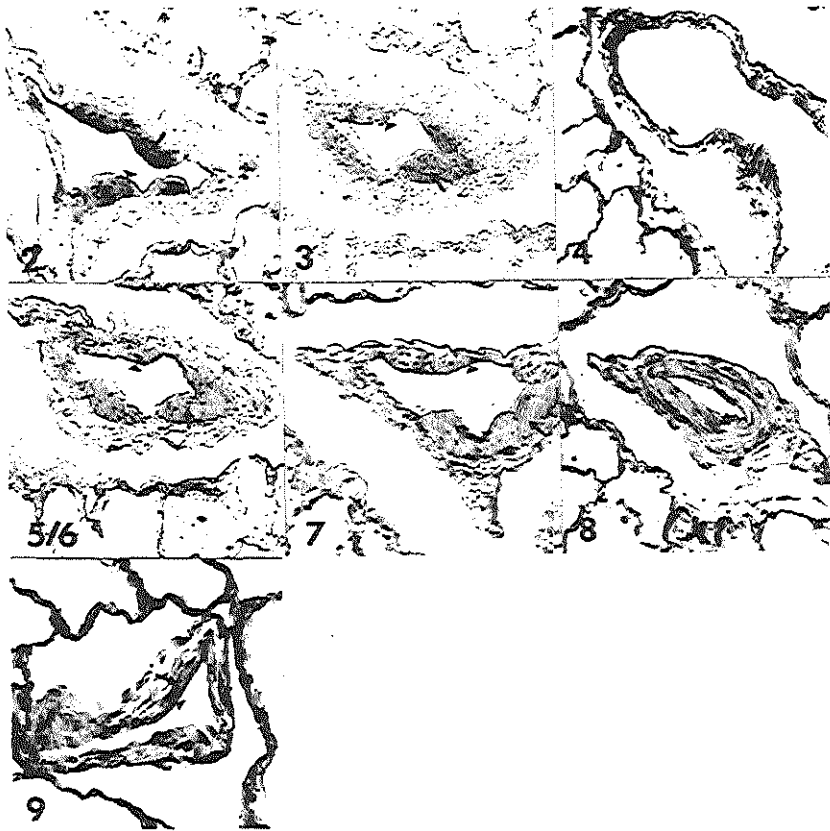
Picture 4: chapter 3: pages 34, 36, 37

Adenylyl Cyclase (subtypes denoted by number in right lower corner) expression in bovine pulmonary artery (100 μ m). Endothelial cell positivity (arrowhead) was the strongest for subtypes VIII and IX. Smooth muscle cells showed diffuse strong positivity of AC II, III and IX in the media (border determined by arrow). AC IV was detected in the adventitial tissue. Immunoperoxidase-ABC; hematoxylin; 240 x.



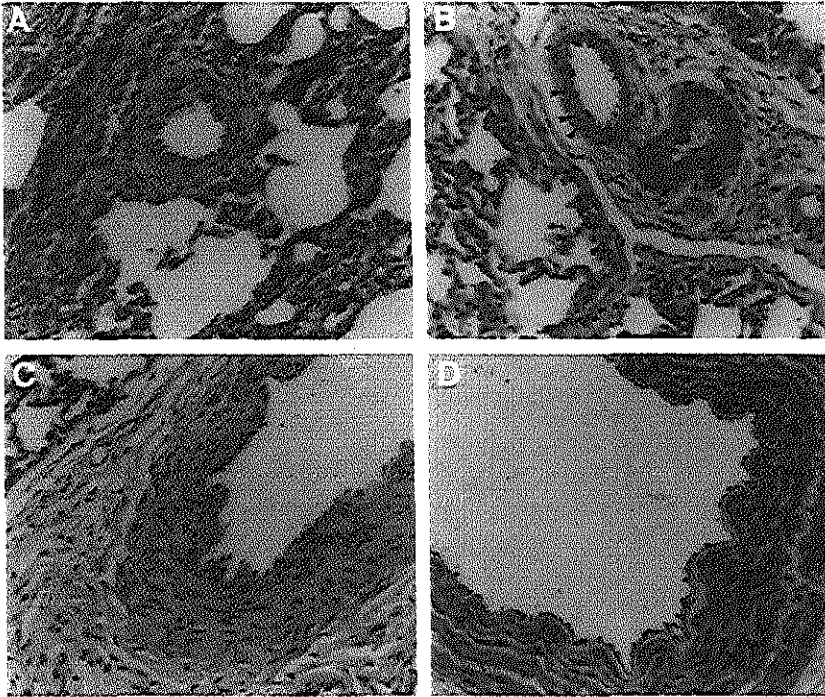
Picture 5. chapter 3: page 35

Adenyl Cyclase (subtypes denoted by number in left lower corner) expression in bovine lung capillaries. Alveolar epithelium (arrowhead) showed positivity of all except subtype VII. Capillary endothelium (arrow) expressed subtypes IV, VII, VIII and IX. Immunoperoxidase-AEC; hematoxylin; 480 x.



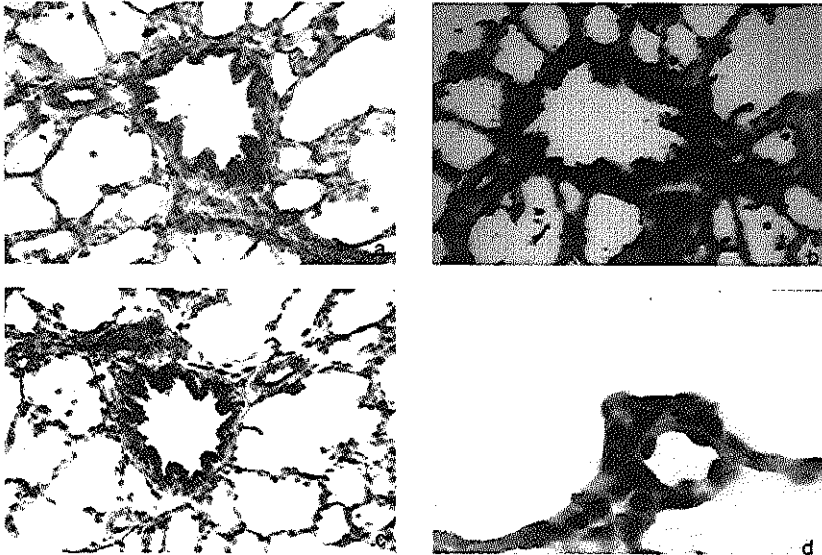
Picture 6: chapter 3: page 37

Adenylyl Cyclase (subtypes denoted by number in left lower corner) expression in veins of bovine lung (100 μ m). Endothelial cell positivity and smooth muscle cell expression is identified by arrowhead and arrow, respectively. Immunoperoxidase-AEC; hematoxylin; 240 x.



Picture 7 chapter 4: page 57

PKC-stimulated adenylyl cyclase (type II) is expressed in pulmonary arteries and arterioles. Diaminobenzadine staining of neonatal and adult animals (brown) was used to determine expression of type II adenylyl cyclase in the intact pulmonary circulation. Antibody specificity was tested by blocking diaminobenzadine staining with peptide inhibitors against primary antibodies (A and C). Medial sections of small (B) and large (D) neonatal pulmonary arteries stained positive with adenylyl cyclase type II- specific polyclonal antibody. Most SMCs in the medial layer of small and large pulmonary arteries stained brown (B and D arrowheads), demonstrating expression of adenylyl cyclase type II. Data with pulmonary vessels from adult animals are not shown. Hematoxylin was used to counterstain cell nuclei (purple).



Picture 8 chapter 5: pages 80 and 81

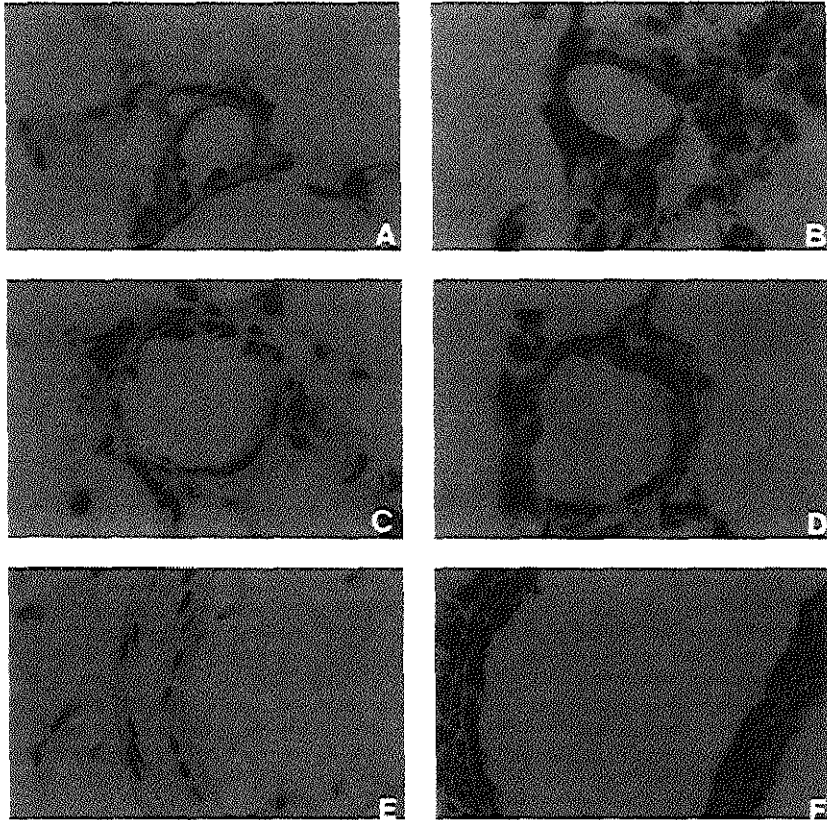
(Phospho-)CREB is expressed in neonatal bovine lung. Diaminobenzidine staining (brown) was used to determine expression (P-)CREB in the intact pulmonary circulation. Antibody specificity was tested by blocking diaminobenzidine with peptide inhibitors against primary antibody (a). Smooth muscle cell cytosol stained positive with total (b) and cell nuclei with phospho-CREB specific (c and d) polyclonal antibody. Hematoxylin was used as counterstain.

a: negative control (16x)

b: CREB (16x)

c: P-CREB (16x)

d: P-CREB (100x)



Picture 9: chapter 6: page 99

Ca²⁺ inhibitable adenylyl cyclase is expressed in endothelial cells throughout the lung. Rat lungs were fixed and prepared for immunohistochemistry as described in Methods. Adenylyl cyclase type(s) V/VI stain brown due to incubation of tissue with a specific polyclonal antibody and streptavidin-biotin-horseradish peroxidase developed with diaminobenzidine. Cell nuclei stain purple due to counterstaining with hematoxylin. [A], [C], and [E] represent peptide controls for the adenylyl cyclase type(s) V/VI polyclonal antibody. [B], [D], and [F] demonstrate adenylyl cyclase type V/VI expression in capillary endothelial cells and intermediate and large vessel endothelial cells, respectively.

List of abbreviations

| | |
|------------------|--|
| AC | adenylyl cyclase |
| ANG II | angiotensin II |
| ATP | adenine triphosphate |
| cAMP | cyclic AMP = 3'5'-cyclic monophosphate |
| CDH | congenital diaphragmatic hernia |
| CREB | cAMP response element binding protein |
| ECMO | extra corporeal membrane oxygenation |
| ERK | extracellular signal regulated kinase |
| ET-I | endothelin I |
| FGF | fibroblast growth factor |
| HIF-1 | hypoxia inducible factor-1 |
| IGF-1 | insulin-like growth factor-1 |
| MAPK | mitogen-activated protein kinase |
| mRNA | messenger RNA |
| NO | nitric oxide |
| PA | pulmonary artery |
| P _a | pulmonary artery pressure |
| P _c | pulmonary capillary pressure |
| PDGF | platelet-derived growth factor |
| PGI ₂ | prostacyclin |
| PMVEC | pulmonary microvascular endothelial cell |
| PPHN | persistent pulmonary hypertension of the newborn |
| PKA | protein kinase A |
| PKC | protein kinase C |
| P _v | pulmonary venous pressure |
| K _{fc} | capillary filtration coefficient |
| RT-PCR | reverse transcriptase-polymerase chain reaction |
| SMC | smooth muscle cell |
| TGF- β | transforming growth factor- β |
| VEGF | vascular endothelial growth factor |

Curriculum Vitae

| | |
|---------------------------|--|
| 1962, March 29th | Born in The Hague, The Netherlands |
| 1974-1980 | VWO- β , Thorbecke Scholengemeenschap, The Hague |
| 1980-1987 | Medical Degree (magna cum laude), University of Amsterdam, Amsterdam |
| 1987-1989 | Resident Pediatric Surgical ICU, Sophia Children's Hospital, Rotterdam |
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