Relationship between aspects of the nitric oxide pathway and vascular responses in the developing lung

Francesca Irene Arrigoni

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Institute of Child Health
University College London

30 Guilford Street
London WC1N 1EH

Abstract

The development of the pulmonary circulation is continuous from the fetal to the mature lung, with nitric oxide (NO) having a role as a major pulmonary vasodilator. Using a porcine model, the relationship of NO with pulmonary development was studied in lungs from 1 week pre-term to adulthood, and in hypoxia induced pulmonary hypertension, from birth and 3 days of age. The effects of breathing on the fetal and newborn lung were also investigated.

Arterial and venous vasoactivity was studied using organ baths, with particular attention being paid to the NO pathway in the arteries. In addition, NO synthase (NOS) activity and protein expression were studied in lung homogenates using the citrulline assay and Western blotting.

At all perinatal ages, vascular reactivity in the veins was greater than the arteries, with a minimal vasoactivity in the fetal arteries. Low contractility was created by the fetal arteries existing in an "un-dilated" state. Despite the basal release of NO, arterial relaxation to ACh was attenuated in the fetus in association with a low NOS activity. This did not correspond with a low NOS protein or the presence of endogenous inhibitors. At birth, arterial contractility improved, related to parturition and the onset of breathing. This was accompanied by an increased NOS activity and basal NO release. However, relaxation to ACh remained absent until 1 day of age.

From 3-14 days of age, changes in arterial contractility were associated with structural development. ACh relaxation improved, not associated with a further increase in NOS activity, but perhaps an increasing contribution of other endothelium dependent relaxing factors.

The enhanced vascular contractility following hypoxia induced pulmonary hypertension from birth corresponded with a change in smooth muscle cell structure. ACh induced relaxation was abolished in association with the alteration of the activities of the NOS isoforms and the production of NO from the smooth muscle.

Thus lung development in the fetus/newborn, postnatal and pulmonary hypertensive pig exists as distinct phases, in which NO has a role.

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Abbreviations

μg	microgram
μl	microlitre
μM	micromolar
ACh	acetylcholine
ADP	adenosine diphosphate
ADMA	asymmetric dimethylarginine
ATP	adenosine triphosphate
BH_4	tetrahydrobiopterin
Ca	calcium
CO_2	carbon dioxide
cAMP	3`, 5`-cyclic adenosine monophosphate
cGMP	3`, 5`-cyclic guanosine monophosphate
dNOS	constitutive nitric oxide synthase
COX	cyclo-oxygenase
COX-1	cyclo-oxygenase-1
COX-2	cyclo-oxygenase-2
DMEM	Dulbecco's modified eagles medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
ECMO	extracorporeal membrane oxygenation
eNOS	endothelial nitric oxide synthase
FCS	fetal calf serum
FPS	fetal porcine serum
sGC	soluble guanylate cyclase
hr	hour
HFV	high frequency ventilation
IFN-γ	interferon gamma
iNOS	inducible nitric oxide synthase
IP3	inositol trophosphate
K^+	potassium
kDa	kilodalton

L	litre
L-NAME	N^G-nitro-l-arginne methyl ester
L-NMMA	N^G-monomethyl L-arginine
L-NNA	N^{ω}-nitro-L-arginine
LPS	lipopolysaccharide
M	molar
Mg	milligram
min	minute
mm	millimetre
mM	millimolar
M_r	relative molecular mass
mRNA	message ribonucleic acid
Na^+	Sodium
NADPH	nicotinamide adenine dinucleotide phosphate
NANC	non-adrenergic non-cholinergic
nNOS	neural nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NS	not significant
O_2	oxygen
^oC	degrees Celsius
P_aO_2	arterial oxygen tension
p	probability
PDE	phosphodiesterase
PG	prostaglandin
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGI_2	prostacyclin
PK	protein kinase
pmol	picomole
PMSF	phenylmethylsulfonylfluoride
PPHN	persistent pulmonary hypertension of the newborn
SDS	sodium dodecyl sulphate

sem	standard error of the mean
SNP	sodium nitroprusside
SOD	superoxide dismutase
TNF-α	tumour necrosis factor alpha
TP	TP (thromboxane) receptor
TXA_2	thromboxane A_2
U46619	9,11-Dideoxy-11α,9α-epoxy-methanoprostaglandin $F_{2\alpha}$
VASP	vasodilator stimulated protein
VEGF	vascular endothelial growth factor
14PS	14 day porcine serum

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Chapter 1. Introduction.

1.1 Pulmonary vasculature.

The development of the lung in humans is a process that starts in early fetal life and continues after birth. The developmental changes that occur encompass both the structural and functional aspects of the pulmonary vasculature, including both arteries and veins, and show marked differences to the structure and function of the mature adult pulmonary vasculature.

In the mature lung, to enable efficient gas exchange, both lung ventilation and perfusion need to be adequately matched. Perfusion of blood in the lungs is controlled by the pulmonary blood vessels, which have thinner walls and greater internal diameters than corresponding branches of the systemic arterial tree. This is because they contain less vascular smooth muscle in their walls and do not become highly muscularised.

Pulmonary arteries subdivide profusely into terminal branches accompanying the airways. The intrapulmonary arterial structure has the same basic structure as the main pulmonary artery, with a media of elastic and muscle fibres and adventitia and intima. As external diameter decreases, the elastic laminae decrease in number, and the muscular wall gets thinner. In contrast, in the veins the wall is made up of irregular muscle bundles containing both collagen and elastic fibres (Wagenvoort et al., 1964). Veins contain an internal but not an external elastic lamina with the thick adventitia containing numerous elastic fibres.

The distribution of the pulmonary veins returning blood to the heart is different to that from the arteries. The veins lie within the interlobular and interlobar connective tissue septae facilitating the reception of blood from many terminal respiratory units. Thin walls indicative of low levels of muscularisation, are a result of the low pressure under which these vessels operate. Between the arteries and the veins lie the capillaries. These form an extensive interdigitating network within the alveolar walls facilitating gas exchange. The pulmonary arteries are partially dilated whilst most systemic arteries are constricted, aiding the distribution of pulmonary resistance, which at rest is evenly distributed between the arteries, capillaries and veins. This contributes to the low resistance to flow in the pulmonary circulation that is 1/10 that of the systemic system. This low resistance permits a total cardiac output of 5L/min to flow through the lung in the adult.

1.2 Fetal pulmonary structure

In contrast to the adult, fetal pulmonary blood flow is low constituting approximately 8-10% of the total cardiac output (Rudolph et al., 1973). This low flow is maintained by blood being diverted away from the lungs via the ductus arteriosus from where 45% of the cardiac output reaches the placenta for gas exchange. Over the last half of gestation pulmonary vascular resistance (PVR) falls progressively (Rudolph, 1979) due to the increased cross sectional area of the lung created by new vessel growth (Levin et al., 1976).

Ultrastructural studies in the fetal pig lung have shown that the thick walled arteries are composed of thick endothelial and smooth muscle cells, both having a low surface area: volume ratio (Hall et al., 1987; Hall et al., 1986). The endothelial cells have deep interdigitating contacts and the smooth muscle cells overlap each other and contain a relatively small proportion of contractile filaments (Hall et al., 1986). Thus the high fetal PVR can be attributed partly to the shape and organisation of cells within the vessel wall. Human fetal arteries are more muscular than the veins and lack elastic tissue (Hislop et al., 1973). The prenatal development of the pulmonary veins parallels that of the arteries, developing at the same time and appearing to grow in a similar pattern, size and number to the arteries. However in the fetal pulmonary circulation the vein wall is thin compared to the arteries and the muscle development lags behind that of the arteries (Hislop et al., 1973). The low muscularity of the vein wall may be a structural adaptation to the low pressures that are present in the vein, permitted by the high resistance in the fetal arteries. This is a relationship that is maintained after birth.

1.3 Postnatal pulmonary structure

By 24 hr after birth, the rapid decrease in total PVR diminishes to 50-60 % of systemic vascular resistance, to reach the adult values within 2-6 weeks of age (Haworth et al., 1981). This decrease in pulmonary pressure is created by structural changes that occur which are observed within the first minutes following birth. Although the amount of smooth muscle present in the vessel wall does not decrease, there is a rapid increase in the surface area: volume ratio of the wall as the cells increase in length. This creates an increase in vessel diameter and lowers pulmonary resistance (Haworth et al., 1987). This increase in length corresponds to the increased stretching which occurs as the lung is expanded. These vessels contain little fixed connective tissue, collagen or amorphous

elastin, which may restrict changes in cell shape and position. Pulmonary artery thickness decreases rapidly during the first days of life, and reaches a mature adult level during the first 3 months of life (Haworth et al., 1981; Hislop et al., 1973). In the elastic and large muscular arteries of the lung, remodelling is characterised by the deposition of connective tissue and the maturation of smooth muscle cells (Hall et al., 1987). These structural changes are associated with the changes in the mechanical properties of the vessel wall. Myofilament density decreases between birth and 14 days of age, reaching a minimum at 3 days of age (Hall et al., 1987), and with maturation the vessel wall becomes stiffer (Greenwald et al., 1982). The progressive growth and remodelling of the vascular bed ensures that as the cardiac output increases with growth, the PVR does not rise.

1.4 Persistent Pulmonary Hypertension of the Newborn (PPHN).

Under certain conditions the adaptation of the pulmonary circulation to postnatal life is prevented by the persistence of hypertension in the lung. PPHN can be secondary to several neonatal disorders including; perinatal hypoxia, sepsis, meconium aspiration syndrome, diaphragmatic hernia, congenital heart disease, severe respiratory distress syndrome or polycythaemia (Greenough et al., 1992). These disorders are grouped as a syndrome because they share several pathological and physiological features. PPHN is typically characterised by a high pulmonary artery pressure, low pulmonary blood flow, and massive right-to-left shunting at the foramen and ductal levels, which can cause profound hypoxaemia. Increased muscularity and luminal narrowing potentiate the pulmonary vascular obstruction and thus contribute further to the hypertension in a vicious cycle.

1.4.1 **Structural changes with PPHN**

The alterations in vascular structure that typify PPHN can be mimicked following exposure of a healthy newborn mammal to chronic hypoxia (Haworth & Hislop., 1982; Allen et al., 1986). Chronic hypoxia in the adult is associated with right ventricular hypertrophy, an increased thickness of the media in normally muscularised arteries and muscularisation of the pulmonary arterioles which normally do not contain any smooth muscle (Aries-Stella et al., 1963). Most studies in humans confirming the effects of chronic hypoxia have been either from individuals living at high altitudes of

approximately 2100m, which creates a fall in alveolar oxygen tension below 75 mmHg, (Anand et al., 1994) or in a variety of clinical conditions such as chronic bronchitis and cystic fibrosis (Hasleton et al., 1968; Shelton et al., 1977; Ryland et al., 1975). A variety of animal models have been used to study PPHN including the calf. It has been demonstrated that exposure to hypoxia in newborn calves induces all the characteristic changes associated with persistent pulmonary hypertension in humans. Pulmonary arteries from hypertensive calves demonstrate a change in smooth muscle cell phenotype resulting in a marked increase in elastin and collagen synthesis and content (Mecham et al., 1987; Crouch et al., 1989). In the smooth muscle cells taken from the media of pulmonary hypertensive newborn calves and rats, cell proliferation and DNA synthesis is greater than normal (Orton et al., 1992; Meyrick et al., 1982). In addition, hypertensive newborn calves exhibit a marked thickening of the arterial adventitia and demonstrate cellular proliferation and extreme extracellular matrix deposition (Stenmark et al., 1987).

1.5 The porcine model of PPHN

The effects of altitude can be mimicked in the porcine lung by the hypoxic hypobaric oxygen chamber. Using a vacuum pump, the partial pressure of oxygen is halved (10.5kPa), and the atmospheric pressure is reduced to half an atmosphere (50.4kPa). This use of chronic hypobaric hypoxia enables the study of pathophysiological functional and structural changes, which are in keeping with human PPHN. The pigs are cyanosed and shunt right to left through persistent fetal channels. At autopsy right ventricular hypertrophy is indicated by an increase in the right ventricular to left ventricular weight ratio (Haworth et al., 1982). Histological examination of the lungs shows an increase in pulmonary artery wall thickness, with muscularisation of small distal pre-alveolar arteries. Electron microscopy shows an increase in smooth muscle cell myofilaments (Allen et al., 1986). Studies in several species have shown consistent changes in the histological appearance of the endothelium and smooth muscle cells with pulmonary hypertension. In neonatal pigs exposed to chronic hypoxia from birth, the smooth muscle cells fail to spread, remaining thick and brick shaped, whilst the smooth muscle cell contractile myofilament density increases more rapidly than normally, preventing complete relaxation in pulmonary arteries (Allen et al., 1986). By 3 days of age the vessels are

fixed in an incompletely dilated state created by an increased myofilament formation and elastin and collagen deposition. Piglets are used to investigate the pathophysiology of PPHN because both their size and arterial structure are similar to human babies. A further advantage of using the pig model for the study of PPHN, is the size of the litter, which is between 8-16 piglets. However the disadvantage to using the pig is the small number of animals produced at a high cost, when compared to the cost of small mammals such as mice and rats. In practise however, the pig is a useful model in which to study human adaptation to the extrauterine environment.

1.6 Regulation of PVR.

At all the stages of the developing lung, the pulmonary vessels respond to a wide variety of physiological stimuli and humoral and exogenously administered vasoactive substances. Functionally the pulmonary circulation is regulated passively and actively. In the mature lung however, passive regulation of blood flow, which includes the redistribution of blood caused by gravity and the recruitment and distension of blood vessels, changes PVR independently of changes in vascular tone.

Active regulation can become important in certain physiological conditions such as in the maintenance of the high pulmonary resistance in the fetus and the transition of the pulmonary circulation at birth. The pulmonary circulation of the fetus requires mechanisms to maintain the high resistance that is required for the large proportion of blood to bypass the lungs. The release of vasoactive substances alters at birth in association with the decrease in PVR that occurs. PVR continues to alter postnatally in addition to acute changes in pulmonary vascular structure.

In the mature lung there are many factors that alter vascular tone. These may be inactivated, altered or removed from the blood or synthesised or released from cells in the lungs. Mediators of vascular tone originate from; plasma (endocrine autocrine factors), platelets (thromboxane A_2 (TXA_2), histamine, 5-hydroxytryptamine (5-HT), adenosine di-phosphate (ADP), adenosine tri- phosphate (ATP)), smooth muscle (Prostaglandin, ATP, ADP), nerves and the endothelium (ATP, NO, Prostaglandins, TXA_2, 5-HT, endothelin, vasopressin, substance P, angiotensin II)(Loesch et al., 1991; Goll et al., 1986; Pearson et al., 1979), (Table 1.1). However, the endothelium produces substances that mediate a large proportion of the intrinsic pulmonary vasoreactivity (Liu et al., 1999).

1.7 Endothelium mediated control of PVR

It has been estimated that endothelial cells constitute 40% of the total adult lung parenchyma playing an important role in the production or metabolism of circulating vasoactive substances. The activity of the endothelium is mediated by transport mechanisms and enzymes that either degrade or take up circulating substances (Gillis 1987; Jaffe et al 1973). On the basis of studies in other vascular beds the endothelium has been shown to have the ability to modulate cell growth as well as tonic responses within the vessel wall (DiCorleto et al., 1984; Campbell et al., 1986; Clowes et al., 1977). Other functions include the regulation of platelet activated blood coagulation and fibrinolyis, mediator production, expression of numerous receptors, and the production of constricting and relaxing factors.

The endothelium, in association with the smooth muscle, regulates the reactivity of the pulmonary circulation. The release of vasoactive agents from the pulmonary endothelium may depend on the maturational state of the lung, the location of the receptor, or the contractile state of the vessel. Experiments *in vitro* have shown that an increase in transmural pressure and flow results in a combination of vasosoconstrictor and dilator factors generated by both smooth muscle cells and endothelial cells. These include the production of endothelin, oxide radicals, NO, arachidonic acid metabolites and bradykinin (Masatsugu et al., 1998; Busse et al., 1998; Dimmeler et al., 1998; Okahara et al 1998; Ryan et al., 1995; Hornig et al., 1997; Katusic et al., 1989). Endothelial regulation of vascular tone may also occur through mechanical deformation of the vessel wall. This imposes stresses of shear and stretch on vascular endothelial cells. In fact endothelial shear has been shown to stimulate calcium activated potassium channels on the endothelium in the systemic circulation (Cooke et al. 1991) as well as in the pulmonary circulation (Storme et al., 1999). This subsequently leads to an increased concentration of Ca within the cell leading to the stimulation of endothelium derived relaxing factors.

The pulmonary endothelium is also known to secrete ET-1 (Yanagisawa et al., 1988), a potent 21 amino acid polypeptide produced by the action of endothelin converting enzyme (ECE) on big endothelin. There is a family of endothelins (ET-1, ET-2 and ET-3), of which ET-1 produces the most potent pulmonary vasoconstriction via the activation of ET_A receptors predominantly found on smooth muscle (Hay et al.,

1993). Binding sites for ET-1 have been described in pulmonary blood vessels in the mature lung in the human, guinea pig, rat and pig (Power et al., 1989; McKay et al., 1991; Davenport et al., 1989; Ihara et al., 1991). ET-1 may also induce pulmonary vasodilation via the activation of ET_B receptors on the endothelium (Hay et al., 1993), although functionally distinct ET_B receptors mediating both vasodilator and vasoconstrictor effects have been located in cultured porcine aortic endothelial cells (Shetty et al., 1993). In addition, the effects of ET-1 on the pulmonary circulation vary with age (Hislop et al., 1994).

The pulmonary vascular bed may also be constricted by the potent arachidonic acid metabolite TXA_2 and other prostanoids (Seily et al., 1986; McMahon et al., 1991). However, in the pulmonary circulation, arachidonic acid and its metabolites play a large role in the maintenance of a low resistance vascular tone (Said et al., 1982; De Mey et al., 1982). Endothelial cells contain prostaglandin H synthase or cyclo-oxygenase-1 (COX-1) (Mitchell et al., 1993) which metabolises arachidonic acid at a basal rate and following endothelial stimulation with flow, induces the production of the vasodilator PGI_2 as has been shown in human cultured endothelial cells (Frangos et al., 1985). The production of PGI_2 may be additionally stimulated via humoral factors which include; angiotensin II, platelet activating factor (PAF) (Gao et al., 1995), bradykinin (Gryglewski 1980; Leffler et al., 1984) histamine, (Truog et al., 1990), ET-1 (Zellers et al., 1994) and ACh (Zellers et al., 1991).

Circulating growth factors and hormones are also known to induce the production of PGI_2. Growth factors that occur in the lung, such as vascular endothelial growth factor (VEGF), upregulate the constitutive form of COX-1 in human vascular endothelial cells (HUVECS)(Bryant et al., 1998). In addition prolonged exposure to the hormone oestrogen enhances PGI_2 synthesis in piglet endothelial cells and rat aortic smooth muscle cells (Seillan et al., 1983; Chang et al., 1980).

Another endothelium dependent relaxant factor also induced in pulmonary arteries is EDHF, or endothelium dependent hyperpolarising factor. EDHF is released from the endothelium in both the systemic and pulmonary system when stimulated by either histamine or ACh (Chen et al., 1989). This causes the subsequent hyperpolarisation of smooth muscle cells and a decrease in vascular tone.

Nervous stimulation of the pulmonary vascular bed also contributes to the maintenance of pulmonary vascular tone. Innervation with parasympathetic/ cholinergic nerves from the vagal nuclei, via the action of acetylcholine on endothelial muscarinic

receptors (Nandiwada et al., 1983) results in vasodilation, as does stimulation of the non-adrenergic non-cholinergic or NANC nerves (Kubota et al., 1988; Maggi et al., 1990). In addition, the vascular endothelium reduces pulmonary vascular tone by diminishing adrenergic contraction in the pulmonary circulation. This is achieved following the uptake and degradation of the neurotransmitters noradrenaline, serotonin (5-HT) and ATP by the endothelium (Said 1982; Alabaster et al., 1970; Dieterle et al., 1978).

In the fetal circulation, humorally stimulated vasoconstriction contributes to the high PVR found in the lungs at this period. This includes the effects of endothelin (Chatfield et al., 1991; Toga et al., 1992), TXA_2 (Reyes, 1993) and leukotrienes which are derived from arachidonic acid via the lipoxygenase pathway (LeBidois et al., 1987). Fetal pulmonary vasodilators are regarded as putative agents that will mediate the transition of the fetal to the newborn pulmonary circulation. PGI_2 is the predominant prostaglandin produced by the fetal and neonatal vascular cells (Remuzzi et al., 1979; Skidgel et al 1984). The importance of PGI_2 at this developmental age has been shown in lambs where the ductus arteriosus prematurely closes following the inhibition of COX-1 (Coceani et al., 1976). During late gestation, towards birth, the synthesis of PGI_2 increases due to the enhanced expression of COX-1 (Brannon et al., 1994). This increase in COX-1 is reflected in the augmented amount of PGI_2 stimulated by bradykinin, the calcium ionophore A23187 or arachidonic acid (Jun et al., 1998). In fetal ovine pulmonary artery endothelial cells (PAEC), an increase in COX-1 protein and mRNA expression is associated with an increase in oestrogen receptors (Jun et al., 1998). Thus the increase in circulating oestrogen in fetal plasma due to rising production by the placenta prior to birth (Carnegie et al., 1978; Gelly et al., 1981; Robertson et al., 1985), is the most likely candidate causing this.

Postnatally, as the lung grows the pulmonary vascular metabolic capacity increases due to an increase in endothelial surface area: volume ratio. Associated with this, in the sheep and pig, vasoactive agents such as prostaglandin E_2 (PGE_2) and PGI_2 in association with other agents such as ADP and ET-1 are increasingly metabolised at birth (Redding et al., 1984; Brannon et al., 1994; Abman et al., 1991;Perreault et al., 1993). In porcine pulmonary arteries, corresponding to the changes in vascular structure postnatally, the contractile responses to $PGF_{2\alpha}$ and KCl alter (Levy et al., 1995) in combination with the sensitivity to ET-1 (Toga et al., 1992; Hislop et al., 1994; Perreault et al., 1993).

Agent	Vasoactive effect	Mediation of relaxation	Other
Histamine	Vasoconstriction via H1receptors on smooth muscle cells Vasodilation via H2 receptors in smooth muscle cells, or H1receptors on endothelial cells	NO , PGI_2, EDHF	Production stimulated by oxygenation Released from mast cells
Acetylcholine (ACh)	M1, M2, M3 muscarinic receptors Contraction induced on smooth muscle, Relaxation induced on endothelial cells	NO , PGI_2, EDHF	Released from nerves
5-HT (Serotonin)	Vasoconstriction 5-HT_1/ 5-HT_2 vasodilation 5-HT_{1C}	NO	Released from; Blood platelets Nerves
Angiotensin II	Vasoconstriction. Activates G proteins in smooth muscle cells/ constriction		Converted from angiotensin I by ACE
Bradykinin	B1 and B2 receptors. Low tone –vasoconstriction High tone-vasodilation	NO , PGI_2, EDHF	Stimulated by oxygenation, shear stress. Degraded by ACE
Vasopressin	Dilates pulmonary vascular bed V1 receptor	NO	Stimulated by Angiotensin II
ANF	Vasodilation via GCA receptor stimulation	PGI_2	Synthesised in pulmonary veins
Endothelin	ET-1 and ET-3 found in the lung Vasoconstriction ET_A Vasorelaxation ET_B	NO and PGI_2	Stimulated by oxygenation, shear stress
Arachidonic acid metabolites			Stimulated by shear stesses, leukotrienes, platelets
TXA_2	Low tone –vasoconstriction High tone-vasodilation		
PGD_2, $PGF_{2\alpha}$, PGH_2	Vasoconstriction		
PGE_2,	Vasodilation		
PGI_2	Vasodilation		Stimulated by oxygenation, shear stress, Bradykinin, ACh, ET-1, arachidonic acid, A23187 etc.
Lipoxygenase products LTB_4, LTC_4,LTD_4	Vasoconstriction		Putative mechanism of hypoxic pulmonary vasoconstriction.
Platelet activating factor(PAF)	Low tone –vasoconstriction High tone-vasodilation	NO and PGI_2	Released by neutrophils, platelets, endothelial cells etc.
Purines Adenosine and , ADP ATP	A_1, P_{2X}-constriction A_2,P_{2Y}- vasodilation	NO and PGI_2	Released by Nerves, and endothelium stimulated by oxygen and shear stresses
VIP	Low tone –vasoconstriction High tone-vasodilation Endothelium dependent receptors on smooth muscle cells	NO and PGI_2	

Table 1.1 **Humoral factors released by the pulmonary circulation**

1.8 Smooth muscle mediated control of PVR

In addition to the PVR being controlled by production/metabolism of vasoactive factors from the endothelium, the pulmonary smooth muscle has the intrinsic ability to respond to external forces and pressures. The myogenic response is defined as the ability of vascular smooth muscle to constrict during an increase in intravascular pressure and to dilate on lowering intavascular pressure (Johansson et al., 1989). In fact this has been demonstrated *in vitro* in the pulmonary resistance arteries from adult cats (Kulik et al., 1988; Madden et al., 1996).

In addition, alveolar oxygen tension is important in minute to minute active regulation of pulmonary vascular tone (Fischer et al. 1976) in both the endothelium (Holden et al., 1984) and the smooth muscle (Madden et al., 1995). Alveolar hypoxia, by causing pulmonary arterial constriction, leads to an immediate and sustained local increase in vascular resistance. This is in contrast to the response of systemic vessels, which vasodilate in response to hypoxia. Thus decreased O_2 tension causes a decrease in local blood flow and hence shifts blood to better oxygenated areas of the lung. The precise mechanism mediating this decrease in PVR is unknown, however a decreased PaO_2has been shown to cause contraction in feline pulmonary arteries by depolarising the smooth muscle cells which creates an increase in intracellular Ca (Madden et al., 1985).

Smooth muscle cells have an equally if not more important role than the endothelium in maintaining the hypertensive fetal PVR. The fetal pulmonary vasculature possesses an intrinsic myogenic tone, which in association with the endothelium regulates the PVR (Storme et al., 1999). Indeed the smooth muscle myogenic tone could explain the transient pulmonary vasodilation and increased blood flow following stimuli such as increased oxygen tension, shear, and several pharmacological agents in ovine fetuses (Abman et al., 1989; Abman et al., 1991; Accurso et al., 1986). Oxygen tension has been shown to regulate fetal pulmonary vascular tone in fetal lambs *in vivo*, whereby the PVR of fetal lambs increases if oxygen tension decreases (Lewis et al., 1976). Conversely, by increasing the PaO_2 of fetal lambs, PVR falls (Assali et al 1968; Heymann et al., 1969). The mechanism for the response to alterations in oxygen tension, and the subsequent high PVR associated with hypoxia may be connected with K^+ channels in the smooth muscle cells. Blockage of the K^+ channel prevents pulmonary vasodilation associated with ventilation (Tristani-

Firouzi et al., 1996). At birth the factors mediating the high PVR in the fetus must be removed, and a decrease in PVR must occur to allow the increased perfusion of the whole lung with blood.

1.9 Control of PVR in the transition of the pulmonary circulation at birth and postnatally

The ventilation of the lungs at birth is one of the stimuli mediating the decrease in PVR creating a subsequent decrease in carbon dioxide and increase in oxygen tension. Using a variety of different techniques an acute increase in oxygen tension, has been shown to induce pulmonary vasodilation in fetal lambs (Lewis et al., 1976; Morin et al., 1988; Assali et al 1968; Heymann et al., 1969). The decrease in PVR caused by oxygen at birth is mediated in conjunction with the gaseous expansion or ventilation of the lungs. The combined effect of both ventilation and oxygenation on the fetal ovine pulmonary artery has been suggested to be greater than the individual effects of either ventilation or gaseous expansion alone (Born et al., 1955; Cassin et al., 1964; Cook et al., 1963; Dawes et al., 1962). However, the initiation of ventilation has in some studies been indicated as the major stimulus for the decrease in PVR at birth (Reid et al., 1990), contributing to more than 65% of the total decrease in PVR (Teitel et al., 1990). The stimulus for the decrease in tension associated with ventilation is most likely the increased shearing on the endothelial cells. Following mechanical stimulation of the lungs, the increased stresses on the lung induce the release of vasoactive factors such as PGI_2 from the endothelium as has been shown in cultured endothelial cells (Edmonds et al., 1969; Frangos et al. 1986). Following the initiation of ventilation the large amounts of PGI_2 produced in the fetus decrease within hours of birth (Leffler et al., 1984).

After the transition from the fetal to the postnatal pulmonary circulation at birth, the endothelium and smooth muscle cells continue to regulate PVR in a fashion that alters with postnatal age. The alterations in pulmonary structure and vascular responsiveness at birth are associated with each other. The low pulmonary resistance in the resting newborn pulmonary circulation is such that most relaxant agonists would not be able to cause further vasodilation. However, during the first week of life, a gradual maturation of vascular cells occurs which allows the endothelium dependent dilation of pulmonary arteries. This includes an increased arterial responsiveness to PGI_2 (Brannon

et al., 1994), associated with a sharp decline in the effects of PGI_2 inhibitors (Redding et al., 1984), which would otherwise cause increases in PVR.

The electrophysiological properties of pulmonary artery smooth muscle cells also change in an age related manner, with a maturational change in K^+ channel expression, from Ca dependent K^+ channels in the fetus to voltage dependent K^+ channels in the adult pulmonary artery smooth muscle cells (Reeve et al., 1998). In association with this the cells from the newborn exist in a depolarised state, unlike those from the older age groups (Evans et al., 1998).

1.10 Control of PVR with PPHN

With PPHN, a combination of structural and functional changes alters the PVR from that observed in the healthy lung. The thicker walled pulmonary arteries that arise following hypoxia induced hypertension in the newborn, increase the cross sectional area of the vessel, and may directly account for an augmented vasoconstriction and subsequent increase in PVR observed (Rudolph et al., 1966). In a similar fashion the thick walls of these arteries occlude the lumen and, the rapid increase in collagen and elastin may prevent these vessels from relaxing (Park et al., 1977). However following chronic exposure to hypoxia, it is a combination of both mechanical and metabolic alterations which alters pulmonary vascular reactivity as has been shown in the isolated perfused lung (Emery et al., 1981).

The importance of the endothelium in regulating PVR is highlighted following endothelial cell injury in human and various types of experimental pulmonary hypertension. This suggests that damage may promote vasoconstriction by upsetting the balance between endothelially produced vasodilators and vasoconstrictors (Rabinovitch et al., 1986; Meyrick et al., 1983). In fact, with pulmonary hypertension an increased proportion of circulating endothelially mediated constrictors has been observed, contributing to the hypertensive state of the lung. In infants with PPHN elevated plasma concentrations of ET-1 have been found (Rosenberg et al., 1993), in addition to the arachidonic acid metabolite TXA_2 (Adatia et al., 1994; Christman et al., 1992). Eddahibi and co-workers illustrated the importance of ET-1 in pulmonary hypertension following the prevention of pulmonary hypertension in the presence of ET-1 inhibitors (Eddahibi et al., 1995). In association with the increased production of endothelium dependent contracting factors, there is a decreased production of endothelium dependent

relaxing factors as has been demonstrated in the isolated pulmonary arteries of neonatal calves which produce diminished PGI_2 and PGE_2 following stimulation (Badesch et al., 1989).

1.11 The role of the veins in mediating PVR

There is now much evidence to suggest that the control of pulmonary vascular responses in the veins is not the same as that found in the arteries. In the mature lung, *in vitro* studies have indicated that the veins possess a greater contractility than the arteries to various agonists. This has been shown in a variety of different species including the pig, rat and cow (Raj et al 1986; Bina et al., 1998; Ignarro et al., 1987) to agonists such as ET-1, TXA_2 and norepinephrine. In a similar fashion venous reactivity in the fetal lung is thought to be greater than arterial reactivity, and has also been demonstrated using ET-1 and norepinephrine (Steinhorn et al., 1995; Toga et al., 1992). This relationship has been shown to be maintained postnatally (Toga et al., 1992; Yoshimura et al., 1989).

A greater vasoreactivity has also been reflected in venous relaxant responses. In 7-13 day old lambs relaxant responses to both PGE_2 and PGI_2 have been demonstrated (Gao et al., 1996), whilst the biological relaxant agonist ANF is produced in greater quantities in veins than arteries from pigs of a similar age (Perreault et al., 1997).

With hypoxia induced pulmonary hypertension, the veins are exposed to different stimuli from the arteries because of the protection which they receive from the haemodynamic stresses imposed on the arteries in the presence of pulmonary hypertension. The venous responses may therefore differ from the arterial ones. It is believed that they contribute, albeit less than the arteries, to the hypoxic pressor response (Fischer et al. 1976). In the isolated lungs of young and adult ferrets, studies have shown that venous pulmonary resistances increase significantly following chronic hypoxia (Raj et al., 1990). In contrast, following pulmonary venous obstruction in the piglet, the early remodelling of the pulmonary vessels has been shown to increase venous distensability. This has been suggested to be caused by the poor cross-linking of elastin, which subsequently protects the lung from venous congestion and diminishes any rise in pulmonary venous pressure (LaBourene et al., 1990).

In addition, in chronically hypertensive calves from high altitudes, the stimulated production of PGI_2 is absent (Badesch et al., 1989), whilst in porcine lungs

the endothelium dependent relaxant responses to bradykinin were observed to be abolished in the veins following exposure to acute hypoxia (Feletou et al., 1995).

Thus in the developing lung the endothelium and smooth muscle directly or indirectly regulate pulmonary arterial and venous responses by releasing a variety of mediators. A number of studies are now emerging that suggest a central role for the vascular effects of NO in the regulation of pulmonary vascular responses in adult lungs as well as in the transitional stages that occur at birth and during development. Moreover a role for NO has also been suggested in PPHN and other related disorders that occur at birth. In order to appreciate the potential for NO in the developmental transition as well as in PPHN, it is useful to first review the aspects of NO biology derived from other biological systems.

1.12 The pharmacology of NO

NO is a noxious free radical gas that acts as a major messenger molecule in the body mediating a variety of biological functions. It is released by endothelial cells or nerves, and can be released as a defence mechanism by macrophages. Its biological functions contribute to homeostatic processes in every organ system in the body. In addition, NO can activate a number of proteins that influence cellular responses including guanylate cyclase, which results in smooth muscle relaxation. However, when NO production becomes excessive, its release can contribute to the processes of inflammation and/or cardiovascular dysfunction.

The ubiquity of NO is largely made possible by the presence of multiple isoforms of the enzyme nitric oxide synthase (NOS) which can be induced, upregulated or suppressed depending upon requirement.

1.13 NO synthesis by different cell types

The actions of endogenously released NO in mammals were first described in experiments using isolated blood vessels. Stimulation of rabbit aorta with ACh showed a resulting relaxation that was dependent on the presence of the endothelium. This endothelium dependent relaxation was in turn mediated by the release of a labile factor, identified as endothelial derived relaxing factor or EDRF (Furchgott et al., 1980). The identity of EDRF was not established until 1987 when it was shown to be indistinguishable from NO (Palmer et al., 1987). NO was further identified as EDRF when it was established as the active metabolite which mediated the smooth muscle

relaxant effects of nitroglycerin and other anti-anginal organic nitrates (Lowenstein et al., 1992). NO was also found to play a role as a neurotransmitter in the central nervous system (Garthwaite et al., 1988) and used by the inhibitory NANC nerves (Gillespie et al., 1972). In addition, it was observed to be the intermediate in the formation of nitrite and nitrate by activated macrophages (Hibbs et al., 1988). The production of NO from diverse cellular origins such as endothelial cells, neurones and inflammatory cells, influenced the progress and direction of future biochemical studies of the enzymes that produced it.

1.14 Synthesis of NO

NO is formed by the conversion of L-arginine to L-citrulline in the presence of nitric oxide synthase (NOS). Mechanisms have been suggested for the formation of NO and L-citrulline from L-arginine (Marletta et al., 1993; Ignarro et al., 1990), with the initial step in NO biosynthesis being the conversion of L-arginine to the intermediate N^G-hydroxy-L-arginine (Marletta et al., 1993), which itself is a substrate for the enzyme (Mitchell et al., 1992; Witteveen et al., 1998). The conversion of N^G-hydroxy-L-arginine to L-citrulline and NO requires nicotine adenine dinucleotide phosphate (NADPH) (Figure 1.1 a), but little else is known.

NOS bears similarities to the structure and activity of cytochrome P450, a haem-protein with recognition sites for L-arginine, NADPH, flavin nucleotides, calmodulin and phosphorylation. As with cytochrome P450, NADPH acts as a source of electrons for oxidation (White et al., 1992). The flavins FAD (flavin adenine dinucleotide) and FMN (flavin mononucleotide) play an analogous role in shuttling electrons from NADPH to the iron haem. In most cases FAD and FMN are so tightly bound to NOS that they are purified along with the protein and so are not required as additional factors. Moreover due to the similarity of the NOS haem moiety to that of cytochrome P450 and the inhibition of NOS activity by carbon monoxide (Ignarro et al., 1990), a role for the iron centre (Fe3+) of the enzyme as the catalytic site is suggested. The formation of NO from L-arginine incorporates molecular oxygen into both L-citrulline and NO suggesting the role of NOS as a dioxygenase enzyme (Leone et al., 1991). For maximal activity NOS also requires tetrahydrobiopterin (BH_4) (Kwon et al., 1989). This couples L-arginine oxidation to NADPH consumption and prevents disassociation from the

ferrous dioxygen complex (Vasquez-vivar et al., 1998), maintaining the integrity of the active enzyme protein (Baek et al., 1993).

NO may be released from 3 distinct isoforms of NOS, which are coded by different genes (Sessa et al., 1993). These isoforms, constitutive(eNOS, nNOS), and inducible by nature (iNOS), have different regulatory mechanisms leading to the synthesis of NO.

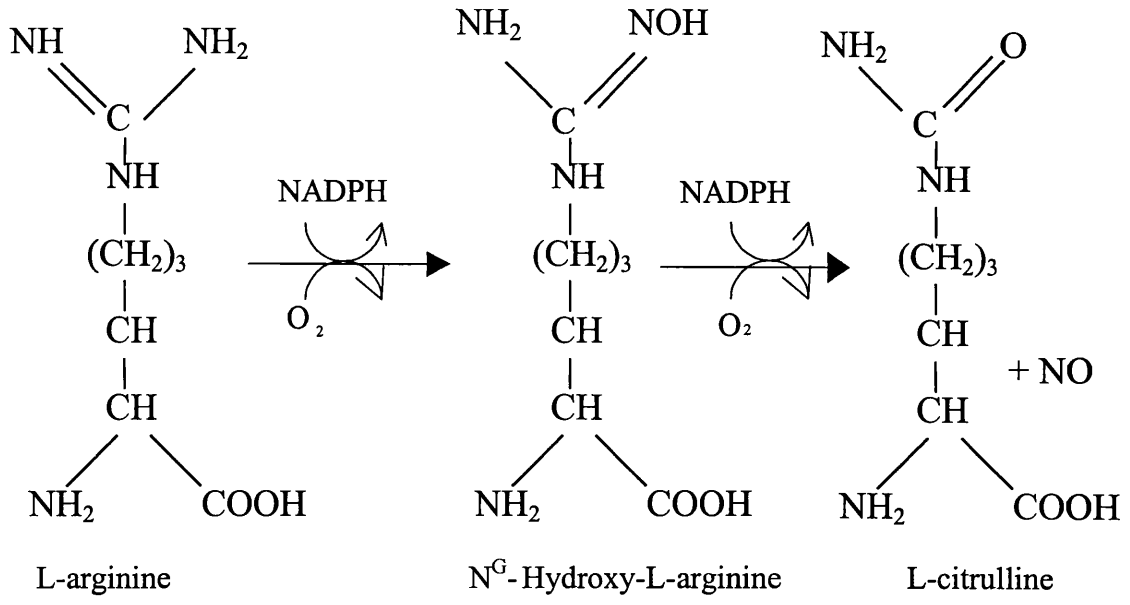

Figure 1.1a **Chemical reaction of the formation of NO.**

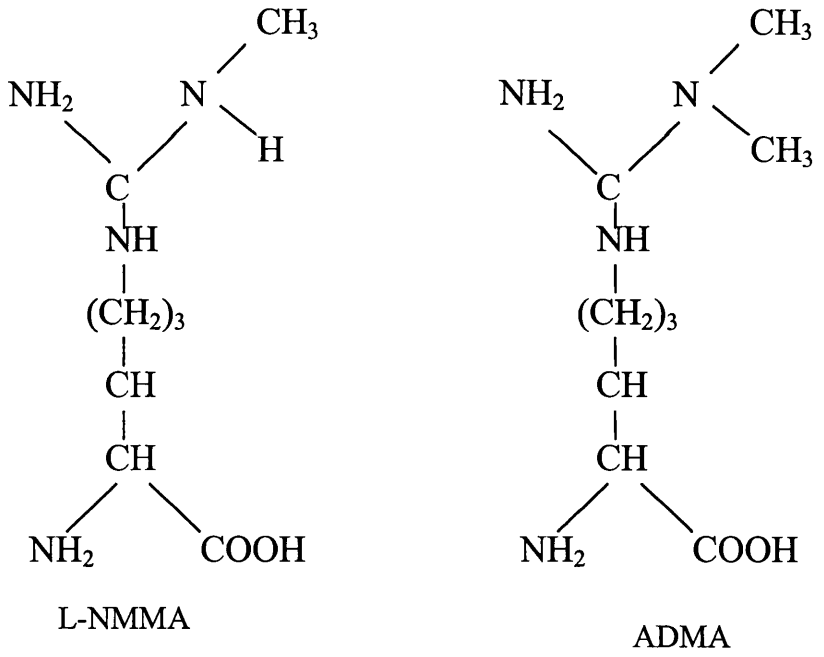

Figure 1.1b **L-arginine analogues that compete with L-arginine and inhibit NOS.**

1.15 Release of NO by nerves: neuronal (n)NOS

nNOS was the first of the NOS isoforms used for the characterisation and purification of NOS. It was named neuronal NOS (nNOS) because of its cellular origin from rat cerebral tissue (Bredt et al., 1990). nNOS is a homodimer with sub-units of approximately 150kDa. It is a soluble protein that requires calcium, calmodulin and NADPH (Bredt et al., 1990; Schmidt et al., 1991) as well as BH_4 (Mayer et al., 1990) for full activity. By utilising these characteristics, the enzyme could be purified using columns packed with 2'5'ADP sepharose (which binds NADPH requiring proteins) and affinity columns for calmodulin.

Once calcium activated calmodulin has bound to the relevant site on the nNOS enzyme, the conformational shape of NOS changes in association with activation. The requirement of nNOS for BH_4 is thought to stabilise the NOS protein (Giovanelli et al., 1991) and/ or act as a redox reagent, like NADPH, which serves as an electron donor (Mayer et al., 1990).

Immunohistochemical localisation of nNOS using antibodies raised by Bredt and Snyder in 1990 was observed in rat brain. Discrete neuronal populations were observed mainly in the cerebellum and the olfactory bulb, areas associated with hormone release and smell, respectively (Bredt & Snyder, 1990).

cDNA for nNOS, cloned and expressed a year later in human kidney 293 cells (Bredt et al., 1991), coded a protein that had structural homology with cytochrome P-450 reductase. nNOS has also been located in non- adrenergic non-cholinergic (NANC) inhibitory neurones, initially purified from the rat anococcygeus (Mitchell et al., 1991), and the bovine retractor penis muscle (Sheng et al., 1991). The release of NO from NANC nerves has also been shown to vasodilate and bronchodilate pulmonary artery and airways respectively alongside the concomitant release of other vasoactive factors (Mitchell et al., 1991; Sheng et al., 1991; Liu et al., 1992; Bredt et al., 1990), (Van der Velden et al., 1999).

1.15.1 **Regulation of nNOS expression**

Numerous stimuli have been shown to modulate the activity of nNOS (Forsterman et al., 1998). nNOS may be up regulated pre- or post-transcriptionally by stimuli such as heat, electrical activation and light (Sharma et al., 1997; Reiser et al., 1997; Goldstein et al., 1997). In addition, the up-regulation of nNOS may occur following exposure to hypoxia. An increase in nNOS levels, as a response to injury, has been observed following cerebral ischaemia (Zhang et al., 1994), with nNOS mediating brain damage by mitochondrial dysfunction and energy depletion (Bolanos et al., 1999). The nNOS is most likely affected at a pre-transcriptional level, with several *in vivo* studies illustrating a time-dependent increase in nNOS mRNA following hypoxia (Shaul et al., 1995; Prabhakar et al., 1996; Guo et al., 1997). This increased expression of nNOS may be a result of hypoxia induced factors acting on the response elements of the nNOS genes, as occurs for other similarly regulated response proteins (Kvieukova et al., 1995).

In addition to hypoxic stress, nNOS can be modulated in association with a number of different chemical agents. In the brain, nNOS levels may increase following the inhibition of glutamatergic transmission in cerebral nerves (Baader et al., 1996) and by increasing stimulation with endogenous ACh (using a cholinesterase inhibitor) in the hippocampus (Bagetta et al., 1993). In addition, sex hormones, including oestrogen and testosterone increase nNOS expression (Luckman et al., 1997; Reily et al., 1997) whilst corticosterone reduces it (Weber et al., 1994). The expression of nNOS may also become reduced in association with the mediators of sepsis, including endotoxin and cytokines (Forsterman et al., 1998).

1.16 Release of NO by endothelial cells: endothelial (e) NOS

eNOS, constitutively expressed like nNOS, was initially thought to be a soluble protein (Palmer et al., 1989; Mulsch et al., 1989). However, subsequent studies clearly showed that the majority of eNOS resides in the particulate fractions of cells (Forstermann et al., 1991; Mitchell et al., 1991). The purified particulate eNOS was found to have a number of similarities to nNOS. For instance eNOS requires calcium, calmodulin, NADPH (Pollock et al., 1991) and BH_4 (Pollock et al., 1993) for full activity. In addition, the amino acid sequence predicted the same regulatory sites as previously published for the nNOS (Sessa et al., 1992). Similar results have been

published using human umbilical vein endothelial cell cDNA (Janssens et al., 1992), with an M_r of approximately 144 kDa. However, both eNOS and inducible NOS (iNOS) are products of distinct genes (Sessa et al., 1992). Bovine endothelial cDNA (Sessa et al., 1992) coded a 4.8 kb transcript which gives rise to a protein with a denatured M_r (molecular mass) of 135 kDa (Pollock et al., 1991), similar in size to nNOS. In addition, eNOS cDNA, unlike nNOS cDNA, encodes for a N-myristylation site (Sessa et al., 1993), which alongside cysteine palmitoylation does not influence catalytic activity, but targets eNOS into calveolae (Liu et al., 1996), the role of which is to compartmentalise, modulate and integrate signalling events at the cell surface (Shaul et al., 1998).

1.16.1 **Regulation of eNOS expression**

In vitro and *in vivo*, the expression of eNOS may be modulated by many factors which include; shear stress (Nishida et al., 1992; Sessa et al., 1994; Xino et al., 1997), hypoxia (Ziesche et al., 1996; Kourembanas et al., 1997) cytokines (Inoue et al., 1995) and sex hormones (Kleinert et al., 1998; MacRitchie et al., 1997). Physical forces of shear and strain upregulate eNOS pre and post-transcriptionally. eNOS activity can increase in the absence of a maintained increase in intracellular Ca by phosphorylation of the NOS enzyme (Busse et al., 1998; Dimmeler et al., 1999; Fulton et al., 1999). In addition, a putative shear stress response element has been described in the promoter region of both the human and bovine eNOS genes (Marsden et al., 1993; Venema et al., 1994).

Hypoxia down regulates eNOS expression as has been shown in pulmonary endothelial cells (Ziesche et al., 1996; Kourembanas et al., 1997; Faller et al., 1999) and in the endothelium from systemic vessels (Forsterman et al., 1998). Furthermore, hypoxia increases the number of genes encoding cell mitogens produced by endothelial cells for example; platelet derived growth factor-B (PDGF-B), ET-1, VEGF. Some growth factors increase eNOS expression in endothelial cells. For example VEGF is known to enhance the production of eNOS mRNA and protein (Kroll et al., 1998; Hood et al., 1998), whilst transforming growth factor β increases eNOS mRNA and protein as a result of enhanced promoter activity (Inoue et al., 1995). However, the expression of eNOS in proliferating cells with respect to resting cells is unclear, with different studies observing diverse stability of the eNOS mRNA (Arnal et al., 1994; Flower et al., 1995). These conflicting observations may reflect the complexity of responses produced by NO in different cells.

In contrast, the effects of different cytokines on the activity and expression of eNOS is clearly defined (Forsterman et al., 1998). For example, tumour necrosis factor-α (TNF-α) can down regulate eNOS (Forsterman et al., 1998) by destabilising mRNA, whilst a combination of interferon and endotoxin can upregulate eNOS expression in bovine aortic endothelial cells (Bucher et al., 1997). This is not however, a consistent observation. *In vivo* a number of studies have shown endotoxin administration to result in the down regulation of eNOS (Liu et al., 1996), an effect that may be attributed to increases in endogenous levels of TNF-α.

As is the case for nNOS, sex hormones have been shown to increase levels of eNOS pre- and post-transcriptionally. Indeed, pregnancy and oestrogen, but not progesterone or testosterone, increase eNOS mRNA, protein and activity (Weiner et al., 1994; Goetz et al., 1994). Similar observations have been made using cultured pulmonary endothelial cells. Here oestrogen increased eNOS mRNA and activity by enhancing the promoter activity via an oestrogen responsive element (Kleinert et al., 1998; MacRitchie et al., 1997), whilst an acute effect of oestrogen on eNOS activity increases the influx of intracellular calcium via stimulation of oestrogen receptors (Lantin-Hermoso et al., 1997).

1.17 Release of NO by cells induced to express NOS: inducible (i) NOS

Inflammatory and infective agents 'induce' cells to express a distinct form of NOS, iNOS. This clear link between infection and NO formation was provided following the measurement of nitrate/nitrite excretion by humans and animals *in vivo* and macrophage cell lines *in vitro* (Green et al., 1981; Stuehr et al., 1987). By inducing macrophages to synthesise nitrates and nitrites following stimulation by LPS, L-arginine was found to be a requirement with L-citrulline formed as a byproduct (Iyengar et al., 1978). This dependence on L-arginine, in association with the formation of citrulline and nitrite, was further observed following the inhibition of mitochondrial respiration, metabolism and DNA synthesis in tumour cells following activation of macrophages with LPS (Hibbs et al., 1988). In addition, substituting the L-arginine with L-arginine analogues could inhibit this nitrite formation alongside the cytotoxic activities of the macrophages (Hibbs et al., 1988).

The induction of iNOS has now been demonstrated in most cell types *in vitro* (Cohen et al., 1998; Wong et al., 1995; Nathan et al., 1997) and in all organs of the rat

in vivo (Mitchell et al., 1993). In addition, there are a number of studies using different cell types, which clearly demonstrate that active iNOS is expressed in human tissues (Cohen et al., 1998; Wong et al., 1995; Nathan et al., 1997; Chester et al., 1998).

iNOS was initially purified from the cytosol of macrophage cell lines, which were activated with LPS and/or interferon-γ (IFN-γ)(Stuehr et al., 1991). The protein found had an apparent M_r of approximately 130 kDa, which was similar in size to both the eNOS and iNOS isoforms. The active iNOS (approximate M_r 250 kDa), required NADPH, BH_4, FAD and FMN, but not exogenous calcium or calmodulin for full activity (Stuehr et al., 1991). Further studies demonstrated that the iNOS enzyme contains activated calmodulin which is extremely tightly bound (Lee et al., 1998; Cho et al., 1992), thereby explaining the observed lack of dependence on exogenous calcium in this isoform (Busse & Mulsch, 1990). The production of NO therefore only occurs after a lag phase, due to the necessary induction of iNOS protein and results in the release of relatively large amounts of NO.

1.17.1 **Regulation of iNOS expression**

iNOS expression is more commonly known to be induced by pro-inflammatory cytokines and/or endotoxin, including interleukin-1-β, TNF-α and IFN-γ and therefore unlike its constitutive counterparts, may be regulated by anti-inflammatory steroids such as dexamethasone (Radomski et al., 1990). Inflammatory cytokines and/or endotoxin will alone or in combination, induce iNOS in a wide range of cell types (Cohen et al., 1998; Wong et al., 1995; Nathan et al., 1997; Chester et al., 1998). Moreover, growth factors such as platelet derived growth factor or VEGF may inhibit (Wong et al., 1995) or promote the induction of iNOS (Kroll et al., 1998). In a similar fashion to eNOS and nNOS, iNOS synthesis may be promoted by oestrogen via E_2 receptors in mouse uterine mast cells, and indirectly inhibited by progesterone (Hunt et al., 1997).

1.18 Classification of NOS isoforms

Because the three NOS isoforms were diverse in their location, stimulation and regulation, they could subsequently be purified and antibodies raised that recognised nNOS, eNOS or iNOS. Using these antibodies, studies revealed that all three NOS isoforms were expressed in other cell types. For example; nNOS was present in epithelial as well as smooth muscle cells of the airway and gut (Forsterman et al., 1998), eNOS was located in bone cells (Forsterman et al., 1998), and iNOS was found to be expressed constitutively in certain cells including those of the macula densa (Cohen et al., 1998; Licino et al., 1999). For these reasons the historical classification of eNOS, nNOS and iNOS has been modified to represent the order of purification of the enzyme. Thus, nNOS becomes type I NOS, iNOS becomes type II NOS and eNOS becomes type III NOS. However, for the purposes of this thesis the original classification will continue to be used. The activation and regulation of the 3 types of NOS is summarised in Table 1.2 below.

Isoform	Intracellular location /mw	Cellular location	Regulation	Mechanisms of regulation
nNOS (NOS I)	Soluble (150 kDa)	Smooth muscle cells, Airway, Gut, nerve cells, neurones, kidney cells, penis retractor muscle	Ca^{2+}/ Calmodulin dependent	Nerve impulses, heat, electrical activation, light, endotoxin, cytokines, hypoxia, humoral reagents, sex hormones
iNOS (NOS II)	Soluble (130kDa)	Macrophages, Brain most other cell types	Ca^{2+}/ Calmodulin independent	Proinflammatory cytokines, endotoxin, growth factors, sex hormones
eNOS (NOS III)	Particulate (135 kDa)	Smooth muscle cells, endothelial cells, bone cells	Ca^{2+}/ Calmodulin dependent and independent	Growth factors, hypoxia, sex hormones, humoral reagents, shear stress

Table 1.2 Localisation and regulation of NOS.

Activation of eNOS and nNOS occurs following stimulation of the cell, whereby an increase in intracellular Ca binds to calmodulin and activates it. The calcium/calmodulin complex then binds to both eNOS and nNOS resulting in activation. iNOS does not require Ca to become activated. However simulation with an inducing agent, such as lipopolysaccharide (LPS) is required. Transduction and transcription factors are activated resulting in the synthesis of new iNOS protein.

1.19 Substrate analogues

By substituting chemical groups on to one or more of the guanidino nitrogens of L-arginine, these analogues have generally proved to be inhibitors of NOS. Guanidines, amidines, s-alkyl isothioureas and other compounds containing the amidine function (-C(=NH) NH2) inhibit NOS (Southan et al., 1997). One guanidino-sustituted analogue of L-arginine in particular, asymmetric dimethylarginine (ADMA), has been detected in high levels in patients with chronic renal failure and pre-eclampsia (Vallance et al.,

1992; Fickling et al 1993)(Figure 1.1 b). Different analogues of L-arginine vary in their ability to inhibit the different isoforms of NOS. This phenomenon was first described with N^Gmonomethyl-L-arginine (L-NMMA) versus N^Gnitro-L-arginine methyl ester (L-NAME). Indeed, L-NAME is a more potent inhibitor than L-NMMA of the constitutive forms of NOS (eNOS and nNOS). By contrast, as an inhibitor of iNOS, L-NMMA is either more potent than L-NAME or of similar potency to L-NAME. In addition to the NOS inhibitors which inhibit the activity of all three isoforms, there are now a number of 'selective' inhibitors for different forms of NOS (Table 1.3) (Nathan et al., 1991; Southan et al., 1995; Moore et al., 1993; Vallance et al., 1992).

Substrate related inhibitors of NOS	
Non-selective	L-NMMA, Asymmetric -dimethy-L-arginine (ADMA), N-iminoethly-L-ornithine, N-amino-L-Arginine, N-nitro-L-arginine, N-nitro-L-arginine methyl ester (L-NAME). 1-H-pyrazole-1carboxyamide HCL(PCA)
iNOS selectivity	Aminoguanidine, Isothioureas, 1400W, 4 methyl PCA, [2-Amino-5, 6-dihydro-6-methyl-4h-1, 3-thiazine. HCl] (AMT.HCl)
nNOS selectivity	N-nitro-L-Arg-p-nitroanaline, 7-nitro indazole (+analogues), [N5-(1-Imino-3-butenyl)-L-Ornithine;L-VNIO] (Vinyl-L-NIO)
Others	
Flavoprotein binders	Diphenylene iodonium, Iodonium diphenyl, Di- 2-thienyl iodonium
Calmodulin binders	Calcineurin, Trifluroperazine, N-(4-aminobutyl)-5-chloro-2-naphthalensulfonamide, N- (6- aminohexyl)-1-naphthalen- sulfonamide
Haem binder	Carbon monoxide, NO
Depleter of BH_4	2,4-Diamino-6-hydroxypyrimidine
Inhibitors of iNOS induction	Corticosteriods, TGF-β-1, -2, -3, Interleukin (IL)-4, IL-10, PGE_2/ Iloprost
Inhibitor of NADPH consumption	Imidazole, Phenylimidazole
Binding NO	Haemo-proteins, Oxidised lipoproteins

Table 1.3 NOS inhibitors

1.20 Effector mechanisms utilised by NO

1.20.1 **Activation of guanylate cyclase**

The use of rapidly acting vasodilators such as nitroprusside and glyceryl trinitrate are well established for treating acute episodes of cardiac failure. The nitrates, by causing peripheral pooling of blood, decrease venous return and therefore the ventricular volume and cardiac output. All organic nitrates relax vascular and non-vascular smooth muscle via the release of NO and activation of soluble guanylate cyclase (sGC) (Murad et al., 1978) causing an increase in intracellular cGMP (Figure 1.2).

By reversibly binding to the haem group in sGC to form nitrosyl complexes, NO activates the enzyme, which leads to cGMP production. Indeed, many of the effects of endogenous NO are produced via the activation of guanylate cyclase, which then stimulates the synthesis of cGMP. In many cases, cGMP mediates the effects of NO via activation of cytosolic G kinases (Clementi et al., 1998). However, cGMP may directly cause the extrusion of Ca from the cell (Hobbs et al., 1996), and has in some cases also been observed to stimulate Ca release from IP_3 sensitive stores in the absence of protein kinase G, with the resulting effect additive to that of IP_3 (Murthy et al., 1998). The physiological responses to cGMP are governed by a family of phosphodiesterases (PDE's) which hydrolyse the cGMP making it inert.

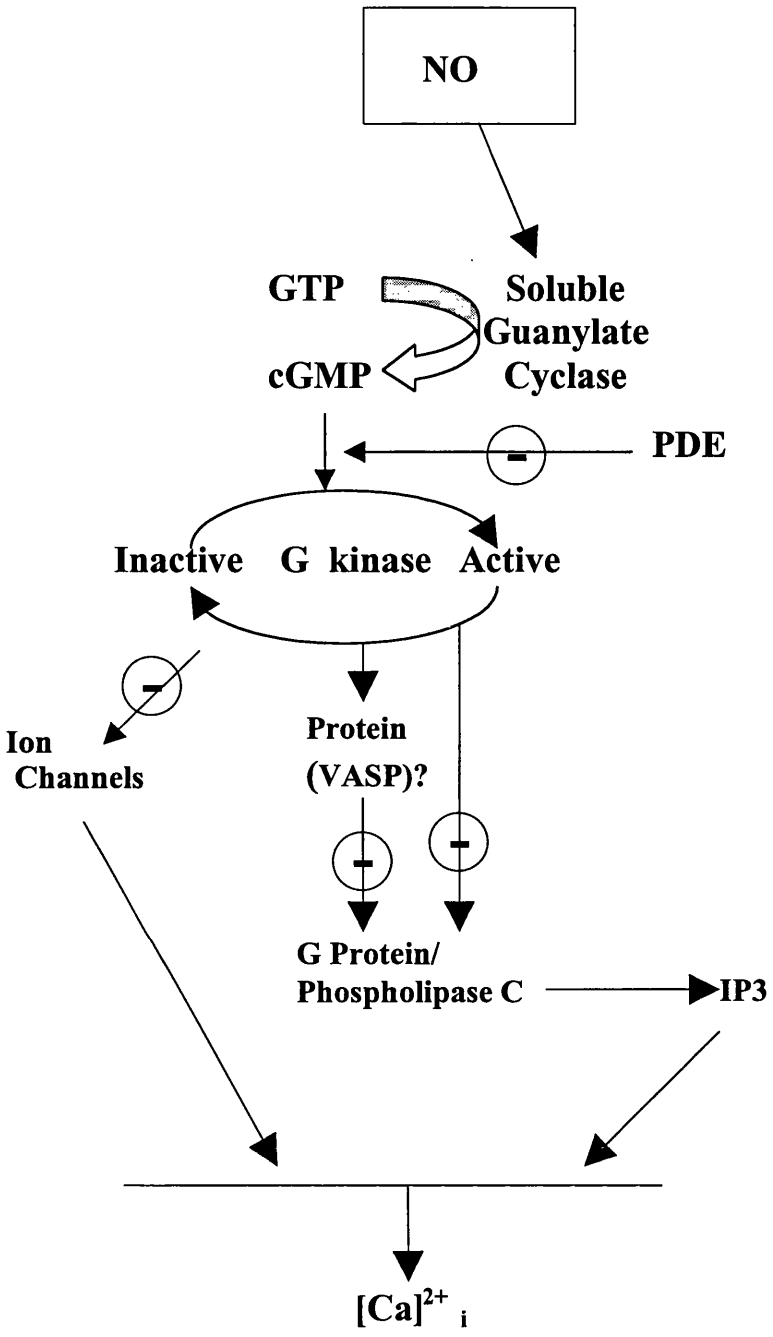

Figure 1.2 Activation of soluble guanylate cyclase by NO.

NO diffuses through and between cells. Once in the cytoplasm, NO activates soluble guanylate cyclase via modification of the haem centre. GTP is then converted to cGMP that can then go on to modulate a number of down stream targets including G kinase (cGMP kinase), which produce a decrease in intracellular Ca, leading to relaxation.

The intracellular levels of cGMP are tightly regulated by phosphodiesterase enzymes that metabolise it. Ca ~Calcium, PDE ~Phosphodiesterase, cGMP~cyclic guanosine monophosphate, GTP~ Guanosine tri-phosphate, VASP~ vasodilator stimulated protein, IP3~inositol tri-phosphate

Numerous responses following the stimulation of protein kinase G lead to a decrease in intracellular Ca (Table 1.4). One is to reduce IP_3 generation, consequently preventing the accumulation of inositol phosphate. Indeed, in blood vessels and platelets NO has been shown to reduce inositol phosphate generation (Rapport et al., 1986; Nakashima et al., 1986). However, the intermediate steps between G kinase activation and inositol phosphate inhibition are not well defined. The activation of G kinase has been suggested to result in the phosphorylation and inhibition of G proteins (Hirata et al., 1990; Light et al., 1990; Nguyen et al., 1991), or the modulation of some forms of phosopholipase C activity (Clementi et al., 1995). In addition, the putative actions of G kinase on G proteins or phosopholipase enzymes may be either direct or indirect via intermediate candidates. One example is the actin-binding protein, Vasodilator Stimulated Protein (VASP), which is an established substrate of protein kinase G (Smolenski et al., 1998).

The inhibitory effects of NO on Ca release may also occur via a G kinase-mediated phosphorylation of the IP_3 receptor, as has been demonstrated in smooth muscle and platelets (Komalavilas et al., 1996; Komlavilas et al., 1994; Cavallini et al., 1996) but not in all cells. IP_3 receptors are one of two families of channels through which calcium is released, the other which is structurally and functionally similar is that of ryanodine receptors. Recently it has been shown that NO can also directly activate ryanodine sensitive calcium stores in skeletal (type 1) and cardiac (type2) tissue by nitrosylating regulatory thiols (Stoyanovsky et al., 1997), modulating the release of Ca.

In addition to the movements of Ca from intracellular stores, there is also evidence to suggest that NO can modulate calcium exchange with the extracellular environment. For instance NO has a dual action on store operated calcium channels. At low levels of NO and cGMP, store operated calcium channels are activated, whilst at high concentrations these channels are inhibited (Xu et al., 1994). NO can also affect the functioning of second messenger operated calcium channels, particularly those linked to muscarinic receptors (Pandol et al., 1990; Mathes et al., 1996; Liu et al., 1997). In addition, NO via G kinase activation, has been shown to activate second messenger operated calcium channels, which have been compared to the receptors of growth factors (Clementi et al., 1995; Pfeifer et al., 1995).

In some cells however, NO does not appear to modulate the intracellular concentration of Ca (Clementi et al., 1998). This effect may be due to a lack of the cGMP/ G kinase mediated pathway in these cells.

Effector	Regulated function	Tissue
cGMP-dependent protein kinase(Soluble)	Voltage gated Ca channel (⇓)	Smooth muscle
	Receptor operated Ca channel (⇑)	Smooth muscle
	Na^+/Ca^{2+} exchanger (⇑)	Smooth muscle
	Plasma membrane Ca ATPase (⇑)	Smooth muscle Ca extrusion (⇑)
	ER Ca^{2+} ATPase (⇑)	Smooth muscle. Uptake of Ca into ER
	Phospholamban phosphorylation (⇑)	Smooth muscle. Increases the activity of the ER Ca pump
	IP_3-triggered Ca release (⇓)	Smooth muscle
	IP_3 receptor phosphorylation (⇑)	Smooth muscle
	G-protein phoshorylation (⇓)	Smooth muscle
	Phosphatase inhibitor phosphorylation(G-Substrate) (⇑)	Cerebellum
	Voltage -gated Ca channel (⇓)	Heart
	Amiloride-sensitive Na^+ channel (⇑)	Kidney
	Ca -activated K^+ channel (⇑)	Pituitary/Smooth muscle
	Intracellular Ca release (⇓)	platelets
	VASP phosphorylation (⇑)	platelets

(⇑) Increased activity (⇓) Decreased activity

Table 1.4 Mechanisms of action of cGMP. Adapted from; Hobbs and Ignarro: The NO/ cGMP signal transduction system

1.20.2 Interactions between superoxide anions and NO: formation of peroxynitrite

The chemistry of NO exists largely due to the arrangement of electrons in the molecule, a single unpaired electron defining the molecule as a free radical. However NO does not exhibit the high reactivity associated with most free radicals, such as the tendency to dimerise, but nevertheless, in the presence of superoxide radical, reactions occur which lead to highly reactive species being formed (Stamler et al., 1992). The combination of NO with superoxide anions leads to the detoxification of both superoxide and NO, but a hydroxyl radical (a potent oxidant) may be formed as a biproduct of the reaction (Beckman et al., 1990). Peroxynitrite, a potent oxidant that can contribute to many of the damaging effects of NO, may also be formed following the combination of superoxide with NO, with the presence of nitrosylated proteins indicative of the reaction that has taken place (Beckman et al., 1996). The relative effects of NO can therefore change depending on the availability of superoxide, which itself is removed by isoforms of superoxide dismutase (SOD) (Chabot et al., 1998). Thus the level of SOD activity present in tissues is a very important component in the overall effect of NOS activation.

It has recently been suggested that NOS activity may independently result in the generation of peroxynitrite. The absence of L-arginine may be thought to be a requirement for this reaction to take place as L-arginine has been suggested to inhibit this process (Pryor et al., 1995). However, other groups suggest that the actions of L-arginine require the concomitant presence of BH_4 to inhibit superoxide production (Vasquez-Vivar et al., 1998). The generation of superoxide may occur at low rates of NOS activity in the absence of calcium and calmodulin, or in their presence, which increases superoxide formation.

1.20.3 Interactions with enzymes

NO can activate or inhibit a large number of enzymes. Indeed, NOS itself can be modulated by NO (Brown et al., 1999). This may occur by NO binding to the haem site of NOS, or indirectly as a result of inhibition of the induction of iNOS (Mitchell et al., 1995). In addition NO can stimulate or inhibit cyclo-oxygenase (Mitchell et al., 1995). The activation of cyclo-oxygenase by NO can occur by NO providing hydroperoxide substrate following the formation of peroxynitrite (Landino et al., 1996). The inhibitory effects of NO on COX may occur as with NOS, via interaction with the haem centre or through nitrosylation (Mitchell et al., 1995). In addition, NO can inhibit the induction of cyclo-oxygenase protein (Swierkosz et al., 1995), and is thought to maintain COX-2

gene expression (Perkins et al., 1999), though the mechanism by which this occurs is unknown. NO, as previously mentioned, activates cGMP dependent kinase. In addition it may also interact with nucleotides, affect iron homeostasis, and through nitrosylation, inhibit the binding of NFK-β to DNA (Upchurch et al., 1996).

1.21 Role of NO in PVR

NO is an endothelium dependent vasoactive agent that mediates smooth muscle cell relaxation in the pulmonary vasculature. The importance of NO in the maintenance of a low vascular tone has recently been studied in humans. This has been demonstrated following a number of studies where NOS inhibitors have been administered to healthy human volunteers, causing an increase in PVR (Stamler et al., 1994; Kiely et al., 1998). NO release which maintains pulmonary vascular tone or basal tone, has also been described in different animal models (Ignarro et al., 1988; Glusa et al., 1993; Crawley et al., 1992). It was described by Ignarro and co-workers who showed the endothelium dependent contraction of quiescent bovine pulmonary arteries to methylene blue (Ignarro et al., 1988).

In the lung, the NOS isoforms, which are differentiated by their mode of stimulation, regulation, activation and cellular localisation, are distributed widely in both the airway and vascular cells (Fischer et al., 1996; Kobzik et al., 1993; Sherman et al., 1999). Constitutive NOS has been located in the pulmonary vascular endothelium and smooth muscle cells although the concentration of NO released from the smooth muscle cells is insufficient to relax an endothelium deprived vascular preparation (Zehetgruber et al., 1993). Pulmonary endothelial cells and vascular smooth muscle cells also contain the soluble iNOS isoform. This has been found in maternal sheep pulmonary vascular smooth muscle and airway epithelium using immunostaining techniques (Sherman et al., 1999) and produces large quantities of NO in response to stimulation (Kobzik et al., 1993;Nakayama et al., 1992; Thomae et al., 1993).

NO may be released from the endothelial cells following receptor or nerve stimulation. These stimulants trigger an increase in calcium in the endothelial cell, which stimulates NOS to produce NO. Endothelium dependent stimulation of NOS by receptors includes; histamine, bradykinin and ACh. Bradykinin is a potent pulmonary vasodilator that may be stimulated at rest and in flow-induced situations and by oxygen (Hornig et al., 1997). Furthermore vasorelaxation induced by bradykinin only occurs when vascular tone is higher than the normal resting state (Frantz et al., 1989; Glasgow

et al., 1997). ACh released from nerve terminals acts on three distinct muscarinic receptors (M1-M3) on both endothelial and smooth muscle cells. The binding of ACh to its receptors causes the direct opening of Ca channels and in the case of endothelial cells causes a subsequent increase in NOS activity. However, ACh binding to muscarinic receptors on smooth muscle cells elicits a vasoconstriction. In addition, ACh stimulates the concomitant release of an endothelial derived hyperpolarising factor (Chen et al., 1989). Similarly histamine also stimulates the release of EDHF concomitantly with NO, via H1 and H2 receptors on the endothelium (Abacioglu et al., 1987), (Table 1.1).

1.21.1 **Role of NO in the control of fetal PVR**

NO plays an important role in the regulation of tone in the fetal pulmonary circulation as has been demonstrated in the rat and sheep models *in vitro* and *in vivo* (Tiktinsky et al. 1992, Abman et al., 1990; Gordon et al., 1993). In fetal lungs a decrease in PVR has been observed following the infusion of ACh (Tiktinsky et al., 1992), whilst the inhibition of NOS increases PVR in association with the attenuation of acetylcholine induced blood flow (Gordon et al., 1993; Shaul at el. 1993). Recent *in vivo* studies showed that following NOS inhibition in fetal ovine lung, partial closure of the ductus led to an immediate and sustained increase in PVR (Storme et al., 1999). In addition, the contribution of NO to this potent myogenic response was revealed as when NOS was not inhibited the haemodynamic increases in PVR following partial ductus closure became transient (Storme et al., 1999).

In association with the production of NO, the levels of pulmonary constitutive eNOS enzyme following immunostaining, have been observed to be higher in the fetus than in other postnatal age groups (Xue et al., 1996; Halbower et al., 1994). In a similar fashion, expression of both endothelial and neuronal NOS protein in fetal and newborn lung increases prior to parturition but postnatally has been observed to decrease (North et al., 1994). In addition to eNOS, the iNOS isoform has also been located in fetal pulmonary vascular smooth muscle (Sherman et al., 1999).

1.21.2 **Role of NO in the transition of the pulmonary circulation at birth**

NO, important in the fetal pulmonary circulation is subsequently believed to play a major role in pulmonary vasodilation associated with the increase in PaO_2 that occurs at birth. *In vivo* studies in the ovine fetus have shown that following NOS

inhibition, the normal decrease in PVR that accompanies oxygenation and ventilation of the lung is attenuated (Abman et al., 1990).

Ventilation and subsequent stresses of shear and stretch are introduced into the lung at birth as the PVR decreases, and blood flow increases. These stimuli may directly upregulate NO production promoting vasorelaxation, in a fashion that does not require an increase in intracellular calcium. Fluid shear stresses are known to increase NO production from cultured endothelial cells, and activate eNOS by separate signalling pathways to the receptor dependent agonists (Fleming et al., 1998, 1997). Shear stress activates the phosphorylating protein Akt (Dimmeler et al., 1999; Fulton et al., 1999) which in turn phosphorylates, and subsequently activates eNOS on serine and tyrosine residues (Corson et al., 1996; Fleming et al., 1998). In addition, fluid shear stresses also upregulate eNOS activity by association with a heat shock protein in cultured endothelial cells (Garcia-Cardena et al., 1998). This shear induced production of nitric oxide has been observed in the systemic vascular bed, but in the pulmonary vascular bed it is uncertain how much a role this plays as the effect of shear induced *in vitro* varies depending on the species studied (Barnard, 1993).

However, at birth, in addition to the ventilatory stimuli, the contribution of NO to the decrease in PVR and increase in flow has been largely associated with the oxygenation of the lung, as many studies in the ovine fetus have shown (Tiktinsky et al., 1993; Shaul et al., 1992). In the fetus, the entire increase in pulmonary blood flow caused by hyperbaric oxygen is blocked following addition of the NOS inhibitor L-NA (Tiktinsky et al., 1993). In addition the relaxant responses to ACh were diminished following a decrease in oxygen tension, and enhanced when oxygenation increased, whilst the responses to nitroprusside remained unaffected (Shaul et al., 1992). Molecular studies measuring eNOS in pulmonary artery endothelial cells from the fetal lamb, have observed that oxygenation increases eNOS mRNA more than ventilation, subsequently increasing protein expression (Black et al., 1997).

In addition, oxygen may indirectly stimulate NO production via factors such as bradykinin (Ignarro et al., 1987), or via non-humoral up-regulation through the production of the oxidoreductases thioredoxin, and thioredoxin reductase (Das et al., 1999), which in fetal baboons upregulates eNOS. Thus the total decrease in PVR at birth may be mediated by NO in combination with other factors such as PGI_2, EDHF and oxygen itself (Figure 1.3).

1.21.3 Role of NO in the control of postnatal PVR

In a similar fashion to the production of other vasoactive substances in the pulmonary vasculature, the production and release of NO postnatally varies in an age-related fashion. Relative to that seen in near-term fetal lambs, both basal and stimulated NO from pulmonary arteries is enhanced postnatally (Abman et al., 1991). However in isolated porcine pulmonary arteries, the relaxant response to ACh is absent at birth, and is only present by 3 days of age (Liu et al., 1992). This is in contrast to other studies in isolated perfused porcine lungs where relaxant responses to bradykinin in the presence of indomethacin show that relaxation at 1 day of age is greater than at 7 days of age (Perreault et al., 1993). In addition, relaxation to bradykinin in the presence of indomethacin, improves from 3-10 days of age and is maintained at this level until adulthood (Zellers et al., 1991).

In correspondence with the increased relaxation produced by certain agents that act via the NO pathway, Hislop and co-workers showed an increased immunostaining from birth for endothelial eNOS in the porcine lung at 3 days of age. However this decreased in the adult to below newborn levels (Hislop et al., 1995). In addition to alterations in endothelial cell function, in the smooth muscle cells the postnatal development of guanylate cyclase (GC) activity and cGMP content also occurs. In isolated porcine pulmonary arteries at birth, a high basal accumulation of cGMP has been observed which by 3 days of age has decreased and is maintained at this level with age (Tulloh et al., 1997). Thus the entire vascular pathway which produces a relaxant response to NO is altered with postnatal development.

1.21.4 Role of NO in the control of PVR with PPHN

In cases of pulmonary hypertension the NO/cGMP pathway is altered. It is believed that hypoxia induced pulmonary hypertension attenuates the relaxant response to ACh through a decrease in the production of NO through the content of NOS. Using immunohistochemistry the levels of eNOS were shown to be reduced in the pig model of hypoxia induced pulmonary hypertension (Hislop et al., 1997). Similarly in hypertensive fetal lambs pulmonary eNOS protein and mRNA content decrease following ductal ligation (Black et al., 1998). This decrease is reflected in relaxant responses to agonists that stimulate the production of NO. In the rat pulmonary artery, ACh relaxant responses have been attenuated following monocrotalline induced hypertension (Altiere et al., 1986). In addition, in porcine intrapulmonary arteries

following exposure to chronic hypoxia from 3 days of age, the newly developed relaxant responses to ACh and A23187 were abolished (Tulloh et al., 1997). Furthermore, eNOS knockout mice display characteristics of pulmonary hypertension (Fagan et al., 1999). Contributing to the observed decrease in NO release, the NOS substrate L-arginine has been found to be deficient in some infants with PPHN (Vosatka et al., 1994; Castillo et al., 1995).

In addition, smooth muscle relaxation to NO has also been observed to be impaired with PPHN. Following exposure to chronic hypoxia from birth and from 3 days of age, relaxations to SNP and exogenous NO were reduced in porcine intrapulmonary arteries (Tulloh et al., 1997). Following 4-5 weeks of exposure to chronic hypoxia in adult rats cGMP levels have been observed to decrease (Rodman et al., 1992). Recent studies have shown that cGMP specific phosphodiesterase activity is markedly elevated in an experimental model of perinatal pulmonary hypertension (Hanson et al., 1998). Similarly, ductus compression increased phosphodiesterase activity in association with a decreased sGC activity in fetal lambs, and decreased protein kinase activity (Black et al., 1998). In contrast, cGMP has been observed to be elevated relative to controls in piglets with PPHN (Tulloh et al., 1997) and in hypertensive lambs, the integrity of the smooth muscle relaxant response was maintained in pulmonary arteries, following normal vascular relaxations to exogenous cGMP (Steinhorn et al., 1995). Thus the precise effects of hypoxia induced pulmonary hypertension on smooth muscle responses is not clear, although in the majority of cases studied some alteration in the cGMP pathway is thought occur.

1.22 The role of NO in the veins

It has been previously shown experimentally that veins are more vasoactive than their paired arteries. This also applies to the production and release of NO observed in the veins from sheep and pigs (Bina et al., 1998; Steinhorn et al., 1993; Gao et al., 1995). Immunohistochemistry of the fetal ovine lung has shown that eNOS is present in veins and arteries (Halbower et al., 1994) and in the porcine pulmonary vein is consistently high but in the arteries varies with age (Hislop et al., 1995). Furthermore, stimulation of NOS using the Ca ionophore A23187 elicits a greater relaxation in fetal ovine veins than arteries (Steinhorn et al., 1995).

In addition to the greater stimulated release of NO, the smooth muscle responses in fetal lambs to NO and the NO donor, SNP, have been shown to be greater than their paired arteries (Steinhorn et al., 1995). This may be due to the greater distribution of sGC in the pulmonary veins of fetal lambs than arteries (D'Angelis et al., 1998) also observed in the mature adult porcine lung (Bina et al., 1998). Therefore, the veins have been shown to maintain the greater ability to relax through the increased production of NO, and the greater smooth muscle response to NO.

Hypoxia induced pulmonary hypertension also affects the pulmonary venous responses to NO. Following chronic hypoxia in the rat, venous relaxant responses to ACh were increased (Lal et al., 1999). However, following pulmonary hypertension, in veins from the fetal lamb the smooth muscle relaxation to A23187, SNP and NO were maintained, unlike relaxation in the pulmonary arteries which was greatly diminished (Steinhorn et al., 1995). This suggests that following hypoxia induced pulmonary hypertension, the alteration in the relaxant responses may be limited to the endothelium. In addition, progressive obstruction of piglet pulmonary veins have been shown to produce large increases in arterial pressure, but not venous pressure (LaBourene et al., 1990) inferring that the pulmonary veins have a greater capacity to dilate than arteries under conditions of hypertension. Thus there is no clear picture of the role of the veins in PPHN.

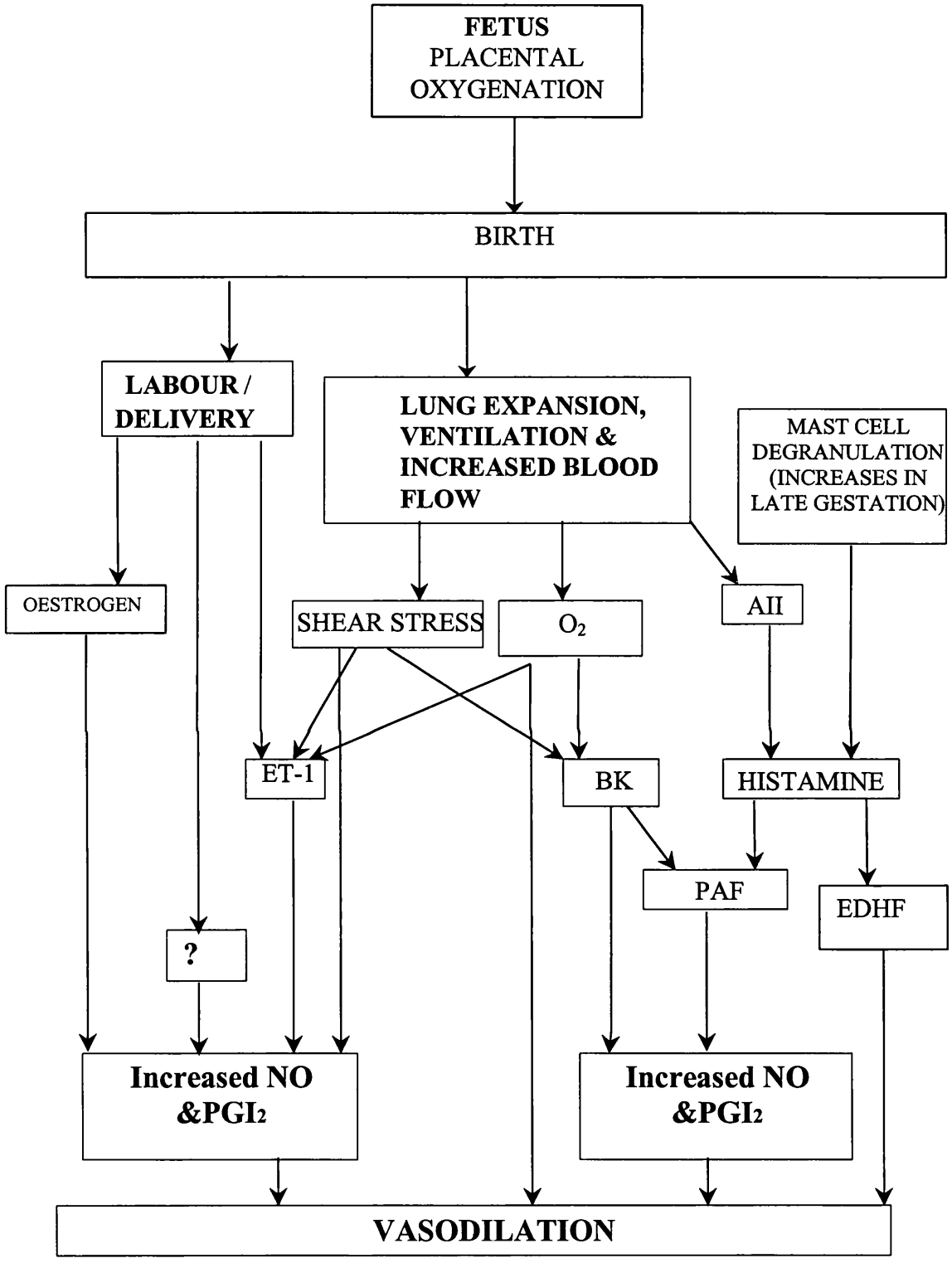

Figure 1.3 Schematic representing the putative pathways that are involved in the pulmonary vasodilation that occurs at birth

1.26 Management of PPHN

Mortality rates in infants with PPHN approach 40% despite current therapeutic modalities (Bartlett et al., 1985). Both extracorporeal membrane oxygenation (ECMO) and high frequency ventilation (HFV) are effective as treatments in some critically ill infants (Bartlett et al., 1985; Toomasian et al., 1988; Boynton et al., 1984) alongside hyperventilation and intravenous vasodilators (Greenough et al., 1992). If treatment of the underlying disease is ineffective, or the cause for the hypertension is idiopathic, then direct attempts to dilate the pulmonary circulation can be made. The use of vasodilators is limited, as they need to specifically dilate the pulmonary circulation only. In addition, intravenous vasodilators may dilate vessels in non-ventilated lung regions and increase intrapulmonary shunting, further impairing gas exchange. One putative agent is ATP, which in low doses in lambs acts as a selective pulmonary vasodilator (Konduri et al., 1991). However because the selective decrease in PVR at birth is mediated by NO and PGI_2, and production of these mediators is decreased with pulmonary hypertension, attempts have been made to use PGI_2 and NO to treat pulmonary hypertension. However, PGI_2 is not a viable vasodilator because of the lack of specificity to the pulmonary vascular system.

1.26.1 **NO therapy**

Inhalation of NO aims to directly affect those areas of the lung which are ventilated, thus avoiding a worsened ventilation-perfusion mismatch. Moreover NO is rapidly inactivated in the blood by haemoglobin, and thus protects the systemic vascular bed from vasodilation (Frostell et al., 1991; Rossaint et al., 1993). Two preliminary studies have shown inhaled NO to be effective in reversing hypoxaemia due to PPHN. The acute effects of NO treatment in severe cases of PPHN reported that inhaled NO at 80 ppm improved post-natal oxygenation without changing Pa CO_2 or systemic blood pressure (Roberts et al., 1992). Moreover an acute improved oxygenation and echocardiographic signs of pulmonary hypertension without systemic hypotension were observed in neonates with PPHN given lower doses of NO (10-20ppm)(Kinsella et al., 1992). In addition, inhaled NO during HFV improved oxygenation more than with either HFV or NO alone. The responsiveness to NO may depend on the specific diseases associated with PPHN (Kinsella et al., 1993). The outcome of NO

administration, and any adverse effects may also depend on the doses given (Kinsella et al., 1999).

1.24 Aims of thesis

The alteration in the structure and function of the lung with increasing maturational age has been extensively studied. However, the differentiation in the pulmonary responses between arteries and veins is not clear in relation to those structural changes. This relationship is important because both types of vessel contribute to the ventilation perfusion matching of the blood in the lung. In addition, the relationship of NO with pulmonary maturation is not clearly defined. It has been demonstrated that NO plays an important role in the functional adaptation of the lung following experimental work and clinical evidence of its application in cases of PPHN. The effects of NO are achieved by highly developed mechanisms for the regulation of its release, most evident in the transition of the pulmonary circulation at birth, and in the developing postnatal lung. A further layer of regulation is provided for by the different transduction mechanisms utilised by NO in different cells. The most important effector pathway for NO is the activation of the soluble form of guanylate cyclase, which also alters with pulmonary development.

Therefore the aims of this thesis were to investigate the alterations in the NO pathway that may have occurred with pulmonary development. This may be done by measuring the pulmonary activity of NOS with age and with PPHN, and by studying the relationship between NOS activity and vascular reactivity with age. Thus the contractile and relaxant properties of arteries and veins were studied from pigs at different stages of pulmonary development and from pigs with PPHN. The relationship of NO with the observed age related changes was further studied by modulating components of the NO/cGMP pathway in experiments measuring pulmonary vascular reactivity. Investigation of the pulmonary vasculature in the hypertensive state gives further insight into the dysfunction of pulmonary vascular development that occurs with PPHN. Finally, the stimuli altering pulmonary vascular reactivity and the subsequent decrease in PVR at birth were investigated in relation to the NO pathway.

Thus, the alterations in the NO-cGMP pathway will be investigated in the transition from the fetal to the neonatal circulation and in postnatal pulmonary

development. In addition, it was intended to try and understand how these mechanisms may have altered in the pathological state of PPHN.

Chapter 2. General methods

2.1 Animal model

In this study, a porcine model was used to examine lung development and adaptation to extrauterine life. Pigs were used for two main reasons (i) previous studies have observed similarities between the postnatal structural adaptation that occurs in the lungs of the Large White pig with that of the human (Haworth et al., 1981) and (ii) extensive information already exists illustrating the changes in pulmonary structure, function and pharmacology associated with this model. Because the porcine model used in this thesis has been on site for many years, principles in the management and handling of the animals were well established. Thus, the animals used in the experiments of this thesis were handled with relative ease and rapidity, with a minimum of stress to the animals. The rapidity of the manner in which the animals were handled was important, as adaptation of the lung to normoxia occurs very quickly at birth and in the 3 or 6 day old hypertensive pigs.

2.1.1 **Lungs from normoxic pigs**

Porcine lungs from Large White pigs, were studied in the following age groups: Fetal (1 week pre-term), breathing fetal pigs (1 week pre-term that had been allowed to breathe spontaneously), newborn (<5min), non-breathing newborn pigs (<5min that had been prevented from breathing spontaneously), 1 day, 3 days, 5-6 days and 14 days old. The fetal pigs, breathing and non-breathing, were collected from Selbourne Biological supplies Ltd., Basingstoke, UK. Lungs from adult pigs were collected from a commercial abattoir.

For fetal controls, animals were delivered by Caesarian section and killed immediately before air breathing had occurred by placing a rubber condom over the snouts of the piglets, then giving them an intra-peritoneal injection of pentobarbitone (Expirol) (100mg/kg). Staff at Selbourne had previously killed the mother by exsanguination following electrocution. For breathing fetal pigs, the pigs were delivered like the controls but allowed to breathe spontaneously without aid for 5 minutes. These piglets had had the membranes wiped from their snouts and were rubbed dry. Within 5 minutes, they had become pink and appeared to have regular respiratory functions and were walking.

Newborn piglets were delivered in the Institute of Child Health, normally after the full term of gestation, and were then killed with an overdose of pentobarbitone (100mg/kg), within 5 minutes of their breathing. Newborn pigs that did not breathe were delivered in a similar fashion but killed immediately before air breathing had occurred by placing a condom over the snout and injecting an overdose of pentobarbitone (100mg/kg).

The older age groups studied were piglets either from the litters of the sow, delivered within the Institute of Child Health and maintained until the relevant age, or they were piglets transported live to the Institute 24 hours before they were used from Selbourne Biological supplies Ltd., Basingstoke, UK. The animals were killed as previously described. Those animals that were too young to feed themselves were gavage fed with modified cows milk.

In all the animals studied the thoracic cavity was opened and the heart and lungs removed en-bloc. The cardiac lobe was snap frozen in liquid nitrogen within 5 minutes of death for studies using whole lung tissue. The remaining lung was stored in cold Krebs-Henseleit solution and transported to the laboratory for further dissection.

The adult tissue from the abattoir was collected within 20 minutes of death. These pigs were killed by electrical stunning followed by exsanguination and the lungs removed. On site the cardiac lobe was removed and snap frozen, but whole lobes were transported to the laboratory in ice-cold Krebs-Henseleit solution by motorcycle courier within 2 hours of death for use in the organ bath experiments.

2.1.2 **Lungs from hypoxia induced pulmonary hypertensive pigs**

Newborn or 3 day old pigs were placed in a hypobaric chamber for 3 days with a continuous supply of modified cows milk. The newborn piglets were delivered normally from the sow at the Institute of Child Health and placed in the chamber within 20 minutes of birth. The 3 day old piglets came from a litter that had been delivered from the sow in the Institute, thus the precise age of the piglets when entering the chamber was known. The chamber contained a heating lamp and straw for bedding. The internal temperature was maintained at 29°C and the air pressure maintained at 50.8 kPa. The chamber was cleaned and food replenished three times daily for a maximum of 20 minutes. The younger aged animals were not able to feed themselves, therefore they were gavage fed with modified cows milk. Animals placed in these chambers develop pulmonary hypertension with right ventricular hypertrophy and a systemic arterial

oxygen saturation of 71±5% with pulmonary hypertension indicated by cyanosis due to right to left shunting through persistent fetal channels (Tulloh et al., 1997).

After 3 days in the chamber, the animals were killed with an overdose of pentobarbitone (100mg/Kg), and the lungs collected for different types of analysis.

Wherever possible other pulmonary tissue was used from each animal by other investigators working within the department.

All animals received humane care in compliance with the British Home Office Regulations and with the *Principles of Laboratory Animal Care* formulated by the National Society of Medical Research and the "Guide for the Care and Use of Laboratory animals" published by the National institutes of Health [DHEW Publication No. (NIH) 80-23, Revised 1985, Office of Science and Health reports, DRR/NIH, Betheseda, MD 20892].

2.2 Assessment of whole lung NOS activity (Ref. chapter 3)

2.2.1 **Preparation of crude lung homogenate**

In order to investigate the activity of the NOS enzyme in the lung, homogenised cardiac lobes were studied, subsequently termed crude homogenates. The following age groups were studied: Fetal controls, breathing fetal pigs, newborn, 1, 3, 6 and 14 day old pigs and adult, together with 3 day and 6 day old pulmonary hypertensive pigs. The cardiac lobes from lungs of the younger developmental age groups and the adult were snap frozen in liquid nitrogen within 5 or approximately 20 minutes of death respectively. The lung tissue was weighed and then homogenised on ice using a polytron grinder in assay buffer (500mM Tris HCl, pH7.5 containing 0.1mM EDTA and 0.1mM EGTA) in a 1gram: 5ml ratio. This was carried out in the presence of the protease inhibitor phenylmethanesulfonyl fluoride (PMSF 1mM) to prevent protein degradation. After the tissue had been successfully homogenised, it was then sonicated over ice in 3 short blasts of 10 seconds duration. 30 µl of the cold homogenate was then taken to be used for the NOS assay and 10µl taken for the protein assay. The rest of the homogenate was stored at –80°C for future experiments.

2.2.2 **Measurement of NOS enzymatic activity**

NOS activity was determined by measuring the conversion of L-[^{3}H] arginine to L-[^{3}H]citrulline (Pollock et al., 1991, Figure 2.1). The homogenate (30µl) was

incubated with a reaction mixture that maximally stimulates NOS (total volume 100 µl). This contained each of the following: NADPH (1 mM), BH_4 (10µM), $CaCl_2$ (2.5 mM), calmodulin (28 units), L-arginine (10 µM) with L-[^{3}H] arginine (1mCi/ml, 55Ci/mMol), and L-valine (500mM). L-valine was required to inhibit arginases, enzymes from the ornithine cycle, which produce L-Citrulline or L-arginine by circumnavigating the metabolism of NO via NOS.

The Ca dependence of the NOS enzyme was evaluated in another reaction mix by the replacement of 2.5 mM $CaCl_2$ with 2.5mM EGTA, a Ca chelator. The amount of L-citrulline produced from NOS, and not via other physiological pathways, was evaluated by addition of 1M L-nitro arginine methylester (L-NAME) to another reaction mix in the presence of Ca. In some incubations Tris buffer was added to adjust the final volume to 100µl. After all the additions, this mixture was left at room temperature for 30 minutes.

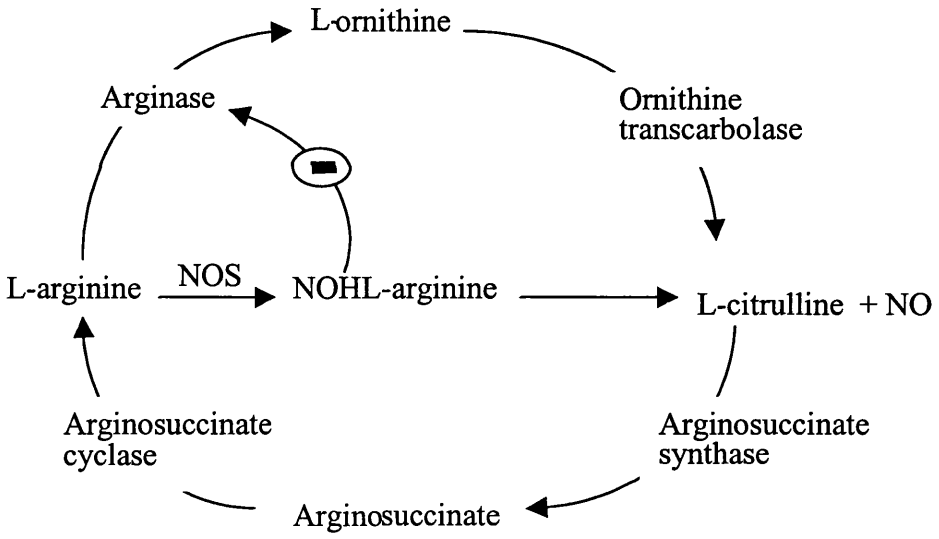

Figure 2.1. The ornithine cycle which produces L-arginine and L-citrulline in the presence of essential enzymes. L-arginine acts as a substrate for both NOS and arginases.

After the incubation period the reaction was stopped with the addition of 1ml of cold stop buffer (20mM HEPES, pH5.5; EDTA 0.1 mM; EGTA. 0.1 mM). This mixture was then passed over a 1 ml column containing Dowex AG 50WX-8 resin (Na^+ form, pre equilibrated in Stop Buffer) and then eluted with 1ml of Hepes buffer. The total volume of approximately 2.1ml, containing L- [^{3}H] citrulline was collected in 20ml scintillation vials and quantified by β-scintillation counting. In addition, by measuring

the activity of the mixture without lung homogenate present (made up to 100 μl), the total amount of activity in the mixture (total) was assessed. Furthermore, by passing a "total" incubate (made up to 1.1 ml) over the column, the effect of the Dowex beads on the binding of L-arginine in the absence of the homogenate (blank) was assessed. As the production of L-citrulline from L-arginine (and therefore NO) occurs at a 1:1 ratio, given the initial amount of L-arginine, the amount of L-citrulline produced may be used as a guide for the activity of NOS.

2.2.3 **Analysis of NOS activity**

NOS is contained not only in the pulmonary vasculature, but also in the airways and surrounding parenchyma. The citrulline assay in these experiments was used to assess activity in the whole lung and did not therefore indicate the cellular origin of the NOS. Thus these experiments were used to identify trends in NOS activity with age, without specifically targeting changes of activity in blood vessels.

In addition, by using whole lung samples, levels of endogenous L-arginine may be present in the un-purified lung homogenate. This would dilute the amount of labelled L-arginine and thus the amount of radiolabelled L-citrulline measured by an unknown amount (Giraldez et al., 1990). Furthermore, levels of L-citrulline may be underestimated following the conversion of L-citrulline to L-arginine in the presence of arginases. Thus these limitations must be taken into account when analysing and interpreting the data.

The activity from the β -scintillation counter was measured as disintegrations per minute or DPM. Each tube contained 1000 pmol of unlabelled L-arginine, plus approximately 3 pmol of L-[^{3}H] arginine resulting in a content of 100,000-200,000 DPM. Therefore in a typical assay each total contained ≈150,000 DPM, each blank contained ≈4,000 DPM and a sample with detectable NOS activity ≈10,000-40,000 DPM.

Therefore:

$$\text{NOS activity (pmol)} = \frac{\text{DPM in sample- DPM in Blank}}{\text{DPM in total}}$$

The activity was then corrected to protein using the following equation:

$$\text{Protein (from assay)} = \text{X } \mu\text{g/ml}$$

Volume of tissue in aliquot = 30µl

Thus in the 30µl aliquot there is $\frac{X}{33.3}$ µg protein

Therefore the citrulline/NOS activity (pmol activity) is divided by the amount of protein present to give a value of pmol /µg protein.

The total NOS activity present was represented as the total L-citrulline converting activity in the presence of Ca, minus any activity in the presence of L-NAME. In a similar fashion the activity in the presence of EGTA also had the activity in the presence of L-NAME subtracted from it.

Total NOS activity was that which was found in the presence of Ca. The total NOS activity was therefore a mixture of activity of both Ca dependent and independent NOS. The proportion of Ca dependent activity was calculated by subtracting the Ca independent activity from the total activity. This data was also represented as a percentage of total NOS activity (100%).

2.2.4 **Protein Assay**

The protein content of samples was measured in a 96-well culture plate using a modified Bradford assay (Bradford, 1976). Bradford reagent was added according to manufacturers recommendations (i.e. 1:20 dilution) to samples, and optical density measured at a wavelength of 570nm. Protein content of samples was calculated against standards of bovine serum albumin (BSA) dissolved in Tris buffer. Increasing concentrations of BSA of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200 µg/ml were used and a standard curve obtained. In each case samples and standard were compared on the same plate and the concentrations assessed using the computer programme Dynatech Revelation 2.0.

2.2.5 **Homogenate preparation for purification of NOS**

Different animals were studied for these experiments. Purification of NOS was achieved using previously described methods with some modifications (Pollock et al., 1991). Lung (cardiac lobe) was homogenised in a 1g: 5 ml ratio in cold buffer solution (buffer 1) comprising of 50mM Tris HCl (pH7.4), EDTA (0.1mM), EGTA (0.1mM),

and 0.1% 2-mercaptoethanol with the following protease inhibitors: 1μM Pepstatin A, 2μM Leupeptin, and 1mM PMSF. Using a polytron grinder, lung tissue from the following age groups was homogenised: Fetal pigs, newborn, 3 day, adult and 3 day hypertensive pigs. The homogenate was then centrifuged at 150 x g for 10 minutes to remove larger particles and then ultracentrifuged at 100,000 x g for 60 minutes at 4°C. The supernatant (soluble fraction) was removed, and then stored at –80 °C. The pellet was homogenised further in buffer 1 containing 1M KCl, which was used to remove associated cytosolic proteins from "true" membrane bound proteins. The homogenate was then centrifuged at 100,000 x g for 30 minutes at 4°C, the supernatant discarded and the pellet re-homogenised in buffer 1 containing 10mM 3-3[(3-cholamidopropyl) dimethylammonio]-1-propaneslphonate (CHAPS) homogenising buffer with protease inhibitors. The homogenate was circulated gently at 4 °C for 30 minutes and centrifuged for a further 30 minutes at 100,000 x g. CHAPS buffer solublises the membrane bound proteins such as eNOS. Samples were then centrifuged at 100,000 x g for 30 min and the supernatant obtained from this taken and stored at –80°C.

2.2.6 **Purification of the NADPH binding proteins (i.e. NOS)**

The total volume from either the soluble or particulate fractions was circulated 4 or 5 times over columns of 0.5ml pre-swollen, 2', 5'-ADP Sepharose beads at 4°C (Pollock et al., 1991). 2', 5'-ADP Sepharose selectively binds NADPH binding proteins, such as NOS. Thus by circulating the sample, NOS bound to the column of these beads provided its NADPH site was intact. The column was then washed with a 1ml solution of NaCl (0.5M) made up in buffer 1. For the column that had had the particulate fraction circulated over it, the column buffer contained 10 mM CHAPS. The strong salt solution dislodged any proteins that were bound non-specifically to the column, and with a subsequent wash of 1 ml column buffer, the salt solution was removed. NOS was eluted with consecutive 400μl, 600μl and 500 μl volumes of 10mM NADPH solution dissolved in column buffer. The NADPH solution competed for the NOS bound to the beads, which was then eluted and collected for immediate NOS activity analysis, using the citrulline assay as previously described. The highest levels of NOS activity were found in either the second or third elutates. A small volume of 150 μl from the relevant sample was saved for the Western blots.

2.2.7 **Data analysis**

Alteration of activity in crude and semi-purified homogenate with age was analysed using one–way ANOVA (GraphPad Prism, GraphPad Software Inc) with Bonferroni's correction. Comparison between the activity in lung homogenate from the 3 day and the 3 day hypoxic animals was analysed using an unpaired t-test (GraphPad Prism, GraphPad Software Inc).

Protein assay concentrations were assessed using the computer programme Dynatech Revelation 2.0. (Dynatech Revelation 2.0, Dynatech labs).

2.3 Western Blots/analysis of eNOS.

In order to assess the amount of eNOS protein in the lung samples, Western Blots were carried out on crude particulate samples. These were taken from the fetal, newborn, 3 day old and adult age groups. The protein concentration of the samples was determined by the Bradford assay using bovine serum albumin as the standard (Bradford, 1976) as previously described. Subsequently, the particulate homogenates were taken in equal concentrations and separated by SDS-polyacrylamide gel (7.5% gel) and electrophoretically transferred to nitrocellulose membranes. A kaleidoscope molecular weight marker was also added as a reference point. The membranes were briefly allowed to air dry for 10 minutes and then blocked for 1 hour with 5% non fat dry milk in Tris buffered saline containing 1% Tween (TTBS). After blocking, the membranes were then incubated with a primary eNOS antibody (H32), raised in mouse, at 4°C overnight in a 1:1000 ratio of antibody: milk. Membranes were washed in TTBS in 5% non-fat milk, before incubation for 1 hour with anti-mouse horseradish peroxidase-conjugated secondary antibody. Membranes were then washed in TTBS and developed using enhanced chemiluminescence substrate (ECL, Amersham.UK).

2.4 Organ bath experiments

2.4.1 **Preparation of tissue for organ bath experiments**

The upper lobes of the lungs were removed for the dissection of the axial intrapulmonary arteries and veins. Tissue from these animals was also used in other experiments within the department. Once adjacent vessels were cleared of connective tissue, rings each 2-3 mm in length were cut from the axial vessel after the first branch. These had a diameter of 1 to 2 mm from vessels from all the young animals, and in the

adult animals, vessels from the same position were studied measuring 3-4mm in diameter.

Each ring of both artery and vein was suspended between 2 tungsten wire stirrups and mounted in organ baths, one stirrup fixed and the other attached to an isometric force transducer, in 5ml of gassed (95% O_2, 5%CO_2) and warmed (37°C) Krebs-Henseleit solution (NaCl (119 mM), KCl (4.9 mM), KH_2PO_4 (1.2 mM), $NaHCO_3$ (25 mM), MgSO4 (1.2 mM), Glucose (11.1 mM) and $CaCl_2$ (2.5 mM)). Care was taken throughout the dissection to maintain an intact endothelial layer. In arteries which had the endothelium removed, the luminal surface had been gently rubbed with a pair of watchmakers forceps. The recording of results was via both Grass model 7 polygraph and Maclab recorders.

2.5 Protocol to study the variation in contractile and relaxant responses (Ref. Chapter 4)

2.5.1 **Length tension experiments**

Initial experiments were carried out in order to assess the optimum applied tension for the arteries and veins from the different age groups studied, as these appeared to vary in size. Arteries and veins with an intact endothelium were used. The wire stirrups holding the vessel rings were widened until the ring was fully supported and produced only a slight increment in tension. The rings were then left to equilibrate for 1 hour after which the rings were again stretched, but not to any significant tension. The position of the clamp adjusting the stirrup diameter was noted to within 0.1mm, using an attached vernier scale. This force (g) generated by the ring (Passive force) was monitored by the force transducer and recorded on a Grass model 7 polygraph. Once the increase in tension had stabilised, the organ bath was drained and a KCl solution (125 mM) replenished the bath. Once the maximal force generated (active) stabilised, the KCl solution was replaced by Krebs-Henseleit solution, permitting the ring to return to a non-contracted state. Passive force was again increased by stretching the vessels by 0.1-0.2mm and the previous steps repeated. This continued until the vessels had been stretched to approximately twice their initial diameter, an arbitrary value set to maintain the diameter of the vessel to within as physiological a range as possible. The diameter at which the vessels produced the greatest contraction to KCl was assessed by observing

the greatest increment in active force from the passive force of the vessel i.e. active force minus passive force.

2.5.2 Measurement of pulmonary vascular reactivity at different ages

Isolated intrapulmonary arteries and veins were studied from healthy, non-breathing fetal piglets (1 week pre-term), newborn animals (<5min), and from piglets aged 1, 3, 6 and 14 days of age and also from adult pigs. After equilibration the vessels were stimulated to contract by adding a solution of 125 mM KCl. Once a stable contraction had been obtained the KCl was washed out and the chambers filled with Krebs-Henseleit solution. The reproducibility of contraction was confirmed by performing a second stimulation with KCl. After re-equilibration in Krebs-Henseleit solution, and adjusting any drift from the baseline tension, cumulative concentration response curves to the thromboxane mimetic U46619 were constructed ($1x10^{-11}$M to $3x10^{-6}$M). Once a maximum response was achieved, vasorelaxation was assessed by the cumulative addition of ACh ($1x10^{-9}$ to $3x10^{-4}$M). At the end of each experiment, in order to test the integrity of the relaxant response, vessels were maximally dilated by the addition of papaverine ($1x10^{-5}$M) to the organ bath.

2.5.3 Data analysis

Contractile data were expressed as an increase in tension above resting tension with any drift accounted for. Relaxation was expressed as percentage decrease in tension from a maximum induced by U46619 to a minimum expressed by papaverine. In each case papaverine was added to completely reverse the contraction induced by U46619. Concentration response curves were compared by two-way ANOVA (2-Way repeated measure test). The maximum response (E_{max}) for U46619, KCl and ACh were compared using one-way ANOVA with Bonferroni's correction as a post-Hoc test. Within any age group, comparisons between arteries with and without endothelium were made using an unpaired t-test, as was comparison between arteries and veins (GraphPad Prism, GraphPad Software Inc). Comparisons between KCl and U46619 contractions were analysed using a paired t-test. Individual tests are stated in the appropriate figure legend. A p value of less than 0.05 was assumed to be significant.

2.6 Protocol to study the variation of tonic responses in the presence of various drugs (Ref. Chapter 5)

2.6.1 **Preparation of tissue**

The tissue was dissected and prepared for the organ bath, and the isolated intrapulmonary arteries studied from the following age groups: Fetal pigs (1 week pre-term), newborn pigs (<5 min), 3 and 14 day old, adult and 3 day old hypertensive pigs.

2.6.2 **Measurement of vascular reactivity**

From each lung five rings from arteries with and without endothelium were obtained, one of each used as a control. The vessels were then incubated for 1 hour in the organ bath as previously described. After the equilibration period the vessels were stimulated with KCl twice, and then a single administration of U46619 (EC_{80}) added to the organ bath. The EC_{80}, the concentration at which the vessel reached 80% of its maximum response, was derived from the data obtained in section 2.5. This concentration varied for each age group studied, and was for U46619 (M): Fetal artery, $1x\ 10^{-6}$; newborn artery, $1x\ 10^{-6}$; 3 day artery, $1x\ 10^{-6}$; 14 day artery, $3x10^{-6}$; adult artery, $1x\ 10^{-6}$; 3 day old hypertensive artery, $3x10^{-6}$). Once a stable tone had been obtained one of the following agents were added to the organ bath in a single concentration; L-arginine (1mM) the NOS substrate, L-NAME (1mM) the NOS inhibitor, 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ) (10μM) the guanylate cyclase inhibitor, and Mn (III) tetrakis (1-methyl-4pyridyl) porphyrin pentachloride (MNTPY) (10μM) a SOD mimetic. The vessels were then left again to equilibrate for 30 minutes. After a stable tone was obtained, ACh ($1x10^{-9}$-$1x10^{-5}$) was added in a cumulative fashion. At the end of each experiment vessels were maximally dilated by the addition of papaverine ($1x\ 10^{-5}$M) to the organ bath.

2.6.3 **Data analysis**

Vascular contractile or relaxant responses following addition of the drugs were presented as a percentage change in U46619 contraction. The increase or decrease in tone following addition of the drugs was compared to the tone obtained to U46619 alone (100%) using a one-sample t-test. Comparison of E_{max} between age groups for responses to any particular drug was compared using one-way ANOVA with Bonferroni's correction as a post-Hoc test. Comparisons of E_{max} between arteries and

veins were made using an unpaired t-test. Dose response curves for acetylcholine in the presence of the agents used were compared to controls using two-way ANOVA and between age groups. The E_{max} to ACh was compared using one-way ANOVA with Bonferroni's correction as a post-Hoc test (GraphPad Prism, GraphPad Software Inc).

2.7 Vessel organ culture (Ref. Chapter 6)

The axial intrapulmonary arteries were dissected free of connective tissue in Krebs-Henseleit solution as previously described, under sterile conditions. The following age groups were studied: Fetal, newborn, 1, 3, 6 and 14 day old, and adult pigs. The artery was cut into segments approximately the same size as those used in the organ bath (diameter 1-2 mm, 3-4 mm in the adult), and then either placed in the organ bath for immediate experiments as in section 2.5, or placed into 96 well plates with 200 µl of Dulbecco's modified Eagle medium (DMEM) for 24 hours of incubation. The DMEM contained 10% heat inactivated Fetal Calf serum, 2% of penicillin (100U/ml) and streptomycin (100µg/ml), and 0.3 % glutamine. All tissue incubations were carried out at 37°C, in an atmosphere of 5% CO_2, 21% O_2 and N_2 (74%). After this 24 hr period of incubation, the contractile responses to U46619 and KCl and relaxant responses to ACh were then studied in the organ baths, as described in section 2.5.

2.7.1 **Collection of serum**

Blood from fetal and 14 day old animals was collected and left to clot. The edge of the container was scraped to loosen the contents. The blood was then centrifuged at 100 x g for 8 minutes. After this time, the supernatant was taken off and this supernatant again centrifuged at the same speed for the same duration to further remove any fraction of supernatant that may have remained. This supernatant (serum) then substituted the fetal calf serum present in the DMEM used for the organ culture as described in the following section.

2.7.2 **Organ culture of arteries in different sera.**

Porcine pulmonary arteries from the fetal and 14 day old piglets were studied. These were incubated for 24 hours in culture medium, as described in section 2.7, with serum from either the fetal or 14 day old pigs substituted for the fetal calf serum. Following incubation, contractile and relaxant responses to U46619 and ACh

respectively were studied, in a similar fashion to the vessels incubated in fetal calf serum, in organ baths containing bubbled and warmed Krebs-Henseleit solution.

2.7.3 **Analysis of data.**

As in section 2.5, contraction to U46619 was expressed as the increase in tension (mg), above resting tension with any drift accounted for. Relaxation was expressed as percentage decrease in tension from a maximum induced by U46619 to a minimum expressed by papaverine. The E_{max} for U46619, KCl and ACh, were compared between age groups using one-way ANOVA with Bonferroni's correction as a post-Hoc test.

Segments of artery had been separated to be used immediately, or to be incubated for 24 hours. Thus the comparison of the E_{max} to U46619, KCl and ACh between fresh and incubated tissue was analysed using a paired t-test. Concentration response curves to ACh or U46619 were compared between incubated and control arteries by two-way ANOVA. The responses from vessels incubated in different sera were compared in the same way (GraphPad Prism, GraphPad Software Inc).

2.8 Protocol to study the contractile and relaxant responses in the vasculature from breathing and non-breathing fetal and newborn pigs (Ref. Chapter 6)

The lungs from the following age groups were taken: Fetal (1 week pre-term) and breathing fetal pigs (1 week pre-term that have been allowed to breathe spontaneously), newborn (<5min) and non-breathing newborn pigs (<5min that have been prevented from breathing spontaneously). Isolated intrapulmonary arteries and veins were dissected free of parenchyma and rings then taken and left to equilibrate for 1 hour in the organ bath as previously described. Concentration response curves to U46619 and ACh were studied as described in section 2.5, with a final dose of papaverine ($1x\ 10^{-5}M$) to the organ bath to allow full vascular relaxation.

2.8.1 **Analysis of data**

Data were presented as in section 2.5. E_{max} responses to KCl, U46619 and ACh were compared between breathing and non-breathing pigs from the same age group

using unpaired t-tests. Concentration response curves from breathing and non-breathing pigs from the same age group were analysed by two-way ANOVA.

2.9 Chemicals and reagents.

2.9.1 **Citrulline Assay**

Dowex 50WX8-200 strongly acidic cation exchanger, NADPH, calmodulin, BH_4, $CaCl_2$, L-NAME, EGTA, arginine (Sigma.UK), L-[^{3}H]arginine (Amersham.UK). Homogenate buffers: EDTA, EGTA, Tris Base, Tris HCl, PMSF, Pepstatin, Leupeptin (Sigma.UK).

2.9.2 **Purification/ separation**

KCl, NaCl, (BDH Chemicals LTD, Poole England), CHAPS (Sigma.UK), 2'5'-ADP Sepharose (Pharmacia, Biotech Sweden)

2.9.3 **Western Blots**

Tween, acrylamide, Tris HCl, Tris Base (Biorad.); eNOS antibodies, H32 (Biotech ltd). Kaleidoscope prestained standards (Biorad.)

2.9.4 **Organ Chamber studies**

9,11-Dideoxy-11α, 9α-epoxy-methanoprostaglandin $F_{2\alpha}$ (U46619), ACh and papaverine (6,7-Dimethoxyl-1-veratryl-isoquinoline) (Sigma.UK). U46619 was made up in ethanol and acetylcholine and papaverine were made up in distilled water. The Krebs-Henseleit solution was composed of: NaCl (119 mM), KCl (4.9 mM), KH_2PO_4 (1.2 mM), $NaHCO_3$ (25 mM), MgSO4 (1.2 mM), Glucose (11.1 mM) and $CaCl_2$ (2.5 mM). L-arginine, L-NAME, ODQ (Sigma.UK), MNTPY (Calbiochem)

2.9.5 **Organ Culture**

Dulbecco's modified Eagle medium (DMEM L-Arginine supplemented), Penicillin-streptomycin (ICN Flow biomedicals), Glutamine (Sigma.UK).

Chapter 3. Changes in NOS activity in the developing and hypertensive porcine lung.

3.1 Rationale

The aim of this study was to investigate NOS activity in whole porcine lung, from the transition of the fetal to the newborn circulation, with postnatal development and in the pathological state of pulmonary hypertension.

The evidence that NO is involved in the changes that occur in the transition of the pulmonary circulation at birth and in postnatal pulmonary development is strong and expanding. Inhibition of NOS in fetal lambs is known to attenuate the decrease in vascular resistance that would normally occur at birth (Abman et al., 1990). Furthermore at the onset of birth, oxygenation, ventilation and endothelial shear stresses are induced, and are thought to play a role in the transition of the pulmonary circulation from fetal to postnatal life (Morin III et al., 1992; Black et al., 1997; Busse at al. 1998; Fleming et al .1999). These factors have been observed to stimulate NO production (Abman et al., 1990; Shaul et al., 1992) subsequently decreasing pulmonary vascular resistance, although the precise mechanisms mediating these increases in NO production are unknown. Therefore in these studies NOS activity was investigated in the lungs from non-breathing and breathing fetal pigs by allowing fetal pigs to breathe spontaneously for 5 minutes.

In the isolated intra pulmonary arteries of the newborn pig the relaxant responses to ACh are absent and present only by 3 days of age (Levy et al., 1995; Tulloh et al., 1997). Immunostaining in porcine pulmonary artery endothelial cells has shown that the eNOS, present at birth, increases at three days post parturition (Hislop et al., 1995) corresponding to the observed initiation of the relaxant responses. These and other studies have led to the suggestion that NOS levels (in endothelial cells) increase rapidly after birth in order to support the low resistance environment of the pulmonary circulation (Xue et al., 1996; Kawai et al., 1995). However, the direct measurement of NOS activity in the lungs from mammals at different stages of development (pre- and post-parturition) has not previously been studied. Thus, NOS activity and protein in porcine lung tissue in the fetus, at birth and at different stages of development up to and including adulthood were measured.

Pulmonary NOS levels have been shown to alter in adult rats with hypoxia induced hypertension (Xue et al., 1996). Indeed, many studies show diverse alterations in NOS RNA, protein and activity in this pathological state (Black et al., 1998; Rodman et al., 1992). In the porcine pulmonary arterial endothelium, a decrease in eNOS immunostaining has been observed following the induction of hypoxia induced hypertension in 3 day old pigs (Hislop et al., 1997), corresponding with the loss of the relaxant response from pulmonary arteries taken from the same pig model (Tulloh et al., 1997). Thus we investigated pulmonary NOS activity in the hypertensive state.

3.2 Results

3.2.1 NOS activity in crude lung homogenates from: Fetal, newborn, 1, 3, 6, 14 day and adult pigs.

In lung tissue from pigs at all ages, the L-citrulline detected was represented as the amount of NOS activity. The NOS activity was predominately dependent upon the presence of calcium (calcium dependent activity as a % of total activity: Fetal, 71±15 (n=15); newborn, 72±13 (n=6); 1 day, 94±4 (n=5); 3 day, 87±8 (n=9); 6 day, 86±5 (n=7); 14 day, 77±10 (n=9); adult, 83±11 (n=7). Table 3.1, Figure 3.1 A&B). The total Ca dependent NOS activity in fetal lung tissue was consistently low compared to that from other age groups (Figure 3.1A), and significantly lower than activity observed at 3 and 14 days of age (one-way ANOVA using Bonferroni's correction).

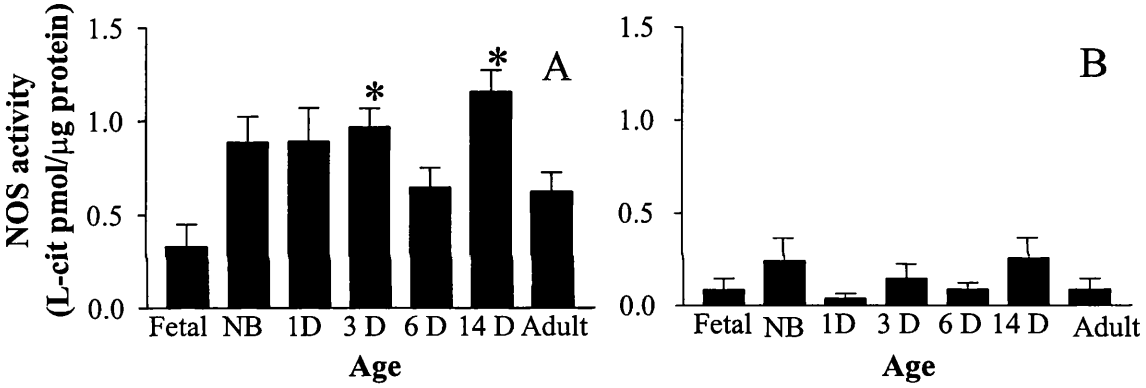

Figure 3.1 NOS activity in crude lung homogenates taken from pre-natal and a variety of postnatal age groups. NOS activity in the presence of calcium (A) and EGTA (calcium free) (B). Values represent mean ± s.e.m : Fetal, (n=15); newborn, (n=6); 1 day, (n=5); 3 day, (n=9); 6 day, (n=7); 14 day, (n=9); adult, (n=7). *$p<0.05$ NOS activity significantly different from fetal values, one-way ANOVA with Bonferroni's Correction

	NOS activity (pmol citrulline/μg protein)					
	+Ca			+EGTA(Ca free)		
Age	**Crude**	**Purified Particulate**	**Purified Soluble**	**Crude**	**Purified Particulate**	**Purified Soluble**
Fetal	0.33 ± 0.12 (15)	0.47±0.5 (6)	0.2 ±0.2 (6)	0.06± 0.06 (15)	0± 0 (6)	0± 0 (6)
Fetal (breathing)	0.4 ± 0.1 (7)			0.22± 0.07 (7)		
Newborn	0.89 ± 0.1 (6)	33.3± 20.5 (5)	8.3±8.3 (5)	0.24± 0.1(6)	15.3± 9.6 (5)	2.6± 2.7 (6)
1 Day	0.89± 0.2 (5)			0.04± 0.02 (5)		
3 Day	0.97 ± 0.1* (9)	235±114.5* (5)	33.2 ± 16.2 (5)	0.15± 0.1 (9)	21.1± 17.9 (5)	8.8± 5 (5)
6 Day	0.65± 0.1 (7)			0.09± 0.04 (7)		
14 Day	1.16± 0.1* (9)			0.3± 0.1 (9)		
Adult	0.63 ± 0.1 (7)	42 ± 23.8 (4)	1.6± 1.6 (4)	0.09± 0.06 (7)	0.2± 0.2 (4)	1.6± 1.6 (4)

Table 3.1 NOS activity in lung homogenates taken from pre-natal and a variety of postnatal age groups, in the presence and absence of calcium. Number in parentheses represents the number of animals used. In crude-homogenised lung and semi-purified: following passage over column of 2'5'-ADP sepharose beads. Values represent mean (pmol/ μg protein) ± s.e.m. *$p<0.05$ NOS activity significantly different to fetal values, one-way ANOVA using Bonferroni's correction.

3.2.2 Comparison of NOS activity in purified lung homogenate with age: calcium dependent & independent activity.

Purification enabled NOS activity to be assessed without the presence of other enzymes/substrate inhibitors which may affect the activity of NOS. Following semi-purification using 2'5'- ADP sepharose beads, activity increased in both the particulate and soluble fractions, relative to the crude un-purified samples. The particulate fractions contained more NOS activity per μg protein than the soluble fractions. However in both particulate and soluble fractions fetal pulmonary NOS activity was consistently low, as was the case for crude samples. At birth NOS activity tended to increase and remain constant with maturation except for a transient increase at 3 days of age, which reached statistical significance in the particulate fraction ($p<0.05$,one-way ANOVA, Table 3.1).

In the semi-purified particulate fraction, NOS activity was almost entirely calcium dependent at all ages studied. However in the newborn age group, calcium dependent activity, represented as a percentage of total activity, was observed to decrease (calcium dependent activity as a % of total activity: Fetal, 100±0 (n=6); newborn, 48.4±18.7(n=5); 3 Day, 88.2±7.3(n=5); Adult, 99.5±0.3(n=4), Table 3.1).

In the semi-purified soluble fraction, fetal lung NOS activity was entirely calcium dependent. By 3 days of age a greater proportion of activity was calcium independent, and in the adult lung NOS activity was totally calcium independent (calcium dependent activity as a % of total activity: Fetal, 100±0 (n=6); newborn, 50±50 (n=5); 3 day, 63.5±22 (n=5); adult, 0.0±0 (n=4), Table 3.1).

3.2.3 Effect of breathing in the fetus on NOS activity in crude lung homogenate: calcium dependent & independent activity

In the fetal pigs that had spontaneously breathed air for 5 minutes, the relatively low level of NOS activity present in the un-purified lung tissue was maintained and tended to be lower than the observed NOS activity in the newborn lung. However there was a trend for the Ca independent portion of this low NOS activity to increase. In fact NOS activity was observed to be approximately half calcium dependent and half calcium independent (calcium dependent activity as a % of total activity; 51.5±15, mean± s.e.m, p=NS). Interestingly, the amount of calcium independent NOS activity was equivalent to that found in the newborn lung (Figure 3.2).

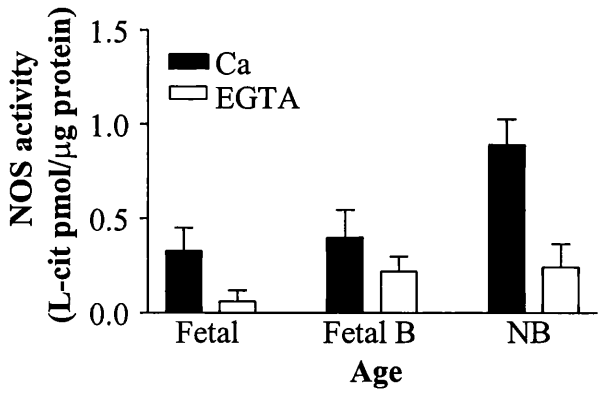

Figure 3.2 Bar chart showing NOS activity in the presence of calcium (Ca) or EGTA. NOS activity in crude lung homogenate of fetal and newborn piglets and breathing fetal piglets (Fetal B). Values represent mean ± s.e.m. Fetal, (n=15); Fetal Breather, (n=7); newborn, (n=6).

3.2.4 **Fetal "all or nothing" responses.**

Having observed large variations in the NOS activity present in samples from individual cases in the fetal age group, the NOS activity values from each lung sample were more closely studied. It was observed that 50% of the crude homogenate samples taken from fetal lungs contained no activity. Following the onset of breathing, the number of fetal lung homogenates containing activity increased (Figure 3.3). In the lungs from newborn pigs, all cases showed NOS activity, as did those from other age groups. Moreover, when active, the NOS activity from lung homogenates of the breathing and non-breathing fetuses was in the same range as values observed in the newborn (Figure 3.3, Table 3. 1).

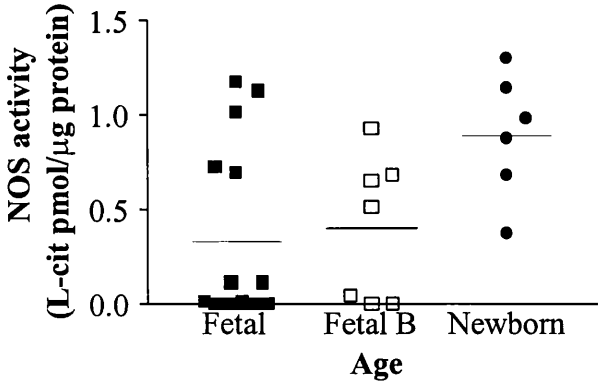

Figure 3.3 NOS activity in the presence of calcium in the porcine lung, from crude lung homogenates of fetal and newborn piglets and breathing fetal piglets (Fetal B). Scatterplot points represent individual cases studied, with the line illustrating the mean.

3.2.5 Effect of hypoxia induced pulmonary hypertension on NOS activity in crude lung homogenate at 3 and 6 days of age: calcium dependent & independent activity

When compared with values from the newborn and 3 day old pigs, total NOS activity in lung homogenates from 3 day old pulmonary hypertensive (3DH) animals did not change. However, there was a significant increase in the amount of Ca independent activity (i.e. iNOS) present in the 3 day old hypertensive compared to their age matched control (calcium dependent activity as a % of total activity; 3 day hypertensive: 36.7±15.7; 3 day control: 88.2±7.3, $p<0.05$ un-paired t-test, Figure 3.4).

In the 6 day old pulmonary hypertensive (6DH) animal, hypertensive from 3 days of age, total NOS activity was similar to that seen in the 6 day controls, with no alteration in the proportion of Ca dependent or independent NOS activity observed.

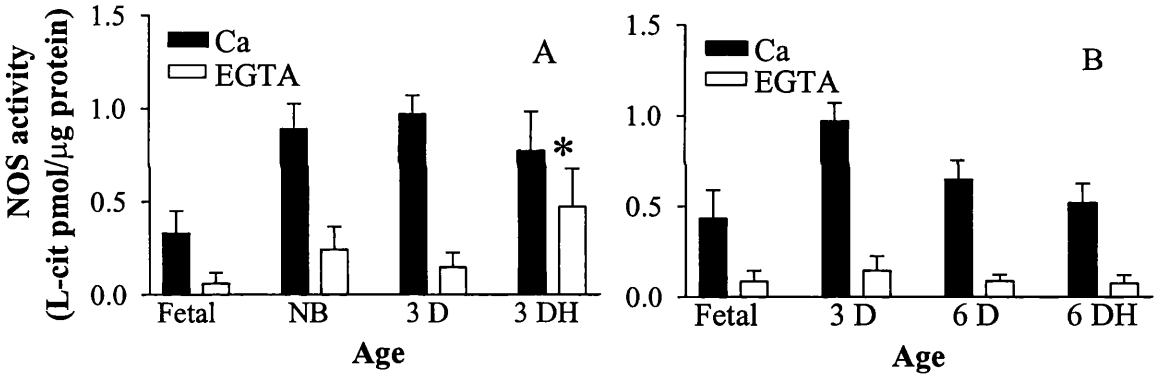

Figure 3.4 Bar chart showing NOS activity in crude lung homogenates in the presence of calcium (Ca) or EGTA (calcium free) in the hypertensive lung. In the fetus, newborn (NB), 3 day old (3D), and 3 and 6 day old hypertensive (3DH & 6DH). A: The activities of crude lung homogenate from 3 day old pigs, kept in the hypobaric chamber from birth, for 3 days (3DH) B: The activities of crude lung homogenate from 6 day old animals kept in the hypobaric chamber from 3 days of age, for 3 days. Values represent mean ± s.e.m. *$p<0.05$ Ca independent NOS activity significantly greater in pulmonary hypertensive animals when compared to age matched controls, unpaired t-test. 3 day, (n=9); 6 day, (n=7); 3 day hypertensive (n=9); 6 day hypertensive (n=9)

3.2.6 Effect of hypoxia induced pulmonary hypertension at 3 days of age on purified NOS activity: calcium dependent & independent activity

Following semi-purification using 2'5'- ADP sepharose beads, the activity in lung homogenates from 3 day old pulmonary hypertensive pigs was very low in both soluble and particulate fractions. Moreover, activity in the particulate fraction of the lung from the 3 day old pulmonary hypertensive pig was significantly lower than that from the 3 day controls ($p<0.05$, un-paired t-test). The proportion of Ca dependent activity in the particulate fraction from the pulmonary hypertensive pigs tended to be equivalent to the other ages studied (calcium dependent activity as a % of total activity: 3DH: particulate, 75±25; soluble, 0±0. Table 3.2).

NOS activity (pmol citrulline/μg protein)						
	+ Ca			+EGTA(Ca free)		
Age	**Crude**	**Purified Particulate**	**Purified soluble**	**Crude**	**Purified Particulate**	**Purified Soluble**
Newborn	0.89 ± 0.1 (n=6)	33.3± 20.5 (n=5)	8.3±_8.3 (n=5)	0.24±0.1 (n=6)	15.3±9.6 (n=5)	2.6±2.7 (n=5)
3 Day	0.97 ± 0.1 (n=9)	235±114.5 (n=5)	33.2 ± 16.2 (n=5)	0.15±0.1 (n=9)	21.1±17.9 (n=5)	8.8±5 (n=5)
6 Day	0.65±0.1 (n=7)	~	~	0.09±0.04 (n=6)	~	~
3 Day Hypertensive	0.8 ± 0.2 (n=9)	3.9±2.2* (n=4)	0.16 ±_0.16 (n=5)	0.5±0.2* (n=9)	0.08±0.08 (n=5)	0.16±0.16 (n=4)
6 Day Hypertensive	0.72±2.1 (n=9)	~	~	0.13±0.05 (n=9)	~	~

Table 3.2 Calcium dependent and independent L-citrulline activities in crude and purified lung homogenates taken from normal and pulmonary hypertensive age groups. Values represent mean (pmol/ μg protein) ± sem. *p<0.05 NOS activity significantly different from 3 day old piglet lung homogenate, un-paired t-test.

3.2.7 Expression of eNOS protein in lung samples from pigs at different stages of development using Western Blots: Fetal, newborn, 3 day and adult

In order to assess the levels of eNOS protein in the lung, Western Blots were carried out on lung tissue from animals in the fetal, newborn, 3 day and adult age groups. At these stages of development detectable levels of eNOS-like immunoreactivity were observed. In contrast to the low levels of enzyme activity observed in fetal lung homogenate, there were comparable levels of eNOS protein to the newborn and adult age groups (Figure 3.5). The highest level of NOS protein detected was from lung homogenates taken from the 3 day old age group.

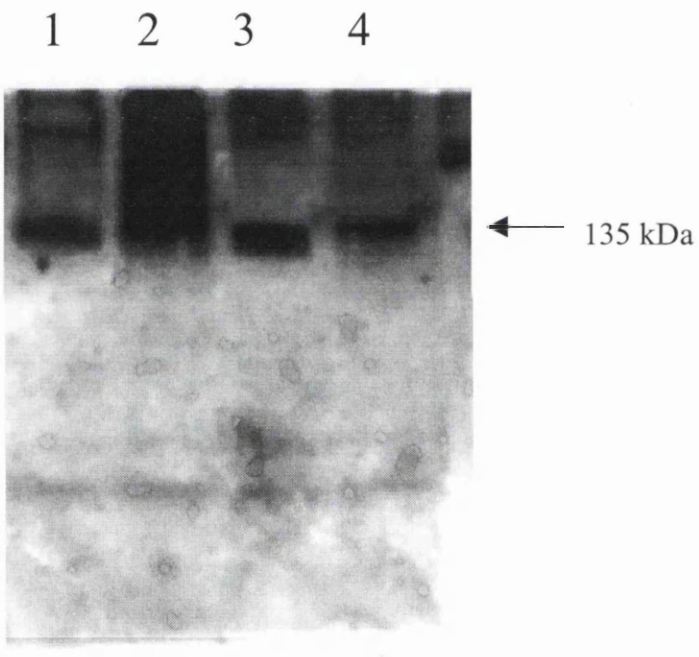

Figure 3.5 Western blot analysis of lung homogenate from pigs in different developmental age groups (n=3).
From left to right: lane 1, newborn; lane 2, 3 day; lane 3, fetal; lane 4, adult.

3.3 Discussion.

Previous immunohistochemical studies using porcine pulmonary arteries and veins showed variations in the levels of eNOS at a variety of developmental ages (Hislop et al., 1995; Hislop et al., 1997). Although providing valuable information, it did not indicate how active the NOS enzymes present were. In this study, the smallest levels of NOS activity were found in fetal lung homogenate, which after birth increased. With hypoxia induced pulmonary hypertension total NOS activity did not alter, but the level of Ca independent NOS activity increased.

3.3.1 **Isoforms of NOS**

The NOS activity in the majority of crude lung homogenates was predominantly calcium dependent, derived from calcium dependent eNOS, nNOS or a combination of both isoforms. All three NOS isoforms are expressed in the developing lung (Shaul et al., 1997;Xue at al. 1996; Buttery et al., 1995; North et al., 1994; Rairigh et al., 1998). In the porcine lung, eNOS levels vary with age (Hislop et al., 1997; Hislop et al., 1995) and nNOS levels decrease after birth (Buttery et al., 1995). However in the studies described in this chapter, the proportion of calcium independent NOS activity from breathing fetuses and 3 day old pulmonary hypertensive pigs was observed to be higher in other age groups. Ca independent iNOS has been detected in the perinatal rat and fetal sheep lung (Xue et al., 1996; Sherman et al., 1999; Rairigh et al., 1998) and has been observed to increase following the induction of hypoxia induced hypertension in fetal sheep (Resta et al., 1999). This Ca independent NOS, may not be iNOS exclusively as eNOS has also been shown to become activated without the requirement of intracellular calcium, by becoming phosphorylated by kinases that are themselves induced by fluid shearing of endothelial cells (Fulton et al., 1999; Dimmler et al., 1999). It is therefore not possible in the systems used in this chapter to exclusively say which of the isoforms of NOS predominate at any one time point. However, it was the intention here to ascertain the gross changes in activity and relate them to other processes that are known to alter during development.

3.3.2 **Fetal/ Newborn Transition**

Fetal lung contained the lowest levels of NOS activity. Functionally, in the sheep, the pulmonary vascular reactivity of the fetus is low, with a lower release of NO

than in postnatal age groups (Shaul et al., 1993). In this chapter, despite low fetal NOS activity, the eNOS protein was similar in the fetal, newborn and adult lung. In contrast eNOS RNA has been observed to increase by 1 day of age from levels observed in the fetal rat lung (Kawai et al., 1995), whilst immunolocalisation studies and RNA assays using lungs from sheep and rats have shown a greater eNOS content in the fetus than in postnatal age groups (Halbower et al., 1994; Xue et al 1996; North et al., 1994). The conflict in the studies observed could be a result of differences between the species studied.

In approximately 50% of the fetal lung homogenates, NOS activity was absent. The activities in the other 50% of fetal lungs were similar to those found in the newborn age group. In addition, within 5 minutes of birth pulmonary NOS activity was present in all the lung homogenates. The NOS activity in the newborn lung may have been stimulated by either the induction of labour or by the ventilation of the lungs. Oxygenation and ventilation are thought to be the most important factors leading to the upregulation of NO/NOS in the fetal pulmonary circulation (Finemann et al., 1995; Titinsky et al., 1993; Cornfield et al., 1992; Tietel et al., 1990). However, ovine fetuses have been grouped into major and minor responders to pulmonary ventilation induced relaxation (Teitel et al., 1990), with fetal responses being either present or absent (Cook et al., 1963). Thus the "all or nothing" responses seen in the fetal lungs for NOS activity are most likely to be a result of acute post-transcriptional modifications that are switched on at birth. This may include a possible change of the conformational shape of the NOS. One candidate may be the increased stresses of shear and strain applied to the lungs at this time. Strains of shear and stretch have been observed to increase the activity of both Ca dependent eNOS (Garcia-Cardena et al., 1998), and Ca independent eNOS (Fulton et al., 1999; Dimmler et al., 1999). In addition, the shear may have increased the content of iNOS, which is also found in the lungs of the fetal lamb (Rairigh et al., 1998). This increase in NOS activity and subsequent increase in NO production would contribute to the decrease in fetal PVR that occurs at this time.

The total NOS activity in crude lung homogenate from breathing fetuses remained low, analogous to levels observed in control fetuses. However, there was a trend in the proportion of Ca independent activity to increase, in addition to the greater number of lung homogenates that demonstrated NOS activity. Thus ventilation and oxygenation did appear to stimulate NOS activity to some degree. Factors at birth other than ventilation and oxygenation of the lungs may upregulate fetal NOS activity. For

example circulating oestrogen, which increases prior to birth, increases eNOS protein and mRNA via stimulation of oestrogen receptors (Goetz et al., 1999; MacRitchie et al., 1997), and may thus increase NOS activity.

However, the increase in NOS activity seen at birth, rather than being due to the up-regulation of NOS may be due to a down-regulation of some inhibitory factor such as ADMA, which increases in pregnancy (Fickling et al., 1993). Such inhibitors could create the low NOS activity in the fetal lung, which may then be inactivated at birth by putative factors stimulated by the onset of breathing. However, following semi-purification, pulmonary NOS activity in the fetus did not increase, making it unlikely that such inhibitors were present.

At birth, there tended to be an increased proportion of Ca independent NOS activity in a similar fashion to lung homogenates from breathing fetal piglets. This increase may have been created by acute post transcriptional alterations in the NOS enzyme. As previously suggested, NOS activity may be acutely increased following the phosphorylation of the tyrosine grouping on the enzyme (Garcia-Cardena et al., 1996), following stimulation by shear stress (Fulton et al., 1999; Dimmeler et al., 1999).

3.3.3 **Postnatal Activity**

In the postnatal porcine lung, NOS activity was predominantly calcium dependent and varied very little with age. Interestingly, lung NOS activity tended to decrease at 6 days of age and was lower in the adult. In line with these observations eNOS immunostaining decreases in the lung endothelium and airways postnatally from 6 days of age onwards (Hislop et al., 1995; Hislop et al., 1997; Buttery et al., 1995), whilst in rat lung, eNOS RNA increases from 1 to 16 days of age but decreases in the adult (Kawai et al., 1995). In contrast, declining eNOS from birth has been reported in sheep (Hallblower et al., 1994) and rat lungs (North et al., 1994; Xue et al., 1996), exposing clear differences in the changing levels of NOS after birth. However, the trend in all these examples is for the lung NOS content to decrease in adulthood. Nevertheless, despite NOS activity decreasing at this age, in this chapter, studies using Western blot analysis show that the NOS protein observed in the newborn and adult lung homogenates were equivalent. Thus the decrease in pulmonary NOS activity in the adult was not likely to be due to a decrease in NOS protein, but more likely to be due to an alteration in the regulation of the enzyme.

In all the postnatal age groups studied, following semi- purification, the specific activity of NOS was greater than that observed in crude lung homogenate. This is, of course, to be expected since NADPH-requiring proteins, i.e. NOS, were selectively isolated. Total activity in the particulate fraction was greater than in the soluble lung fractions, which is typical of eNOS (Mitchell et al., 1991; Pollock et al., 1991; Fosterman et al., 1991). Furthermore, mostly Ca independent NOS activity was located in the soluble fraction of the lungs, which is typical for iNOS (Busse et al., 1990; Stuehr et al., 1991). From both fractions the patterns of NOS activity with age were similar in as much as NOS activity from fractions in the adult and newborn age groups were alike, with the greatest NOS activity at 3 days of age. The NOS activity in the newborn, 3 day and adult lung thus corresponded to the observed NOS protein levels in the Western blots. Moreover, the observed increase in pulmonary NOS activity at 3 days of age also corresponded with previously reported increases in eNOS immunostaining in the pig lung (Hislop et al 1997; Hislop et al 1995). This increase in activity also corresponded with the initiation of NO mediated pulmonary arterial relaxation at this age (Liu et al., 1992; Tulloh et al., 1997), in association with a large amount of cell growth and other adaptive changes in structure of the pulmonary vasculature (Hall et al., 1987).

3.3.4 **Hypoxia induced pulmonary hypertension**

In lung homogenates from pigs with hypoxia induced pulmonary hypertension at 3 days of age, when compared to the 3 day old control animals, the proportion of Ca dependent NOS (i.e. eNOS, nNOS) decreased. This corresponded with a decreased pulmonary immunostaining for eNOS (Hislop et al., 1997) and the absence of pulmonary arterial relaxation to ACh in hypertensive pigs at this age (Tulloh et al., 1997). Other investigations have also found that in the pig, hypoxia induced pulmonary hypertension from birth decreased the pulmonary content of eNOS and subsequent exhaled NO (Fike et al., 1998). Moreover eNOS protein, RNA and activity decreased in the lungs of fetal lambs, hypertensive following ductal ligation (Villamor et al., 1997; Black et al., 1997). In contrast however, eNOS content has been reported to increase with hypoxia induced hypertension in the lungs from the fetal (North et al., 1994) and adult rat (Xue et al., 1996; Shaul et al., 1997). Perhaps the difference between animal models studied creates these inconsistencies. In line with a decrease in the proportion of Ca dependent NOS (eNOS) activity, Ca independent NOS increased with pulmonary hypertension at 3 days of age. This had previously been observed in the adult rat lung,

where following chronic hypoxia, the transcription of iNOS increased (Resta et al., 1999). In addition, levels of Ca independent NOS activity in the lungs from the 3 day old hypertensive piglet and the neonate were similar. With hypoxia induced pulmonary hypertension the arrest of the postnatal decrease in surface area to volume ratio, and the subsequent "spreading" of the endothelial cells (Allen et al., 1986), may affect the endothelium mediated metabolism of vasoactive agents. In addition, the alterations in the proportion of Ca independent activity in the 3 day old hypertensive pig lung may not be iNOS, but a Ca independent eNOS, activated by an increase in endothelial shear (Busse et al., 1998, Fulton et al., 1999, Dimmler et al., 1999) which might arise following the onset of birth and pulmonary hypertension.

In contrast to the 3 day old hypertensive pig, in the 6 day old hypertensive animal, the levels of calcium dependent and independent NOS activity did not alter significantly from the lungs of 6 day old controls. In contrast, immunohistochemical studies have shown an increase in concentration of eNOS, in this pig model of pulmonary hypertension at 6 days of age (Hislop et al., 1997). However this anomaly between the content of NOS and subsequent activity could be created by the differences between studying arteries and whole lung, or it could simply be an example of the NOS protein not directly representing NOS activity. The differences in NOS activity in the 3 day and the 6 day old hypertensive piglets appeared to be created by the age at which the animals were exposed to hypoxia. Thus the reduction in the proportion of pulmonary Ca dependent NOS activity may not only be induced by hypoxia (Faller et al., 1999) but also by the susceptibility of the lung to be affected by hypoxia after 3 days. In pigs exposed to hypobaric hypoxia from birth, pulmonary arterial wall structures do not mature, unlike hypoxia induced hypertension from 3 days of age (Allen et al., 1986). Therefore the immature state of the vasculature in the 3 day old pulmonary hypertensive pigs may be affecting the NOS activity in the whole lung.

In conclusion, alterations in NOS lung activity were observed to occur between the fetal and newborn age groups, and in the postnatal development of the lung. The transition from the fetal to the newborn circulation increased NOS lung activity, stimulated to some extent by the initiation of breathing. This fetal/newborn difference was not likely to be created by alterations in either the lung content of the NOS protein or the presence of inhibitors, since the low level of NOS activity present in the fetal lung was still apparent when the tissue was subject to purification steps that would have removed such factors. However, the difference may have been created by a

stimulated increase in the proportion of Ca independent NOS, induced by the increased shearing on the blood vessels by breathing. By 3 days of age NOS activity in the lung was observed to be at its highest level, perhaps necessary for the adaptations in structure and function which predominate at this age.

Hypoxia induced pulmonary hypertension from birth was observed to increase levels of Ca independent NOS activity. This may have been a result of increased shear forces on the endothelial cells, and subsequently the increased the proportion of calcium independent NOS. These alterations in NOS activity in the pulmonary hypertensive animal probably contribute to the abnormal vascular reactivity observed in previous studies using this model. However, exactly how this observed alteration in NOS activity in lung development and hypertension relates to the alterations in the responses of the pulmonary vasculature remains to be studied.

Chapter 4. Alteration of tonic responses in arteries and veins with pulmonary maturation.

4.1 Rationale

The aim of this study was to compare the contractile and relaxant responses of intra pulmonary axial arteries and veins of the developing porcine lung in vitro, at a variety of developmental ages and in the pathological situation of hypoxia induced pulmonary hypertension.

Adaptation to extrauterine life occurs not only with the immediate transition from the fetal to the extrauterine environment at birth, but also during the first weeks of life as the pulmonary vascular resistance continues to fall. In porcine and ovine pulmonary arteries, the reactivity to different vasoactive agents in the perinatal period alters with lung maturation (Liu et al., 1993; Tulloh et al., 1997; Zellers et al., 1991). These responses are important in relation to the structural changes that occur with age (Haworth et al., 1982). In addition, contractile responses have been found to alter depending on the initial tension of the pulmonary vessels. This physiological resting tension may vary between age groups and also between arteries and veins. Thus initial experiments were required to obtain the physiological optimum resting tensions of the vessels, to be applied to the vessels prior to the addition of any agonists.

Only a few studies to date have concentrated on the changes in contractility that take place in the arteries between birth and adulthood (Levy et al., 1995) and with few notable exceptions, the role that the pulmonary veins might play in postnatal adaptation has received relatively little attention (Gao et al., 1995). In fact most studies to date have observed the changes in relaxation that occur with age. Endothelial dependent relaxation in the pig alters with postnatal pulmonary adaptation (Liu et al., 1993; Zellers et al., 1991), and is mediated in a large part by NO (Steinhorn et al., 1993; Tulloh et al., 1997). NO is perceived to be particularly important in the transition from the fetal to the newborn pulmonary circulation, following the attenuation of the postnatal decrease in pulmonary vascular resistance with NOS inhibition (Abman et al., 1990). In addition, in the previous chapter NOS activity increased after birth. Therefore in this chapter the contractile and relaxant responses of pulmonary arteries and veins in piglets from fetal to adult life have been compared and the endothelial dependence of the contractile responses of the arteries examined.

In pig lungs following chronic hypoxia induced hypertension, the normal structural adaptations that occur with maturation are altered and arterial remodelling ensues (Allen et al., 1986). Associated with this is the reduction in endothelial function and responses to endothelium dependent vasodilators (Tulloh et al., 1997). In fact even in the mature lung, exposure to chronic hypoxia in the adult rat has been shown to decrease pulmonary arterial NO levels (Archer et al., 1989). The decrease in the pulmonary endothelial mediated release of NO, corresponds to decreased eNOS levels found in arteries from the pulmonary hypertensive pig at 3 days of age, using immunostaining techniques (Hislop et al., 1997). In fact studies in chapter 3 showed a decrease in proportion of Ca dependent NOS at 3 days of age in the same model of hypoxia induced pulmonary hypertension.

The responsiveness of pulmonary veins following hypoxia induced hypertension in the perinatal period has not been extensively studied, with conflicting evidence for the role of the pulmonary veins in the hypertensive lung (Steinhorn at al., 1995; LaBourene et al., 1990; Lal et al., 1999). Thus the contractile and relaxant responses of adjacent pulmonary arteries and veins were addressed in the porcine model of hypoxia induced hypertension.

4.2 Results

The relationship between both the contractile and relaxant responses of the pulmonary vasculature with development and the relationship of these responses between arteries and veins has not been extensively studied. To directly observe the vasoactive properties of these vessels, organ bath studies were carried out, recording the vascular responses following the direct application of pharmacological agents. Preliminary studies were carried out to discover the initial tension at which these vessels would optimally respond to an agonist within a physiological range of diameters.

4.2.1 Length Tension studies

In order to establish a standard resting tension, preliminary studies at all ages investigated the effects of passive stretch to the vessels and active tension produced following the addition of a high concentration of KCl. The lengths of the rings studied were consistent with those taken for other organ bath experimentation.

4.2.2 *Passive tension (g) achieved with increment in vessel diameter.*

This was studied in arteries and veins from the following age groups: Fetal, newborn, 3 and 14 day old, adult, and 3 day old pulmonary hypertensive pigs. A linear relationship existed between the applied increase in vessel diameter and the resulting increase in passive tension at all ages, in both arteries and veins. Little alteration in this relationship occurred with age. Beyond a certain diameter, the passive force in vessels was observed to decrease. This occurred in the arteries from the 14 day old and 3 day old pulmonary hypertensive animals. In the veins this was also observed to occur, but only from the lungs of fetal pigs (Figures 4.1- 4.4, A-F Part i).

4.2.3 *Active tension (g) achieved with increment in vessel diameter.*

The addition of KCl (125mM) created a tension or active force in the pulmonary vessels. On subtraction of the initial resting tension (Passive) from this force (Active), a tension was formulated that varied with the applied increase in diameter of the vessel (Figure 4.1 - 4.4, A-F Part ii). The greatest tension produced was named the optimum tension and was maintained within a range of vessel diameters, with the range depicted as a shaded box. This generally occurred as the highest and flattest part of the curve or

as an area about the peak of the curve (Figure 4.1-4.4, shaded area). The optimum resting tension in arteries at any one induced vessel diameter was smallest in the fetal, newborn and 3 day old arteries and largest in the adult.

Beyond the upper extremities of this range, vessel tension was observed to decrease at most ages in both vessel types. The exception to this was in the arteries at 3 days of age and adulthood where the tension was either maintained or increased. Similarly in the veins from pigs at 14 days of age and adulthood, tension did not decline at the outer limits of the physiological diameter applied to these vessels. Like the arteries, the optimum resting tension in the veins at any one vessel diameter was smallest in the fetal, newborn and 3 day old age groups and largest in the adult. Furthermore in veins from the younger age groups it was observed that the production of an optimum tension, required a smaller increment in diameter.

4.2.4 *Determining optimum resting tension from optimum active tension.*

The range of vessel diameters induced by stretching, at which there was an optimum tension denoted as a shaded area (Figure 4.1 & 4.4A-F Part ii, shaded area), was applied to the graph of passive tension plotted against increase in diameter. This area included the passive tensions that fell within this range of applied diameters (Figures 4.1- 4.4 A-F Part i). The maximum and minimum passive forces that could be applied to a vessel to produce optimum active tension were thus extrapolated. For both arteries and veins there was a certain degree of overlap in the passive forces required to produce an active tension between the age groups. Thus a common optimum resting tension for the arteries of 1 gram, and for the veins of 300 mg was deduced for all age groups except for veins taken from the adult pig. These required a larger increment in diameter before an optimum resting tension was produced and maintained, thus a tension of 1 gram was applied to these vessels.

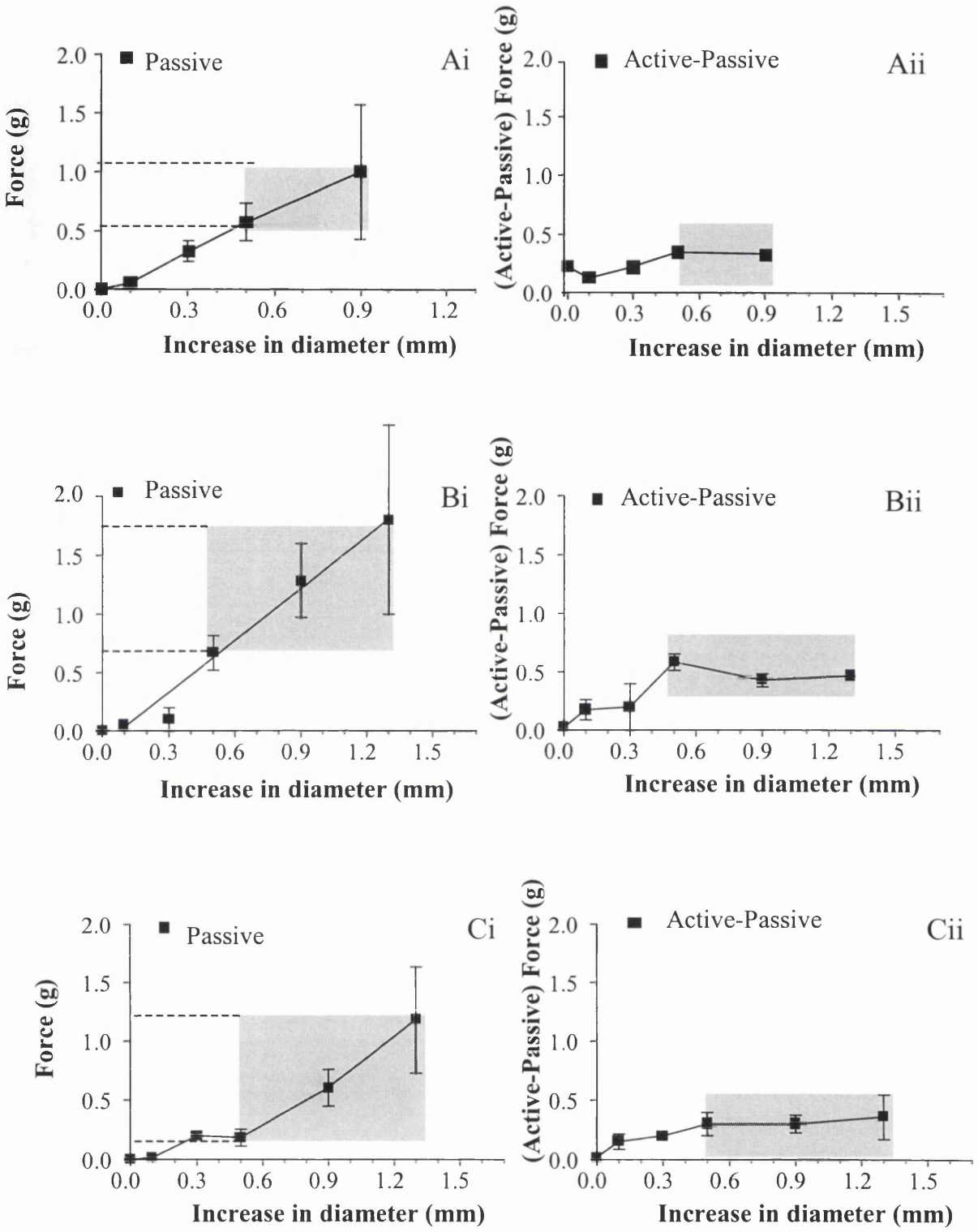

Figure 4.1 Paired graphs showing; i: the increase in force with increasing diameter of the vessel (Passive force) and ii: the difference between passive force and that created with the addition of KCl (125 mM) (Active force).
Pulmonary artery from the following age groups; A: fetal (n=12), B: newborn (n=7), C: 3 day old pigs(n=9). Shaded area: Range of applied vessel diameters that are required to produce optimal force on addition of KCl (125 mM)

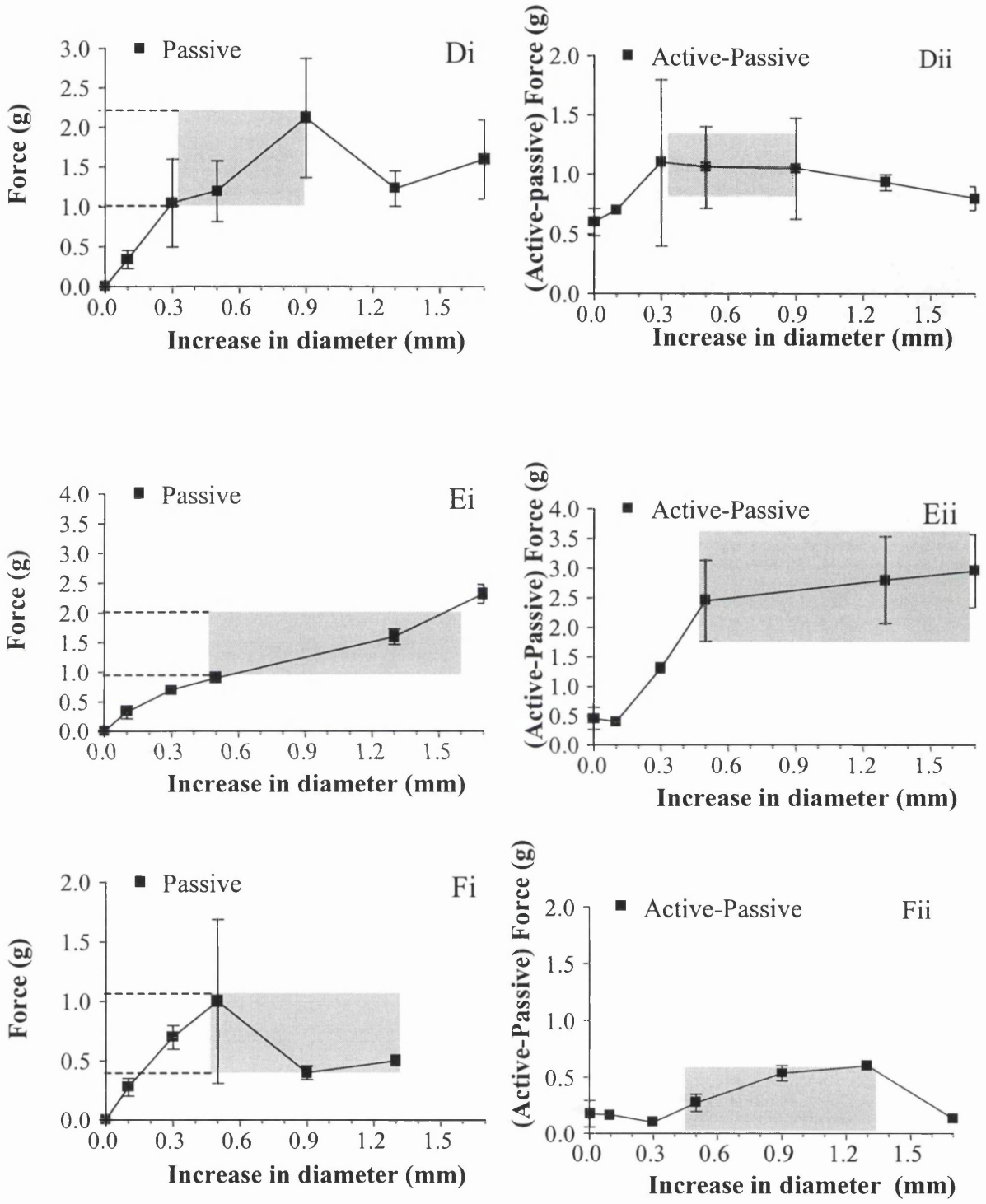

Figure 4.2 Continuation of figure 4.1 showing; i: the increase in force with increasing diameter of the vessel (Passive force) and ii: the difference between passive force and that created with the addition of KCl (125 mM) (Active force).

Pulmonary artery from the following age groups; D: 14 day old pig (n=5), E: adult (n=4), F: 3 day old hypertensive pig (n=7). Shaded area: Range of applied vessel diameters that are required to produce optimal force on addition of KCl (125 mM)

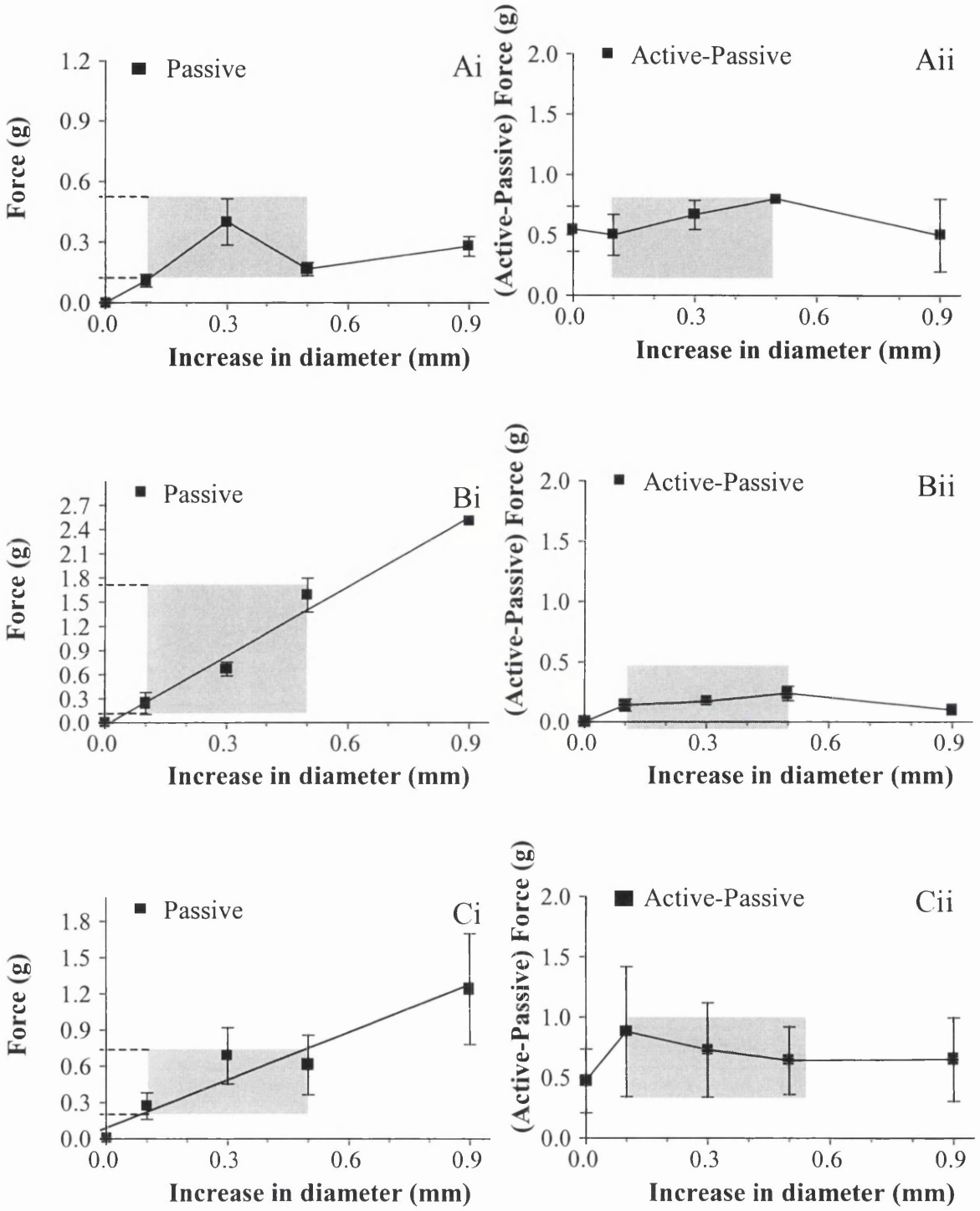

Figure 4.3 Paired graphs showing; i: the increase in force with increasing diameter of the vessel (Passive force) and ii: the difference between passive force and that created with the addition of KCl (125 mM) (Active force).

Pulmonary vein from the following age groups; A: fetal (n=12), B: newborn (n=7), C: 3 day old pigs(n=9). Shaded area: Range of applied vessel diameters that are required to produce optimal force on addition of KCl (125 mM)

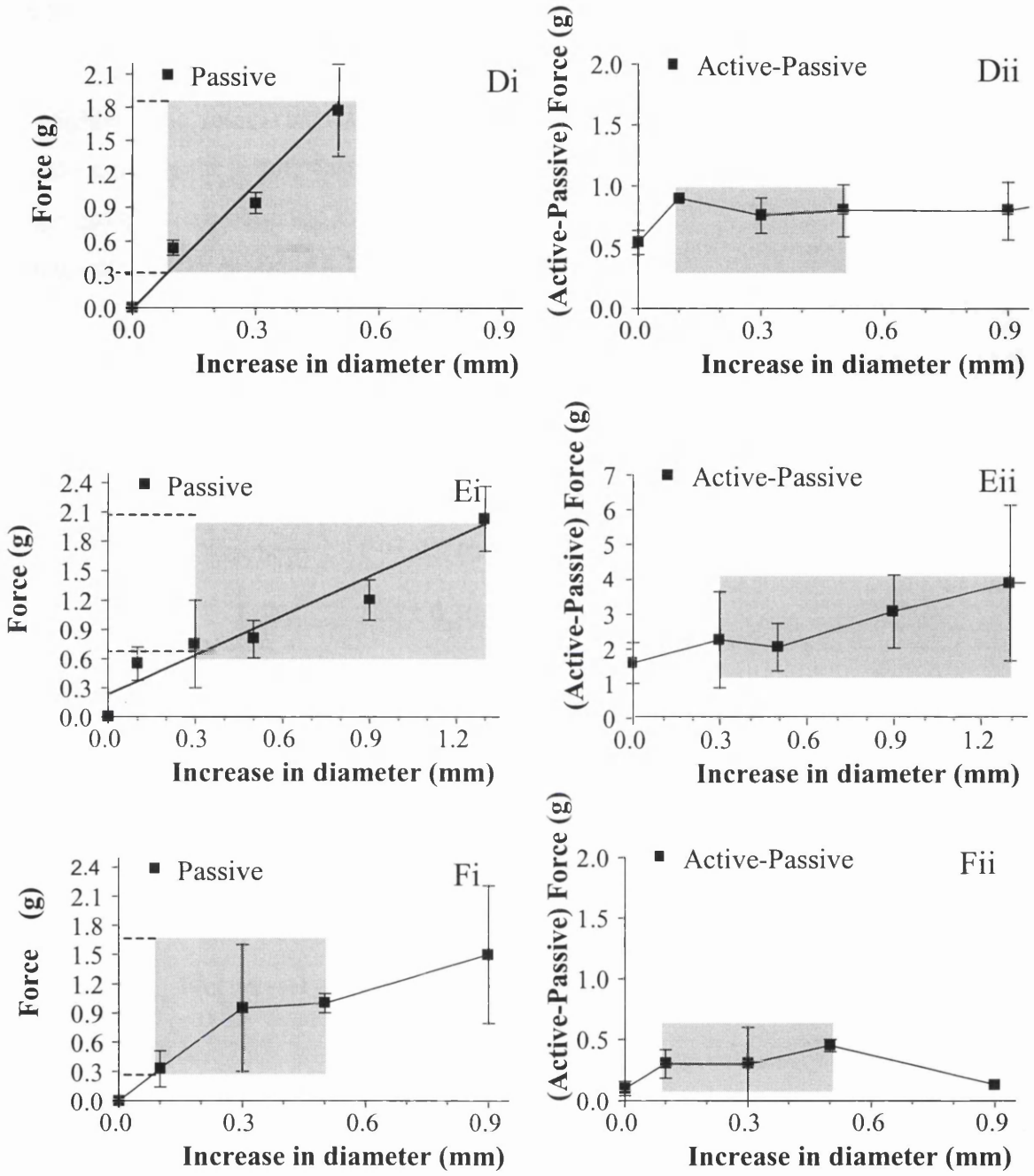

Figure 4.4. Continuation of figure 4.3 showing; i: the increase in force with increasing diameter of the vessel (Passive force) and ii: the difference between passive force and that created with the addition of KCl (125 mM) (Active force).

Pulmonary vein from the following age groups; D: 14 day old pig (n=5), E: adult (n=4), F: 3 day old hypertensive pig (n=7). Shaded area: Range of applied vessel diameters that are required to produce optimal force on addition of KCl (125 mM)

4.2.5 *Alteration of vessel ring weight with age.*

Between the younger postnatal age groups, no significant differences in the weights of the vessel rings were observed. This was an observation true of both arteries and veins. In the adult, vessel rings of equivalent length to those taken from younger age groups were observed to be significantly heavier in both arteries and veins ($p<0.05$ one-way ANOVA, Table 4.1).

Weight **Age**	**Artery(mg)**	**Vein(mg)**
Newborn	1.9± 0.2 (n=6)	0.5± 0. 1(n=1)
1 day	2.07± 0.3(n=4)	1.3± 0.3 (n=4)
3 day	2.4± 0.5(n=6)	1.8±0.26 (n=4)
6 day	1.3± 0.4 (n=6)	0.9± 0.3 (n=2)
14 day	2.7±0.3(n=15)	1.8± 0.14 (n=13)
Adult	18.6± 5.5(n=6)*	16.5± 0.45(n=2)*

Table 4.1. Wet vessel weights of arteries and veins following time spent in organ chamber. *$p<0.05$ vessel weight significantly greater than all other age groups, one-way ANOVA.

4.2.6 Contractile responses in vessels from normoxic pigs

4.2.7 *Porcine pulmonary artery contractile responses to U46619 and KCl with age*

The arterial contractile responses to U46619 and KCl followed similar patterns with age, showing a minimum response in the fetal age group that increased at birth, and a large increase in the contractile response between 14 days of age and adulthood.

The contractile responses of vessels to KCl, which were lowest in the arteries from the fetal age group, had within 5 minutes from birth increased ($p<0.05$, unpaired t-test), and were maintained with age. The greatest response to KCl occurred in the arteries from the adult age group ($p<0.05$, one-way ANOVA, Table 4.2). In a similar

fashion to the responses of the arteries to KCl, the contractile responses to U46619 in the fetal age group were of the lowest magnitude of any age studied, except for vessels taken at 3 days of age ($p<0.05$, two-way ANOVA. Figure 4.5). Postnatally, within 5 minutes of birth, the arterial contractility to U46619 (E_{max}), transiently increased from fetal levels, ($p<0.05$, unpaired t-test). This decreased again to a minimum at 3 days of age at which age the concentration dependent responses to U46619 were observed to be significantly less than that at all other postnatal ages ($p<0.05$, two-way ANOVA, Figure 4.5). In all the perinatal age groups the arterial contractility to U46619 (E_{max}) was less than that in the adult ($p<0.05$, one-way ANOVA, Table 4.2). With age, arterial contractility (E_{max}) to U46619 increased but the sensitivity (Log EC_{50}) was observed not to alter (Table 4.2).

4.2.8 *Contractile responses of porcine pulmonary artery with age, following endothelium removal*

Endothelium removal did not affect the arterial responses to KCl in most age groups. However it did cause a significant reduction of arterial contractility to KCl at 3 and 6 days of age ($p<0.05$, un-paired t-test, Table 4.2). Following the endothelium removal, arterial concentration dependent responses to U46619 were diminished in most postnatal age groups, attaining significance at 3 and 6 day old and adult pigs (Figure 4.5, $p<0.05$ two-way ANOVA). In the fetal age group, the arterial contractility tended to be enhanced following removal of the endothelium. Between the age groups, in arteries with the endothelium removed, contractility to KCl was lower at all ages compared to that from the adult ($p<0.05$, one-way ANOVA, Table 4.2). Similarly the contractility (E_{max}) to U46619 was lower at all ages compared to the adult age group with the concentration dependent responses to U46619 reaching statistical significance at 1, 3 and 6 days of age ($p<0.05$, one-way ANOVA, Figure 4.5).

4.2.9 *Porcine pulmonary venous contractile responses to U46619 and KCl with age*

In the veins there was a gradual increase in maximum contractile response to both KCl and U46619 with age, with no age producing a significantly different maximum response from another. The smallest contractile responses to KCl tended to be observed in the fetal age group. To U46619, the smallest venous contractile

responses were from the fetal and newborn age groups (Table 4.3), with the concentration dependent responses of U46619 in the fetal veins being significantly lower than that produced in any other age group ($p<0.05$, two-way ANOVA, Figure 4.5, Table 4.3). The trend for venous contractility to U46619 was to increase with age, reaching a maximum at 3 days of age and to remain at this level until adulthood. U46619 produced a greater concentration dependent contraction in the veins from the 3 day old animals than from any other age group studied ($p<0.05$, two-way ANOVA, Table 4.3).

Compared to arteries, the venous responses to KCl and U46619 were greater in most of the age groups studied until adulthood. The venous responses to KCl were significantly greater than the arterial responses in the fetus, and at 3, 6 and 14 days of age ($p<0.05$,un-paired t-test). In a similar fashion the venous contractility to U46619 (E_{max}), at all ages except the newborn and adult, were significantly greater than that of the arteries, reflected in the concentration dependent responses to U46619 at these ages ($p<0.05$ two-way ANOVA, Figure 4.5).

		Arterial responses			Arterial responses without endothelium			
AGE	n	**KCl (mg)**	**U46619 Emax(mg)**	**U46619 LOG M EC_{50}**	n	**KCl (mg)**	**U46619 Emax(mg)**	**U46619 LOG M EC_{50}**
Fetus	10	250 ± 32*†	424 ± 32†*	-6.3 ± 0.03	5	287.5±62.5†	593.8±108.1	-6.4±0.07
Newborn	12	519±97†∝	778.2±134†∝	-6.5 ± 0.08	10	536.4±152†	437±76	-6.4±0.19
1 day	6	439 ± 80†	568 ± 105†*	-6.4 ± 0.02	6	354.4±95.9†	529.8±141.5†	-6.0±0.332
3 day	11	476 ± 46*‡†	470 ± 75†*	-6.2 ± 0.09	6	217.5±44†	269.2±57.53†	-6.02±0.614
6 day	9	366 ± 57*‡†	544 ± 87†*	-6.9 ± 0.08	5	154±48†	300.7±111†	-5.9±0.148
14 day	16	543 ± 61*†	758 ± 133†*	-6.2 ± 0.04	9	570±146†	813.6±222	-6.8±0.169
Adult	6	2149 ± 659	2611± 937*	-6.1 ± 0.3	4	2650±550	1650±150	-6.2±0.29

Table 4.2 Vaso-activity of porcine pulmonary artery with and without endothelium to KCl (125 mM) and U46619, (E_{max}) and Log EC_{50}, with age. (Data represent mean (mg) ± s.e.m)*$p<0.05$ E_{max} significantly different to veins from same age group, un-paired t-test. † $p<0.05$ E_{max} significantly different to adult one-way ANOVA ‡ $p<0.05$ Arterial E_{max} significantly greater than E_{max} of arteries with endothelium removed, from same age group, un-paired t-test. ∝$P<0.05$ E_{max} significantly different from fetal, unpaired t-test

	Venous Responses		
AGE	KCl (mg)	U46619 E_{max}(mg)	U46619 Log M EC_{50}
Fetus (n=8)	399 ± 59*	730 ± 173*	-6.9 ± 0.7
Newborn(n=9)	548 ± 119	756 ± 130	-6.9 ± 0.1
1 day(n=7)	558 ± 146	865 ± 217 *	-7.3 ± 0.3
3 day(n=10)	1111 ± 236*	1686 ± 277 *	-8.1 ± 0.09
6 day(n=10)	791 ± 118*	1178 ± 135 *	-7.7 ± 0.13
14 day(n=13)	1209 ± 128*	1647 ± 207 *	-6.8 ± 0.1
Adult(n=7)	1022 ± 298	1734 ± 464	-6.8 ± 0.06

Table 4.3 Vaso-activity of porcine pulmonary vein to KCl (125 mM) and U46619, (E_{max}) and Log EC_{50}, with age, (Data represent mean ± s.e.m). * $p<0.05$ E_{max} significantly different to arteries from same age group, un-paired t-test. Difference in contractile response between age groups p=NS.

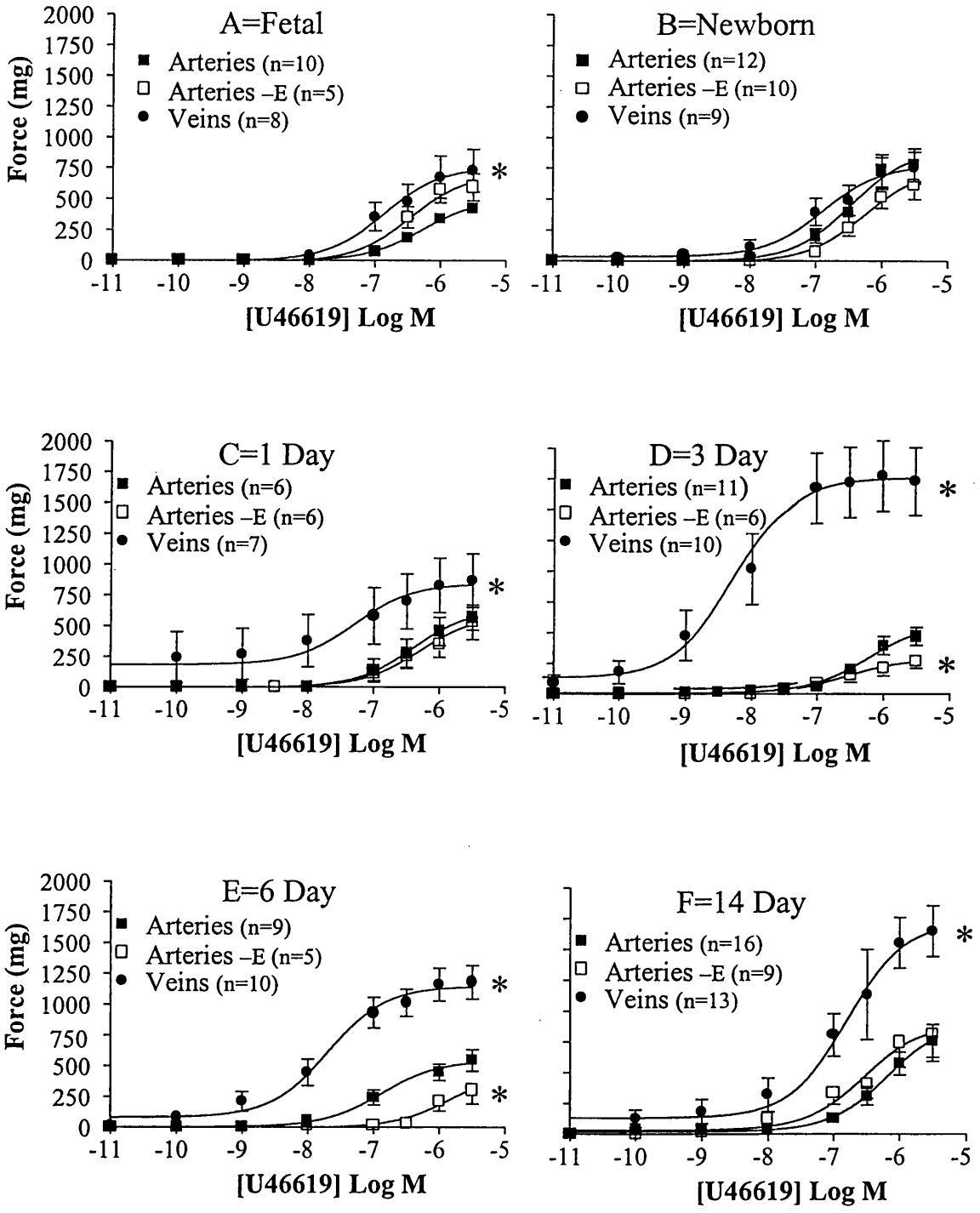

Figure 4.5 Concentration response curves to U46619 in arteries (closed squares), arteries without endothelium (-E) (Open squares) and veins (closed circles). From the following age groups; A: Fetal piglets, B: Newborn piglets, C: 1 day old piglets, D: 3 day old piglets, E: 6 day old piglets, F: 14 day old piglets. (Data represent mean ± sem). *$p<0.05$ Concentration response curves significantly different from arterial response curves, two-way ANOVA.

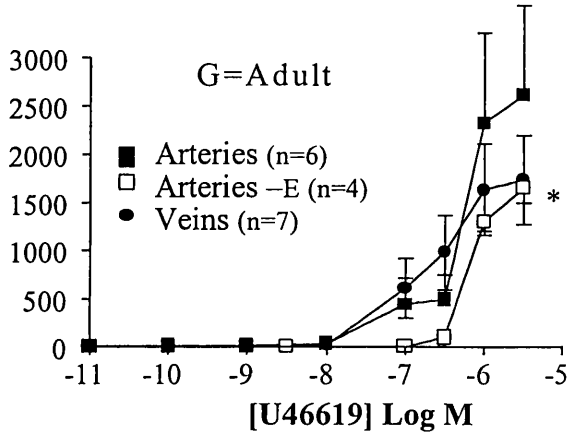

Figure 4.6 Concentration response curves to U46619 in arteries (closed squares), arteries without endothelium (-E) (Open squares) and veins (closed circles). In the following age group; G: adult pigs (Data represent mean ± sem). *$p<0.05$ Concentration response curves significantly different from arterial response curve, two-way ANOVA

4.2.10 Relaxant responses in vessels from normoxic pigs

4.2.11 *Arterial relaxant responses to ACh with age*

Relaxation to ACh was observed in both arteries and veins to occur in a concentration dependent manner. Once a maximum response to ACh had been reached, it was maintained at that level, or in the case of the arteries from the 14 day old age group, contracted. The arterial relaxations to ACh were smallest in the fetal and newborn age groups and greatest at 3 and 14 days of age (Figure 4.7). The greatest arterial relaxation to ACh was attained at 14 days of age, although this did not reach significance, and only ever reached a maximum relaxation of 30% of the relaxation to papaverine. The concentration at which the maximum relaxation took place did not follow any pattern with age and values remained within 1.5 Log M of each other.

4.2.12 *Arterial relaxant responses to ACh without endothelium*

Following removal of the endothelium, ACh induced negligible arterial relaxation at all ages except at 6 days of age. (Values for arterial relaxation minus endothelium expressed as a % of papaverine relaxation, mean ± sem: fetal (n=5), 5.4±5.4; newborn (n=10), 0.5±0.5; 1 day (n=6), 2±1; 3 day (n=6), 0±0; 6 day (n=5), 15±7; 14 day (n=9), 6±6; adult (n=4), 0±0). At 6 days of age the concentration dependent response to ACh was similar in endothelium intact and denuded arterial responses (Figure 4.7).

4.2.13 *Venous relaxant responses to ACh with age*

With increasing concentration of ACh, venous tone decreased. However, in some cases at high concentrations of ACh, contraction was induced. This was particularly notable in the tone of the veins taken from the newborn pig. Venous relaxation was observed to increase with age from the fetal to the 14 day old age group reaching a maximum at 3 days of age. However, the smallest relaxant responses were observed in both the fetal and adult pulmonary veins, although this did not attain significance.

On comparison with age matched arterial responses, venous concentration dependent relaxant responses to ACh were significantly greater than arterial responses at all ages, ($p<0.05$, two-way ANOVA, Figure 4.7& 4.8). Moreover, the smallest

venous relaxation to ACh (E_{max}), in the fetal age group, was greater than the largest relaxation to ACh (E_{max}) in arteries from the 14 day old age group. Interestingly, venous relaxation was present in both the fetal and newborn veins, where it had been virtually absent in the arteries (Figure 4. 7, Table 4.4). It was found, in a fashion similar to the arteries that in the veins there was no age related concentration of ACh at which the vessels maximally relaxed.

	Arteries		Veins	
AGE	[ACh] Log M to cause E_{max}	Relaxation(E_{max}) as % papaverine	[ACh] Log M to cause E_{max}	Relaxation (E_{max})as % papaverine
Fetus	-5.5	5±3*	-6	35±10
Newborn	-5	6±4*	-6	53±12
1 day	-4.5	18±10*	-5.5	62±12
3 day	-5	22±5*	-4	88±10
6 day	-4	17±6*	-4.5	48±8
14 day	-5	31±8*	-5	62±7
Adult	-4	23±11	-5	34±8

Table 4.4 Comparison of porcine pulmonary arterial and venous relaxation to ACh (E_{max}). (Data represent mean ± s.e.m). *$p<0.05$ E_{max} significantly different to veins from same age group $p<0.05$, un-paired t-test.

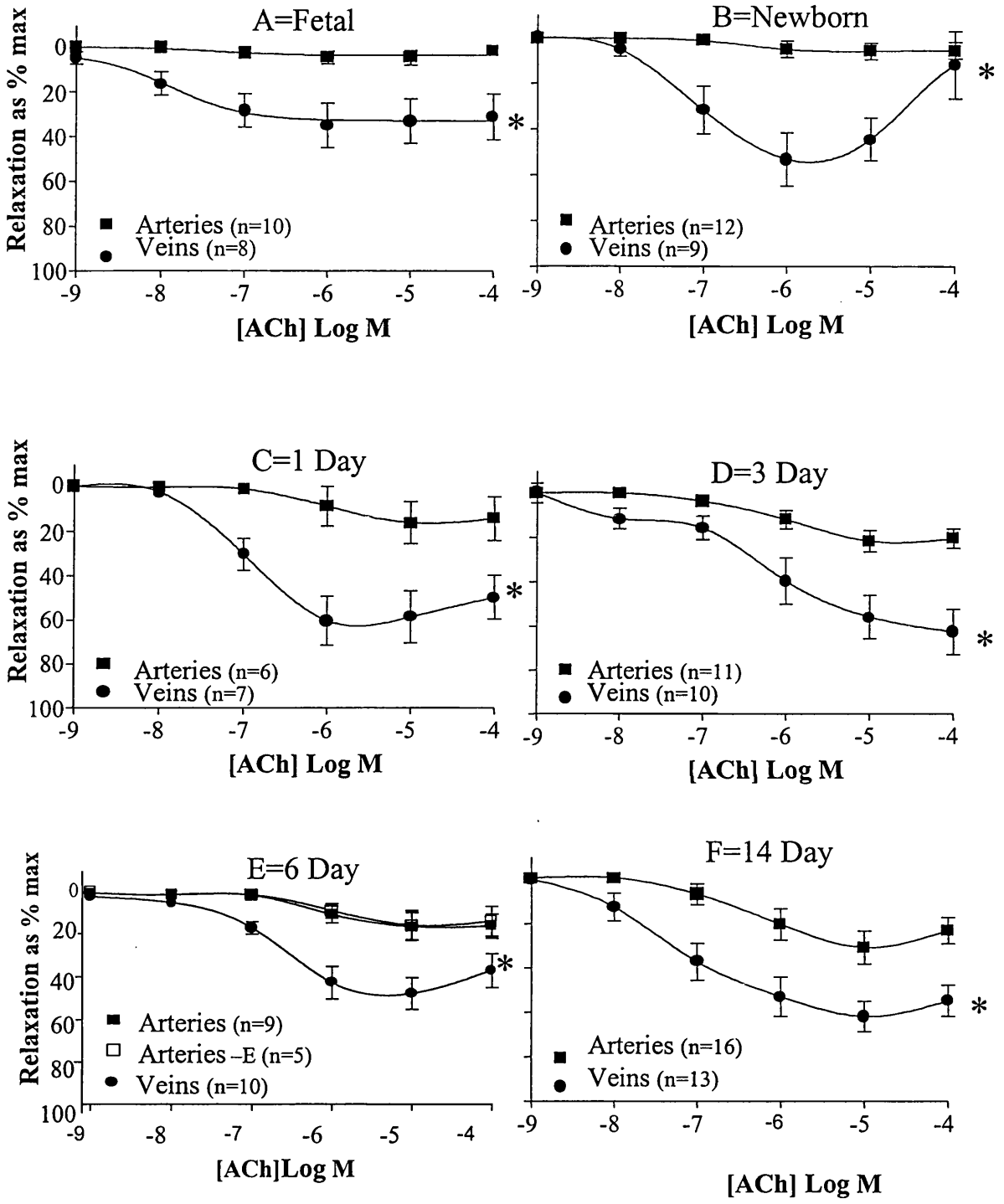

Figure 4.7 Concentration response curves to ACh in porcine pulmonary arteries (closed squares) veins (closed circles) with age and in arteries without endothelium at 6 days of age (Open squares). (Data represent mean ± s.e.m, expressed as a % of maximum relaxation to papaverine). In the following age groups; A: fetal piglets, B: newborn piglets, C: 1 day old piglets, D: 3 day old piglets, E: 6 day old piglets, F: 14 day old piglets. *$p<0.05$ Concentration response curve to ACh significantly different between arteries and veins, two-way ANOVA.

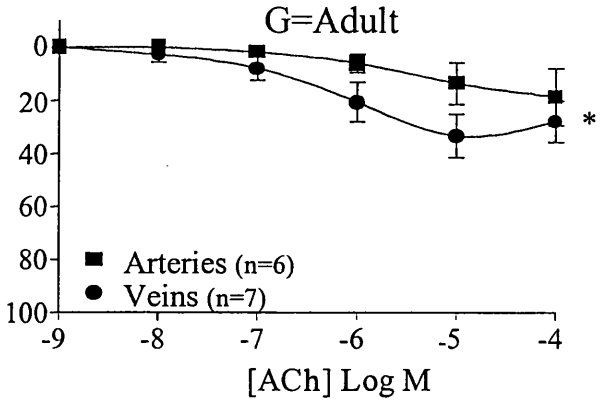

Figure 4.8 Concentration response curves to ACh in porcine pulmonary arteries (closed squares) and veins (closed circles). (Data represent mean ± s.e.m, expressed as a % of maximum relaxation to papaverine). In the following age group; G: adult pigs. *$p<0.05$ concentration response curve to ACh significantly different between arteries and veins, two-way ANOVA.

4.2.14 Hypoxia induced pulmonary hypertension

4.2.15 Contractile responses in vessels from hypoxia induced pulmonary hypertensive pigs

4.2.16 *Porcine pulmonary arterial contractile responses to U46619 and KCl in 3 and 6 day old pulmonary hypertensive piglets*

When compared to age matched controls, arterial contraction to KCl in vessels from hypoxia induced pulmonary hypertensive pigs were not significantly different. In contrast, the concentration dependent responses to U46619 were significantly greater in the arteries from 3 day old hypertensive pigs than age matched controls ($p<0.05$, two-way ANOVA, Figure 4.9), and more similar to contractile responses observed in the newborn. The sensitivity of the arteries from the 3 day old hypertensive animals to U46619 did not change from control values. The arterial contractility to U46619 (E_{max}) in the 6 day old hypertensive pig did not significantly alter relative to age matched controls (Table 4.5, Figure 4.9).

In the 3 day old pulmonary hypertensive pig following the removal of the endothelium, the contractility to KCl was unaltered. However, a significant increase in the arterial concentration dependent response to U46619 was observed ($p<0.05$, two-way ANOVA, Figure 4.9). At 6 days of age however, contractile responses from endothelium intact and denuded arteries remained similar.

When compared to age matched controls, arteries without the endothelium from both 3 and 6 day old hypertensive animals, tended to have an increased contractility to KCl (Table 4.5, Figure 4.9). The concentration dependent responses to U46619 in arteries without the endothelium were observed to be significantly elevated in the 3 and 6 day old hypertensive animals when compared to age matched controls ($p<0.05$, two-way ANOVA, Figure 4.9, Table 4.5).

4.2.17 *Porcine pulmonary venous contractile responses to U46619 and KCl in 3 and 6 day old pulmonary hypertensive piglets*

Hypertension in the 3 day old pig lung significantly reduced venous contractile responses to KCl when compared to the age matched controls ($p<0.05$, un-paired t-test). Following analysis of the whole curve, venous contractile responses to U46619 were significantly reduced ($p<0.05$ two-way ANOVA) and were similar in value to the contractile responses of the newborn pig (Figure 4.9, Table 4.6).

In hypoxia induced pulmonary hypertensive pigs at 6 days of age, no significant alterations in venous contractility were observed to either KCl or U46619 when compared to age matched controls (Table 4.6)

Much like the 3 and 6 day controls, in both sets of pulmonary hypertensive piglets the contractility to both U46619 and KCl in the veins was greater than arteries, reflected in the concentration response curves to U46619 ($p<0.05$, two-way ANOVA, Figure 4.9).

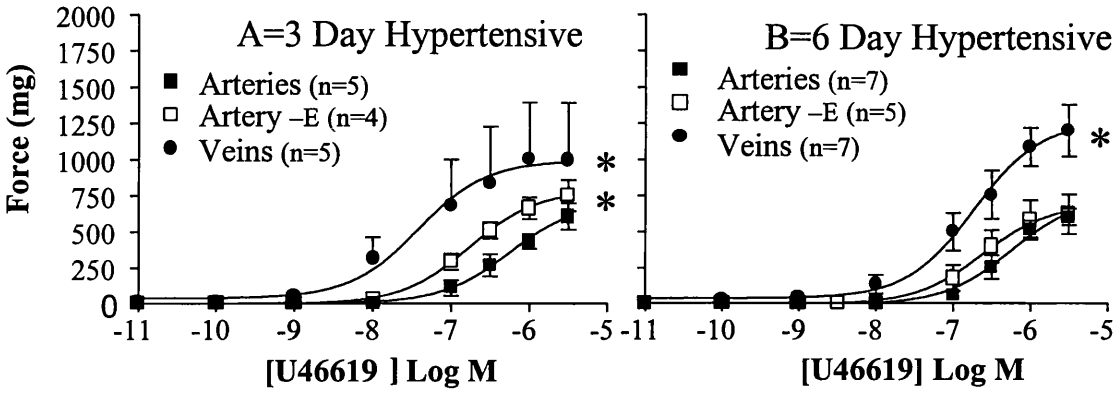

Figure 4.9 Concentration response curves to U46619 in porcine pulmonary arteries (closed squares), arteries without endothelium (-E) (Open squares) and veins (closed circles). (Data represent mean (mg) ± sem). In the following age groups; A: 3 day old pulmonary hypertensive piglets, B: 6 day old pulmonary hypertensive piglets. *$p<0.05$ Concentration response curve significantly different from arterial contraction, two-way ANOVA.

	Arterial responses				Arterial responses without endothelium			
AGE	n	**KCl (mg)**	**U46619 E_{max}(mg)**	**U46619 LOG EC_{50}**	n	**KCl (mg)**	**U46619 E_{max}(mg)**	**U46619 LOG EC_{50}**
Newborn	12	519±97	778.2±134	-6.5 ± 0.08	10	536.4±152	437±76	-6.42±0.19
3 day	11	476 ± 46*‡	470 ± 75	-6.2 ± 0.09	6	217.5±44	269.2±57.53	-6.02±0.61
6 day	9	366 ± 57*‡	544 ± 87	-6.9 ± 0.08	5	154±48	300.7±111	-5.91±0.15
3 day hypertensive	5	379 ± 112	610 ± 92*	-6.3 ± 0.04	4	345.7±59	751.5±107†	-6.76±0.02
6 day hypertensive	7	396.4± 66.0	603.6±58	-6.2±0.15	5	290±117.7	622.5±137†	-6.55±0.2

Table 4.5 Vaso-activity of porcine pulmonary artery with and without endothelium to KCl (125 mM) and U46619 (E_{max}) and Log EC_{50} with hypoxia induced hypertension. ‡$p<0.05$ E_{max} of arteries significantly different to arteries without endothelium from same age group, unpaired t-test* *$p<0.05$ E_{max} significantly different to veins from within same age group, unpaired t-test †$P<0.05$ Emax of hypertensive pig significantly different to arteries from same age group

Venous Responses			
AGE	**KCl (mg)**	**U46619 E_{max}(mg)**	**U46619 Log EC_{50}**
Newborn(n=9)	548 ± 119	756 ± 130	-6.9 ± 0.1
3 day(n=10)	1111 ± 236*	1686 ± 277	-8.1 ± 0.09
6 day(n=10)	791 ± 118*	1178 ± 135	-7.7 ± 0.13
3 day hypertensive(n=5)	526 ± 123†	1000 ± 391†	-7.4 ± 0.1
6 day hypertensive(n=7)	771.4± 184.8	1200± 179	-6.8± 0.19

Table 4.6 Vaso-activity of porcine pulmonary vein to KCl (125 mM) and U46619 (E_{max}) and Log EC_{50}, with hypoxia induced pulmonary hypertension. (Data represent mean ± s.e.m) * $p<0.05$ E_{max} significantly different to arteries from same age group, unpaired t-test †$p<0.05$ E_{max} of hypertensive pig significantly different to age matched controls, unpaired t-test.

4.2.18 Relaxant responses in vessels from hypoxia induced pulmonary hypertensive pigs

4.2.19 *Porcine pulmonary arterial relaxant responses to ACh in 3 and 6 day old pulmonary hypertensive piglets*

Pulmonary arteries from hypertensive 3 day old pigs did not respond following the addition of ACh, thus producing a significantly decreased relaxant response when compared to their age matched controls ($p<0.05$, two-way ANOVA, Figure 4.10, Table 4.7). At 6 days of age, the relaxant responses in the hypertensive artery also tended to be diminished when compared to controls (p=NS), although not as greatly as in the 3 day old pulmonary hypertensive pig.

Removal of the endothelium made little difference to the small relaxations observed in the arteries from the 3 day old pulmonary hypertensive piglet (mean as % of

maximum relaxation to papaverine ± sem: artery minus endothelium (-E), 6.4±6.7; artery plus endothelium (+E), 1.63±0.99).

4.2.20 *Pulmonary venous relaxant responses to ACh in 3 and 6 day old pulmonary hypertensive piglets.*

Compared to the age matched controls, the venous relaxant responses to ACh were significantly reduced in the 3 day old pulmonary hypertensive pig. At 6 days of age, there was no difference between venous responses in control and hypertensive pigs (Figure 4.10, Table 4.7).

In a similar fashion to their age matched controls ACh was more potent in the pulmonary veins than arteries in both hypertensive age groups. In the 3 and 6 day old pulmonary hypertensive animals a significantly greater relaxation in the veins was observed from that of the arteries ($p<0.05$, two-way ANOVA, Figure 4.10, Table 4.7).

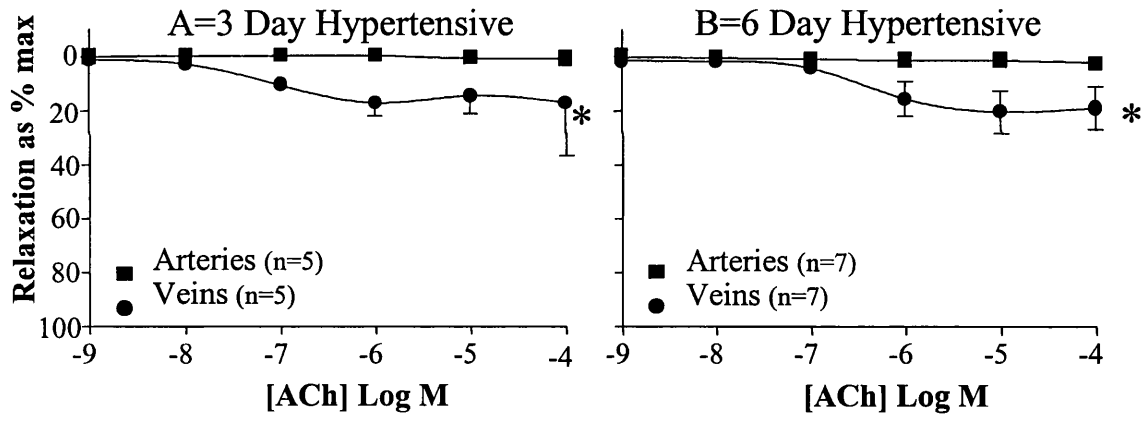

Figure 4.10 Concentration response curves to ACh in porcine pulmonary arteries (closed squares) and veins (closed circles), (Data represent mean ± sem). In the following age groups; A: 3 day old hypertensive piglets, B: 6 day old hypertensive piglets. *$p<0.05$ Concentration response curve to ACh significantly different between arteries and veins, two-way ANOVA.

	Arteries		Veins	
AGE	[ACh] Log M to cause E_{max}	Relaxation (E_{max})as % papaverine	[ACh] Log M to cause E_{max}	Relaxation (E_{max})as % papaverine
Newborn	-5.0	6±4*	-6	53±12
3 day	-5	22±5*	-4	88±10
6 day	-4	17±6*	-4.5	48±8
3 day hypertensive	-4	2±1*†	-4.5	24±17†
6 day hypertensive	-4	8.0±2*	-5	36±8

Table 4.7 Comparison of porcine pulmonary arterial and venous relaxation to ACh with hypoxia induced pulmonary hypertension, (Data represent mean ± sem). * $p<0.05$ concentration response curve to ACh significantly different to veins from same age group, two-way ANOVA. †$p<0.05$, concentration response curve in hypertensive pig significantly different from age matched control, two-way ANOVA.

4.3 Discussion

This chapter described the patterns of vasoactivity between arteries and veins from the porcine lung and how these responses were altered with development. Previous studies have not explored this relationship extensively, and have tended to focus primarily on relaxant responses of the pulmonary vasculature. In addition, this chapter showed alterations in vascular reactivity of arteries and veins induced by a pathological condition resembling PPHN. A lot is known about the structural and functional alterations that occur in the arteries of this porcine model of PPHN, however, the effects on the venous vascular responses have not been extensively studied. Therefore, the alteration in the vasoactivity of arteries and veins was studied in the pathological condition of PPHN, by exposure of piglets to chronic hypoxia from birth and from 3 days of age.

In this chapter the relationship between the passive tension of the vessel and induced diameter was studied. Passive tension reflects the passive physical properties of both the contractile and non-contractile components of the tissue in intact vessels (Sparks et al., 1961). The active tension represents smooth muscle properties in the vessel wall, which when stretched beyond a certain length become unable to produce an active tension (Bergel et al., 1961; Greenwald et al., 1982; Miyagi et al 1995). These data have previously been described in arteries in the developing porcine lung (Greenwald et al., 1982), but not veins. Despite having similar ring weights, the veins from the perinatal age groups, in contrast to the arteries, were found to require a lower application of tension to the vessel before reaching a maximum response. This was most likely due to the structure of the vein wall, which is known to contain less muscular and elastic tissue than its comparative artery (Hislop et al., 1973).

4.3.1 Contractile Properties

The changes with age of both the contractile and relaxant responses in adjacent conduit pulmonary arteries and veins were studied. For most ages, the maximum force of contraction produced by U46619 in both arteries and veins tended to be greater than that produced by KCl. KCl stimulates vascular smooth muscle to contract by depolarising cells, resulting in an influx of calcium (Karaki et al., 1984). U46619 induces contraction following activation of TP receptors that are linked to the inositol phosphate pathway. This leads to the release of calcium from intracellular stores

(Strader et al., 1995). However, in porcine pulmonary arteries U46619 also inhibits the actions of cGMP via the activation of a protein kinase C, thus preventing a cGMP mediated decrease in intracellular Ca (Perez-Vizcaino et al., 1997). Therefore, these two independent mechanisms of contraction to U46619 may explain the greater contractility of U46619 than KCl and also the consistent levels of contraction to U46619 in pulmonary arteries and veins at different ages. There was a trend for the responses to KCl to remain consistently lower than those to U46619, in a similar age related pattern. Given the differences in the mechanisms of action of the two agonists, the changes in response to U46619 with age are not likely to be due solely to differences in the density or effectiveness of the thromboxane receptors but also to the structure of the vessel walls.

For the pulmonary arteries, the smallest contractile responses to U46619 and KCl were produced in the fetus, and within 5 minutes of birth contractility was observed to increase. Following removal of the endothelium the difference between arterial contractility in the fetus and newborn pigs was absent as the fetal contractions to U46619 were enhanced. However, fetal arterial responses to KCl were not enhanced following removal of the endothelium, suggesting that it was the structure of the vessel wall that contributed to the low contractility to agonists displayed at this age. In fact in the fetal ovine pulmonary artery, smaller amounts of the contractile protein actin have been measured in the pulmonary arterial smooth muscle cells than postnatally (Belik et al., 1991).

The increase in fetal arterial contractility to U46619 and not KCl, following removal of the endothelium, may have been caused by an increase in the interaction between the thromboxane mimetic and its receptors, perhaps created because of a change in TP receptor sensitivity.

Within five minutes after birth, the contractile responses to U46619 were greatly enhanced decreasing again to a minimum at 3 days of age. These contractile responses followed a similar pattern to previous studies using $PGF_{2\alpha}$ in endothelium intact and denuded porcine pulmonary arteries (Levy et al., 1995). Thus, the alterations in contractility with age are not agonist related and could be characteristic of smooth muscle cell and arterial wall composition as previously suggested. Indeed on removal of the endothelium the lowest contractile responses to U46619 postnatally were still observed at 3 and 6 days of age. Porcine pulmonary arteries show a significant decrease in pulmonary arterial smooth muscle cell contractile myofilament volume density

between birth and 14 days of age, with the lowest density occurring at 3 days of age (Hall et al., 1987). During the same period of time, there is a marked increase in connective tissue deposition, associated with an increase in structural stiffness of the arteries (Greenwald et al., 1982; Hall et al., 1987). Thus, postnatally in the perinatal period, the structural changes that have been observed could explain the vascular responses obtained from this chapter. This may also explain the large increase in contractility observed at adulthood, which could be related to the larger amount of connective tissue and muscle cells in the artery walls (Greenwald et al., 1982).

In the present study, the contractile responses of veins to U46619 increased between fetal life and 3 days of age, by which time the contractility was maximal and maintained until adulthood. This maximum venous contractility at 3 days of age appeared to compensate for the very low arterial responses to U46619 at this age, and was perhaps indicative of a rapid upregulation of the thromboxane receptor expression in the veins. Further investigations including receptor ligand binding studies are necessary in order to ascertain whether changes in response to U46619 are due to changes in thromboxane receptor density. In a similar fashion to arteries, the veins taken from adult pigs had greater levels of contractility than the younger age groups studied, again because adult veins are thicker than the younger age groups and contain more connective tissue, as observed in both the porcine and human lung (Wagenvoort et al., 1964). In young piglets, as in human infants, the vein wall is particularly thin (Hislop et al., 1973) containing little connective tissue relative to smooth muscle cell content. These walls are visibly thinner than the corresponding arteries and this may therefore facilitate the greater response to contractile agonists than arteries. An increased venous reactivity to U46619 has also been observed in the isolated perfused lungs of newborn (0-4 day old) lambs, where the increase in pulmonary vascular pressure is mediated by veno-constriction (Yoshimura et al. 1989). The greater venous reactivity has also been shown in the mature lung, with greater veno-constriction mediated by the veins from the ferret lung (Raj et al.1990). Thus this occurrence has been observed with a variety of species and developmental ages and highlights the importance of the venous contractile reactivity in the lung.

4.3.2 Relaxant Properties

Arterial relaxation to ACh was absent in most vessels that had the endothelium removed, thus confirming the endothelium dependence of the response to ACh. Surprisingly however, at 6 days of age the relaxant responses to ACh were endothelium independent. This is not likely to be due to the continuing presence of the endothelium, as the same technique had removed the relaxant responses at all other ages studied. This suggests that at 6 days of age, there is a production of relaxing factors from the smooth muscle. One possible candidate for the production of such a factor following stimulation with ACh is PGI_2. Indeed, COX (COX-2) is induced in vascular smooth muscle by a number of physiological and pathophysiological stimuli (Mitchell et al., 1998). Thus, it could be speculated that at 6 days of age COX-2 is induced in the smooth muscle, which produces PGI_2 when stimulated with ACh. This could be easily tested by performing experiments in the presence of a COX inhibitor such as indomethacin. Interestingly, it was also at 6 days of age, that there was a transient decrease in eNOS activity in whole lung homogenate. For whatever the reasons, this demonstrates that at 6 days of age the mechanisms that mediate the vasoactive responses of the pulmonary arteries differ from other age groups. However, it is not within the scope of this study to investigate this occurrence further.

As has previously been observed in the pig model (Zellers et al., 1991; Liu et al., 1992), arterial relaxations to ACh in this chapter increased with maturation. In pulmonary arteries from the fetal and newborn pigs, vasodilation to ACh was absent and never exceeded 40% of the maximum relaxation in the older animals. This was in contrast to the effect of papaverine, which was able to fully reverse the U46619 induced contraction in all preparations, including fetal and newborn pulmonary arteries. In mature vessels, papaverine causes vasodilatation by a different signal transduction pathway to ACh, and increases cAMP content by inhibition of phosphodiesterases. Thus the low levels of arterial relaxation that were observed in all the age groups to ACh could be contributed to, either by the inhibition of the cGMP pathway by thromboxane as has previously been observed in porcine pulmonary arteries (Perez-Viczaino et al., 1997), or by the lack of some component of the NO / cGMP pathway, particularly in fetal and newborn pulmonary arteries. In fact ACh induced vasodilation is commonly known to be absent in the newborn and fetal pulmonary arteries of the pig, sheep and rabbit (Tulloh et al 1997; Liu et al 1992;Steinhorn et al., 1993; Abman et al., 1991) using pre-contractile agonists other than U46619, such as $PGF_{2\alpha}$. In those studies,

vessels could relax to NO and its donors such as SNP, suggesting that beyond the endothelial cell, in the fetal and newborn arteries, sGC has the potential to be activated and that it is the production of NO which is limited. Indeed in the same model porcine lung, stimulation with ANP, which stimulates particulate cGMP, relaxes fetal and newborn arteries (Matsushita et al., 1999). Using immunostaining techniques, eNOS has been found in the pulmonary arteries of both the newborn pigs (Hislop et al., 1995) and fetal lambs (Halbower et al., 1994) and with age is more abundant in the newborn than adult porcine vessels (Hislop et al 1995). However, in the previous chapter (chapter3), using Western blots, concentrations of eNOS were detected that were equivalent in the fetal, newborn and adult porcine lung. However, NOS activity was present in the newborn and adult lung, but absent/ low in the fetal lung (Chapter 3). Therefore in the fetus, the lack of vasodilation to ACh is perhaps created by a lack of NOS activity.

The failure of the newborn vessel to dilate to ACh, is not due to a lack of NOS activity (chapter 3), and, may be related to the smaller number and/or reduced sensitivity of muscarinic receptors stimulated by ACh. In fact, postnatally in the porcine lung, the number of M1 and M3 receptors that mediate relaxation have been observed to be lower at birth (Hislop et al., 1998). The increase in muscarinic receptor number with age in turn may account for the increase in arterial relaxation to ACh that occurs, particularly at 3 days of age. This increase at 3 days of age occurs in association with increased eNOS levels (Hislop et al., 1995) and activity (chapter 3).

However, the lack of relaxation to ACh may not only be related to the receptor, as pulmonary arteries from the newborn pig also have diminished relaxant responses to the Ca ionophore, A23187, in vitro when compared to older postnatal age groups (Tulloh et al., 1997). A23187 activates the release of NO without the use of receptors. This implies that in vitro the diminished release of NO from the endothelial cells following ACh stimulation is also limited somewhat by the availability/ activity of NOS. The NOS activity of the newborn lung (chapter 3), was maximally stimulated in the presence of all required substrates and cofactors, such as L-arginine and BH_4. These substances may have been limiting in the organ bath experiments undertaken in this chapter. If however, the production of NO was not diminished in the newborn age group, a reaction with superoxide radicals may have inactivated it. Superoxide has been shown to be in excess at birth, and has been thought to compromise the arterial relaxation to ACh in newborn (1-2 hr) rabbits (Morecroft et al., 1998). However, since newborn porcine and ovine pulmonary arteries respond well to exogenous NO (Tulloh

et al 1997; Liu et al 1992; Steinhorn et al., 1993; Abman et al., 1991), superoxide may play a minor role. However, further investigation into the roles of these factors is required, to observe if they play any part, not only in the fetal and newborn relaxant responses, but also in the responses from the older postnatal age groups, which maintain a stable level of relaxation with age.

In contrast to the arteries, relaxation to acetylcholine in the veins produced similar responses at all ages that were consistently greater than the arterial responses. The lack of variation in the responses of the veins in these developmental age groups is reflective of the expression of eNOS, which in piglet pulmonary veins is consistently high while that in pulmonary arteries varies with age (Hislop et al., 1995). The greater relaxant responses were evident, particularly in the fetal and newborn age groups. At these ages the low levels of venous relaxation were still greater than the largest arterial relaxation at 14 days of age. Indeed the production of NO has been observed to be greater in veins than arteries of newborn sheep (Steinhorn et al., 1993) with a greater expression of eNOS in veins than arteries from fetal lambs (Halbower et al., 1994). The greater sensitivity of pulmonary veins to NO than arteries from fetal sheep has been associated with strongly positive immunostaining for sGC (D'Angelis et al 1998), whilst fetal veins stimulated with acetylcholine produce higher levels of cGMP than in arteries (Gao et al., 1995). In the other postnatal age groups studied in this chapter, the veins also relaxed more than their corresponding arteries. The same has been found in the 7 day old sheep lung, with maximum relaxations to ACh greater than in matched arteries (Gao et al., 1995). The venous relaxant response in the adult however, appeared diminished relative to some of the younger age groups, This may have been due to thicker vessel walls, which may have prevented the vessels from further relaxation. The greater venous relaxant response to ACh than the age matched arteries, is most likely related to NO production as in the adult pig lung venous NOS activity has been observed to be greater than corresponding arterial NOS activity in addition to an increase in cGMP content (Bina et al., 1998).

Thus the production and effect of NO on venous smooth muscle appears greater than its effects in the arteries, possibly due to an increased number of elements of the NO/ cGMP pathway. In the arteries, more age related changes to both contractile and relaxant agonists were observed. These arteries which receive blood from the heart are thus, with age, adapted to control the amount of blood reaching the lung and

subsequent ventilation-perfusion matching in the lung at a time when the lung structure and size is continually changing.

Agonists are more potent in the veins than the arteries. This may perhaps imply that the veins play a role in preventing the onset of pulmonary oedema in abnormal situations, and thus under normal conditions are quiescent.

4.3.3 Effects of hypoxia induced pulmonary hypertension

As previously reported in piglets exposed to chronic hypoxia from birth, the normal decrease in pulmonary vascular resistance is prevented and pulmonary artery medial thickness is increased alongside other pulmonary adaptations to the hypoxic response (Haworth, 1982). In the present chapter, hypoxia induced pulmonary hypertension caused an increase in contractility. This increase may have been induced by the increased in smooth muscle cell size in the media as has previously been observed (Rudolph et al., 1966; Haworth et al., 1982; Allen et al., 1986). However, the functional response of the endothelium to regulate PVR may have altered, as has been reported in a variety of species following chronic hypoxia (Archer et al., 1989; Feletou et al., 1995; Tucker et al., 1976; Johns et al., 1989). In this chapter removal of the arterial endothelium from pulmonary hypertensive animals, hypertensive from birth, caused a significant increase in contractility, perhaps indicating the removal of an endothelially derived relaxing factor. NO has been observed to be released basally in chronically hypoxic pulmonary hypertensive rats with inhibition of NO inducing a greater vasoconstriction than occurred in age matched controls (Isaacson et al., 1994). However, as the increase in contractility to U46619 seen here with pulmonary hypertension, did not occur with KCl, the response could be related to an increase in TP receptor density.

The relaxant responses to ACh in arteries taken from the hypertensive pig were decreased when compared to the 3 day control. Previous data have shown that in this porcine model the relaxant response to ACh is abolished following exposure to hypoxia from birth (Tulloh et al., 1997). It was shown in chapter 3 that this was associated with a decrease in proportion of Ca dependent NOS activity in whole lung, and others have shown a reduction in arterial eNOS expression (Hislop et al., 1997). Similarly, most data using ovine lungs have shown a decrease in eNOS protein and mRNA in fetal hypertension following ductal ligation (Black et al., 1997, Shaul et al., 1997). The

resulting decrease in NO production is accompanied by decreased activity of sGC in fetal lambs with pulmonary hypertension (Steinhorn et al., 1995).

At 6 days of age, in the pulmonary hypertensive piglet exposed to hypoxia from 3 days of age, the arterial maximum contractile response (E_{max}) to both KCl and U46619 tended to be greater than in controls. However in contrast to the responses observed in the 3 day old pulmonary hypertensive animals, the difference was not statistically significant. In addition, removal of the endothelium did not enhance the contraction to U46619. Like those responses from the 3 day old hypertensive pig, arterial relaxation to ACh was attenuated. This was not due to an alteration in NOS activity, which in chapter 3 was observed to be similar in 6 day old pulmonary hypertensive pigs to the age matched controls. In fact, by immunostaining, levels of eNOS have been shown to increase in this porcine model of pulmonary hypertension at 6 days of age (Hislop et al., 1997). The lack of relaxant response to ACh may be a direct effect of the long term effects of exposure to low levels of oxygen on the metabolic activities of the cells. In addition, it was demonstrated that in contrast to the arteries, following hypoxic exposure the capacity of the veins from the 3 day old hypertensive animals to contract to U46619 had decreased relative to controls. Similarly in the isolated veins of the adult rat, chronic hypoxia impairs the contractility of smooth muscle (Zhao et al., 1995). In contrast, in whole lung from the ferret, an increase in veno-constriction has been observed following hypoxia induced pulmonary hypertension (Raj et al., 1990). This anomaly may be due to the use of different animal models studied, and the different techniques studying whole lung and isolated tissue.

In the data from this chapter, venous relaxant responses at 3 days of age to ACh were decreased in the pulmonary hypertensive lung. In a similar fashion, veins from the fetal hypertensive ovine lung have shown attenuated ACh relaxant responses following ductal ligation, but responded to the Ca ionophore and NO donors (Steinhorn et al., 1995). Thus the endothelial cell does partially mediate the diminished venous responses to ACh in this disease. In contrast to the 3 day old pulmonary hypertensive pig, in the veins from the 6 day pulmonary hypertensive pigs neither contractility nor relaxation were different from that found in age matched controls. As the changes in vascular relaxation from the hypoxia induced pulmonary hypertensive pig at 3 days of age and not 6 days of age suggested the development in the arteries was arrested, this may have also applied to the veins. It is unlikely that the change in venous response observed in the 3 day old pulmonary hypertensive pig was induced by hypoxia alone, as both 3 and

6 day old pulmonary hypertensive pigs were exposed to the same low oxygen tension for the same duration of time. Therefore the reactivity of the veins alters, depending on the age at which the animal was exposed to hypoxia, and the subsequent structural changes that arise. However, changes in venous structure with hypoxia induced pulmonary hypertension have not been extensively studied, and further examination of this relationship between structure and function is required.

In conclusion, the contractile patterns to U46619 and KCl observed in pulmonary arteries and veins with age and the hyporesponsiveness observed in the fetal and newborn pulmonary arteries and veins are most likely mediated by the structural alterations in the vessels that occur with development. Comparison between arteries and veins however showed that the significantly larger contractile responses in the veins are evidently not due to a greater vessel size, but may be related to the thinness of the vessel wall permitting easier cell/cell interaction. The differences in the venous contractile response are unlikely to be receptor mediated, as contractions to both KCl and U46619 were considerably greater than arteries. Further studies would be required to elucidate the mechanisms differentiating the responses between these vessels and investigating the effects of different contractile agonists.

Variation in the relaxant responses to ACh with age, particularly in the arteries, might be due to the maturation of a mixture of elements of the NO/cGMP pathway. These may include maturation of the receptors, transmission of the signal from the receptors, activity of the NOS enzyme, production of NO, and the response of the smooth muscle to NO. Furthermore, a combination of one or all of these elements depends upon other factors such as rate limiting substrates for NOS, deactivation of NO, and activity of other enzymes in the cGMP pathway. From this chapter, it may be suggested that some of these factors alter in the arteries with development more than the veins, which relax in a similar fashion with age. The changes in vasoactivity observed in the pathological state of hypoxia induced pulmonary hypertension appears in this model due to the lack of maturation of the lung following induction by chronic hypoxia and the maintenance of the newborn/fetal state at 3 days of age. This in turn appears to alter the production and activity of NO.

Thus in order to investigate these age and hypertension related vascular differences further, the modulation of the NO /cGMP pathway is required in order to begin to understand why the dysfunction of the relaxant response to ACh occurs in these animals.

Chapter 5. The effect of modulation of the NO/cGMP pathway on tonic responses in arteries from different stages of development.

5.1 Rationale

In the previous chapter (chapter 4), it was observed that the development of the arterial responses varied more with age than the veins. Thus the development of arterial vascular reactivity would be more likely to cause age related changes in PVR. Data presented in chapters 3 and 4 suggested that some of the differences in vaso-reactivity seen in pulmonary arteries might be due to changes in the NO/cGMP pathway with time. The aim of this chapter was to therefore address the changes in arterial vascular reactivity with age, in the light of the modulation of NO. The alterations in contraction and relaxation to a variety of modulators of the NO/cGMP pathway were studied in arteries from selected age groups and the effects of hypoxia induced pulmonary hypertension on this pathway were observed.

Although L-arginine is not rate limiting in endothelial cells normally (Mitchell et al., 1990), little is known about its metabolism with development. Thus in order to address the possible lack of L-arginine as an explanation for the reduced relaxant response in pulmonary vasculature at some stages of development, the exogenous substrate was added to organ baths.

To determine the biological activity of NO released basally (i.e. without the presence of ACh), L-NAME was added to vessels pre-contracted with U46619. However, the biological activity of NO may be greatly limited by the production of superoxide anions (Gryglewski et al., 1986). Thus in order to address the possibility that the low levels of endothelium dependent relaxation seen in some vessels are mediated by an increased superoxide production, or reduced expression of SOD at certain stages of development, experiments were performed in the presence of the cell permeable SOD mimetic MNTPY.

Finally the level of sGC activity involved in any "basal" versus ACh-induced release of NO was assessed by performing experiments in the presence of the inhibitor ODQ (Schrammel et al., 1996).

In addition to studying the effects of L-arginine, L-NAME, MNTPY or ODQ on the vascular responses of pulmonary arteries from the fetal, newborn, 3 day, 14 day and

adult age groups, the same approach was used to understand events occurring in vessels from animals with hypoxia induced pulmonary hypertension.

5.2 Results

Age groups were selected that had, in the studies from the previous chapter, appeared to be important developmentally. The age groups studied were: Fetal, newborn, 3 and 14 day old and adult. Tissue was contracted to approximately 80% of E_{max} for U46619, this level of contraction allowed the observation of constrictor as well as dilator actions of drugs.

5.2.1 **Contractile responses in vessels taken from normoxic pigs**

Organ chamber studies were carried out and the contractile responses to KCl (125mM), and U46619 (EC_{80}) observed. The EC_{80} was observed for the arteries to be (M): Fetal artery, $1x10^{-6}$; newborn artery, $1x\ 10^{-6}$; 3 day artery, $1x\ 10^{-6}$; 14 day artery, $3x10^{-6}$: adult artery, $1x\ 10^{-6}$; 3 day old hypertensive artery, $3x10^{-6}$. Once tone to U46619 had been established, individual drugs were added and the resulting alteration in tone of the vessel observed. Concentration response curves to ACh were then constructed.

The arterial responses to KCl and U46619 seen in this chapter altered with age, in a fashion similar to the trends observed in chapter 4.

5.2.2 *Effect of: L-Arginine, L-NAME, MNTPY, or ODQ on porcine pulmonary arteries preconstricted with U46619*

Following the addition of L-arginine, the tonic responses remained unaltered in all of the age groups studied. In contrast, with the addition of L-NAME, the arterial tension increased in most of the age groups studied, although statistical significance was only achieved in the vessels from the fetus, newborn and 3 day old age groups ($p<0.05$, one sample t-test), with the greatest increase in tension seen in arteries from the newborn pig (Figure 5.1). Following the addition of MNTPY, arterial contraction was not altered in any of the age groups studied. ODQ increased the levels of arterial contraction at most ages, with statistical significance being achieved in all age groups except the newborn ($p<0.05$, one sample t-test, Figure 5.1)

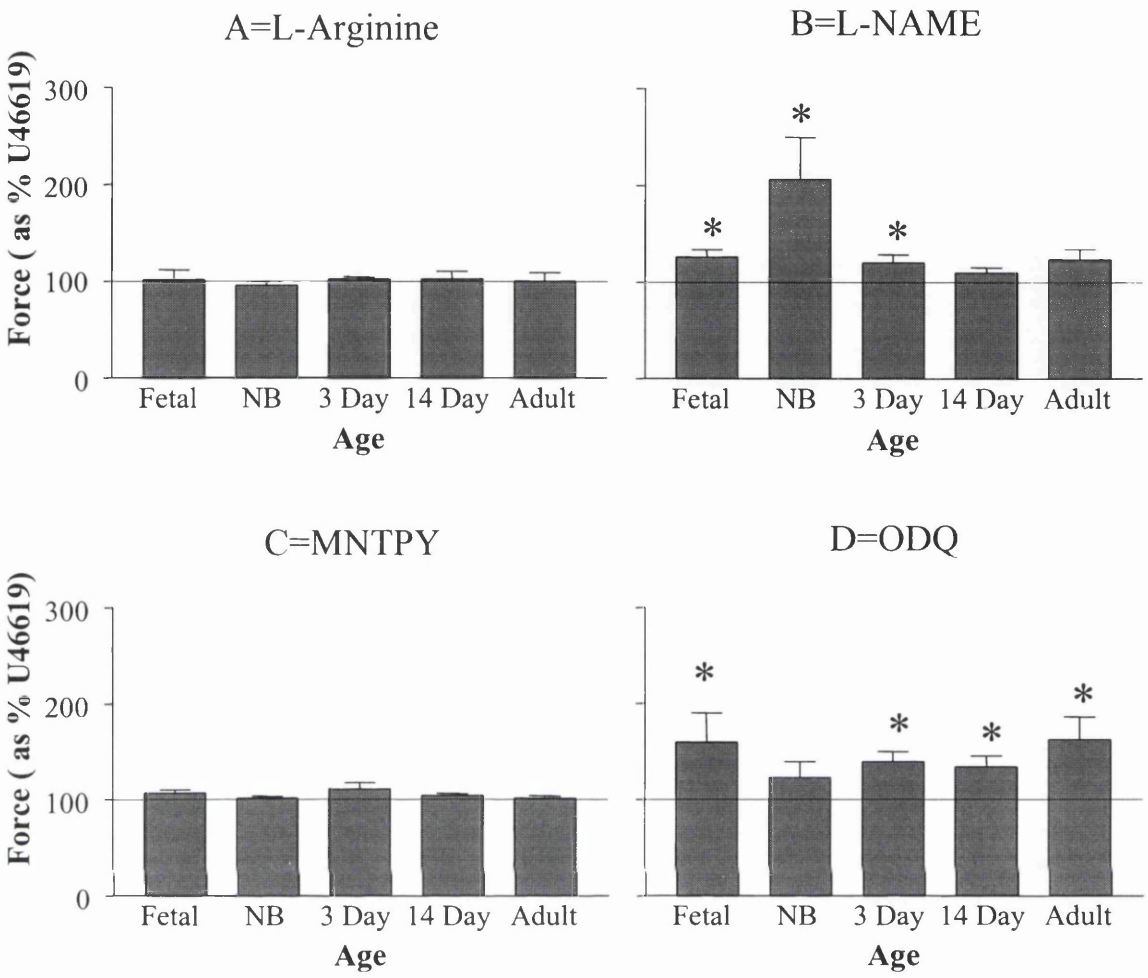

Figure 5.1 Pulmonary artery (n=6): Effect of: L-arginine (1mM) L-NAME (1mM), MNTPY(10μM), or ODQ (10μM), on the vasoconstriction of porcine pulmonary arteries to U46619 (EC_{80} straight line) at a variety of developmental ages. * $p<0.05$ tone significantly different to that induced by U46619 (EC_{80}), one sample t-test . Data expressed as mean (% of U46619 contraction after U46619 (100%) contraction) ± sem.

5.2.3 *Effect of: L-Arginine, L-NAME, MNTPY or ODQ on porcine pulmonary artery, without the endothelium, pre-constricted with U46619.*

No effect on tone was produced at any age group following the addition of L-arginine to arteries without the endothelium. In a similar fashion the addition of L-NAME and MNTPY did not affect contractile tone. In contrast, ODQ induced contraction in vessels from fetal and 3 day time points (*$p<0.05$, one sample t-test (Figure 5.2).

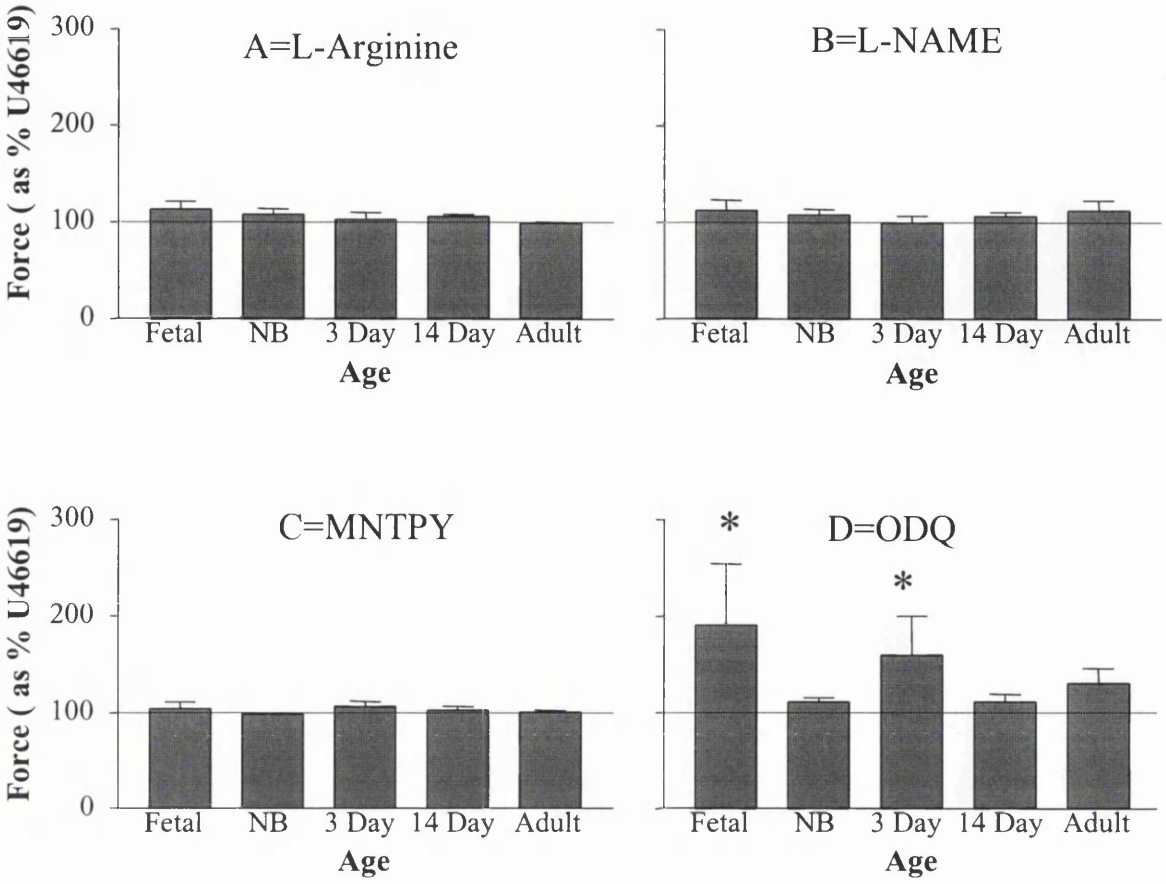

Figure 5.2 Pulmonary arteries without the endothelium (n=6). Effect of: L-arginine (1mM) L-NAME (1mM), MNTPY(10μM), or ODQ (10μM),on the endothelium independent vasoconstriction of porcine pulmonary arteries to U46619 (EC_{80} straight line), at a variety of developmental ages. Data expressed as mean (% of U46619 contraction after U46619 (100%) contraction) ± sem.

5.2.4 **Relaxant responses in vessels taken from normoxic pigs**

In a fashion similar to previous data groups (chapter 4), relaxation to ACh in arteries increased in an age dependent manner, a maximum relaxation being observed at 14 days of age with a decrease apparent at adulthood.

5.2.5 *Effects of: L-Arginine, L-NAME, MNTPY, or ODQ on relaxant responses of porcine pulmonary arteries, induced by ACh*

The dilator effects of ACh (E_{max}) following the addition of L-arginine, were similar to values in the untreated vessels (control) in all the age groups studied (Table 5.1, Figure 5.3). At all ages the relaxation to ACh tended to diminish in the presence of L-NAME, with concentration dependent responses attaining statistical significance at 14 days of age ($p<0.05$, two-way ANOVA, Figure 5.4). However, the arterial responses to ACh in the fetal, newborn and adult age groups did not reach statistical significance, possibly due to the low or undetectable effect of ACh in the younger age groups. MNTPY did not influence the ability of ACh to induce relaxation of pulmonary arteries from any age group. Following the addition of ODQ, the levels of relaxation induced by ACh was significantly reduced in the fetal and 3 day old age groups ($p<0.05$, two-way ANOVA, Figure 5.3). In the pulmonary arteries from the adult pig, concentration dependent relaxation to ACh was significantly enhanced ($p<0.05$, two-way ANOVA, Figure 5.4).

5.2.6 *Effects of: L-Arginine, L-NAME, MNTPY, or ODQ on relaxant responses of porcine pulmonary arteries without endothelium, induced by ACh*

Endothelium removal eliminated responses to ACh ($3x10^{-6}$) in all age groups studied, the presence of any of the drug treatments did not alter this.

	Arterial Responses									
AGE	**Control [ACh] Log M to cause E_{max}**	**Control Relaxation as % papaverine**	**L-Arginine [ACh] Log M to cause E_{max}**	**L-Arginine Relaxation as % papaverine**	**LNAME [ACh] Log M to cause E_{max}**	**LNAME Relaxation as % papaverine**	**MNTPY [ACh] Log M to cause E_{max}**	**MNTPY Relaxation as % papaverine**	**ODQ [ACh] Log M to cause E_{max}**	**ODQ Relaxation as % papaverine**
Fetus (n=6)	-5	12.8±9.9	-5	11.1±4.1	-8	6.8±7.6	-8	6.1±6.7	-7	2.7±3.7
Newborn (n=6)	-4	9.92±5.1	-4	11.6±6.1	-4	3.9±4.5	-4	6.1±6.3	-4	12.5±12.6
3 day (n=6)	-4	26.15±12.1	-4	24.2±9.2	-5	11.0±7.9	-4	37.71±11.3	-5	8.7±8.3
14 day (n=6)	-4	36.26±13.2	-4	44.4±15.5	-4	22.3±9.4	-4	35.5±9.6	-4	36.5±10.2
Adult (n=6)	-4	17.87±7.3	-5	21.9±5.7	-5	11.1±6.8	-5	16.8±5.1	-4	34.5±9

Table 5.1 The effect of L-arginine (1mM), L-NAME (1mM),, MNTPY (10μM), and ODQ(10μM) on porcine pulmonary arterial relaxation to ACh (E_{max}) after precontraction with U46619 (EC_{80}) with age.

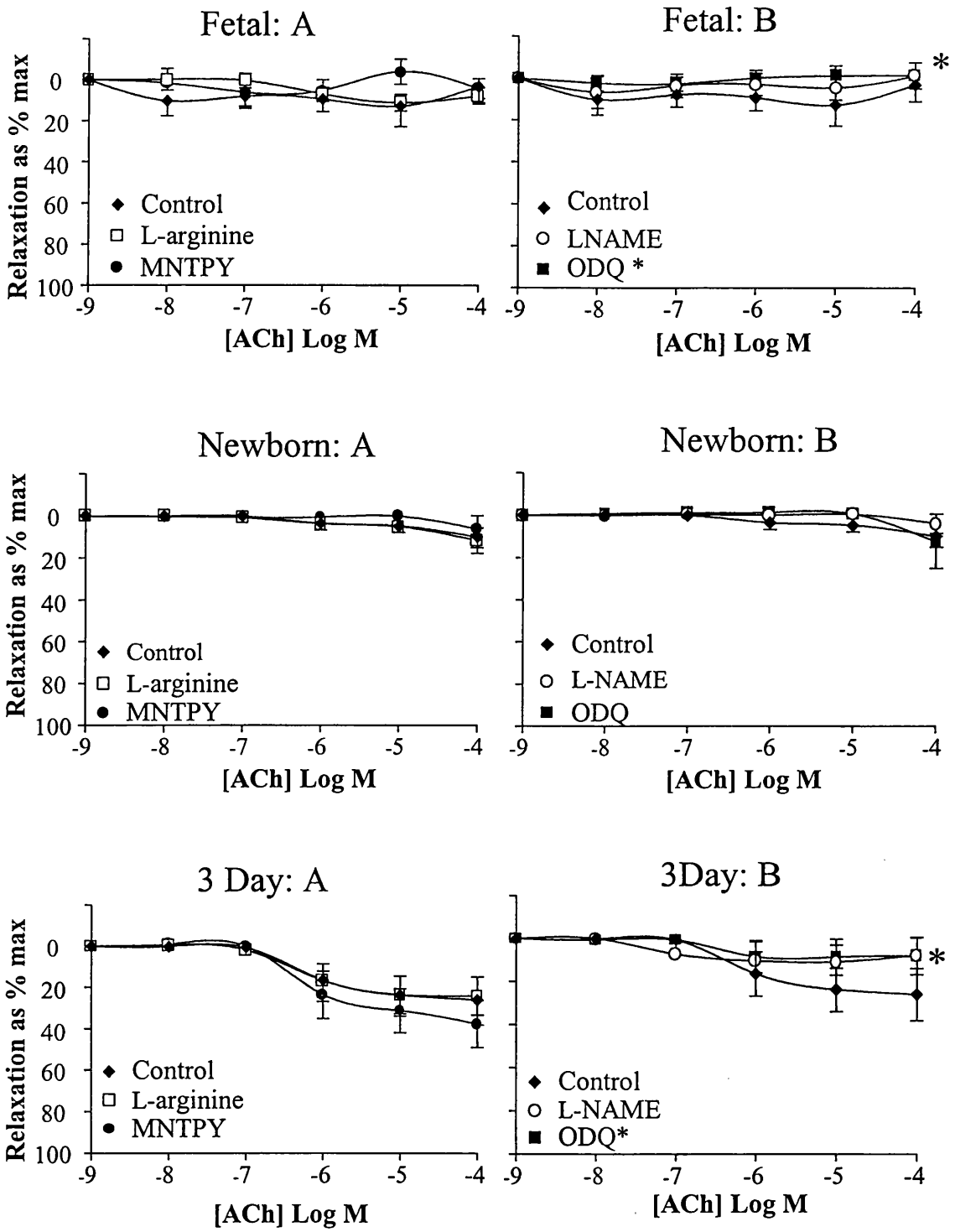

Figure 5.3 The change in porcine pulmonary arterial tone induced by ACh in the presence of various drugs in the fetal (n=6) , newborn (n=6) and 3 day old pig (n=6). The effect of A: Control, L-arginine (1mM) and MNTPY; (10μM) B: Control, L-NAME (1mM) or ODQ (10μM). *$p<0.05$ concentration response curve statistically different between control arteries and arteries in presence of drug ,two-way ANOVA.

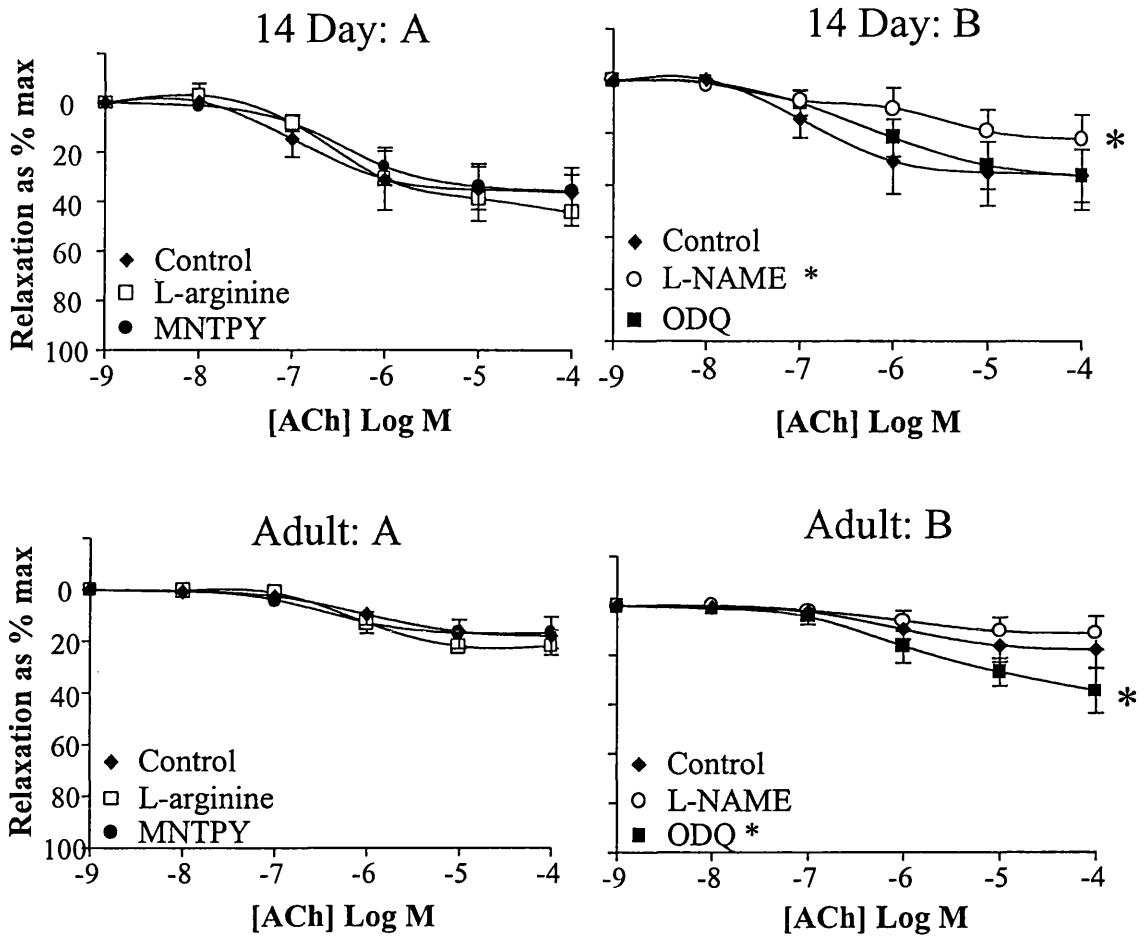

Figure 5.4 The change in porcine pulmonary arterial tone induced by ACh in the presence of various drugs in the 14 day old (n=6) and adult pig (n=6). The effect of A: Control, L-arginine (1mM) and MNTPY (10μM); B: Control, L-NAME (1mM) or ODQ (10μM). *$p<0.05$ concentration response curve statistically different between control arteries and arteries in presence of drug ,two-way ANOVA.

5.2.7 Hypoxia induced pulmonary hypertension

The data from this chapter has shown that the vasoactivity of arteries with relation to the NO/cGMP pathway is continually developing with age. To further study the modulation of the pathway from birth to 3 days of age, the same experiments were undertaken in vessels from the 3 day old hypoxia induced pulmonary hypertensive pigs.

5.2.8 Contractile responses in arteries from hypoxia induced pulmonary hypertensive pigs

The responses to KCl and U46619 seen in the pulmonary arteries from the hypertensive pig lung in this chapter altered in a fashion consistent with that observed in chapter 4.

5.2.9 *Effect of: L-Arginine, L-NAME, MNTPY or ODQ on porcine pulmonary arteries preconstricted to U46619 (EC_{80}) in hypertensive pigs.*

Similarly to the 3 day old control, the contractile response to U46619 in pulmonary arteries taken from hypertensive pig lungs was not altered following addition of MNTPY or L-arginine (Figure 5.5). Following the addition of L-NAME or ODQ the contractility to U46619 in the pulmonary arteries from pulmonary hypertensive pigs significantly increased to a similar level to that attained in age matched controls.

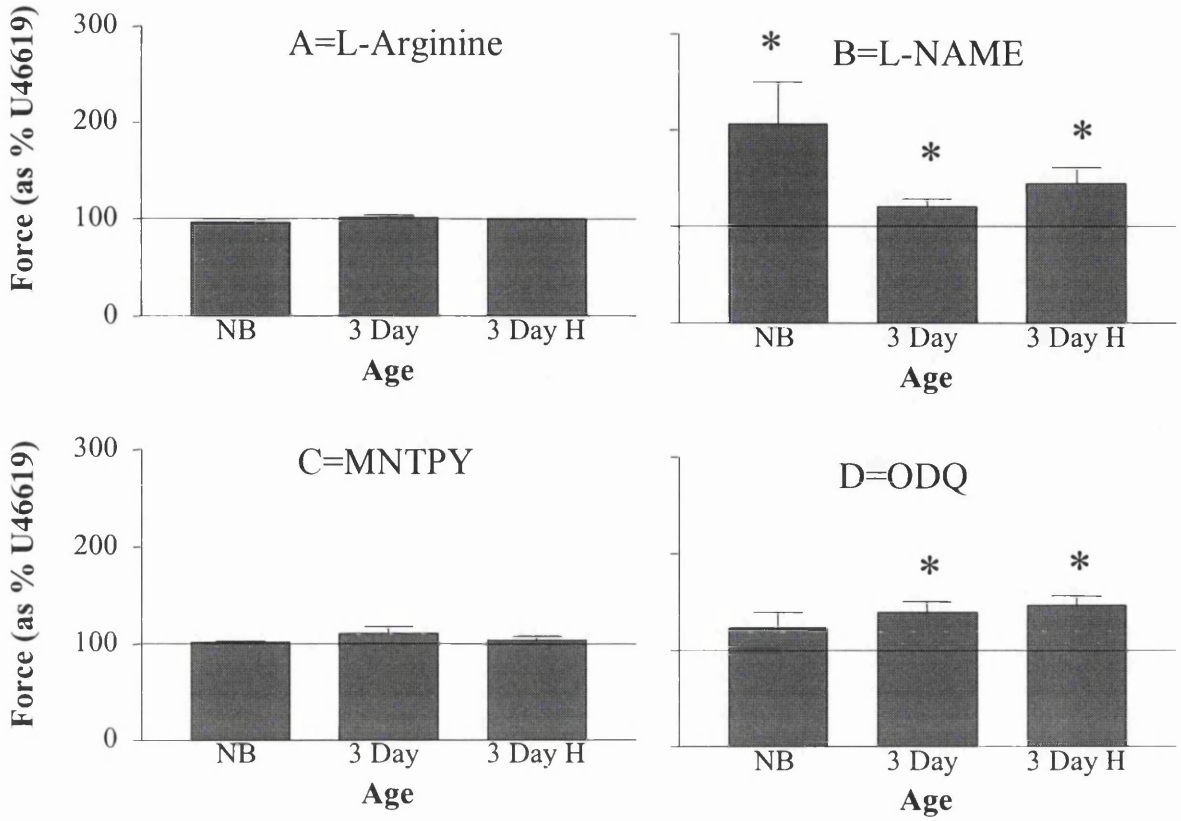

Figure 5.5 Effect of the agonists: L-arginine (1mM) L-NAME (1mM), MNTPY(10μM), or ODQ (10μM),on the vasoconstriction of porcine pulmonary arteries to U46619 (EC_{80} Straight line) with hypertension (n=6). Comparison of responses in the newborn, 3 day old and 3 day old hypertensive (3 Day H) pigs. A: L-arginine, B: L-NAME, C: MNTPY, D: ODQ
* $p<0.05$ tone significantly different to that induced by U46619 (EC_{80}), one sample t-test. Data expressed as mean (% of U46619 contraction after U46619 (100%) contraction)±s.e.m.

5.2.10 *Effect of: L-Arginine, L-NAME, MNTPY or ODQ on porcine pulmonary arteries without endothelium preconstricted to U46619 (EC_{80}) in hypertensive pigs.*

The levels of contraction produced by U46619 in arteries without the endothelium, from 3 day old pulmonary hypertensive animals, did not alter following addition of L-arginine or MNTPY, in a fashion similar to values observed with vessels from 3 day controls and in the newborn. With the endothelium removed, the addition of L-NAME significantly increased contraction in arteries from these pulmonary hypertensive animals, ($p<0.05$ one sample t-test, Figure 5.6), this was in contrast to the negligible effect of L-NAME on the arterial contraction in the 3 day old controls. ODQ

significantly enhanced the contractile responses of arteries from pulmonary hypertensive animals in an analogous fashion to the responses observed in the 3 day control ($p<0.05$, one-sample t-test).

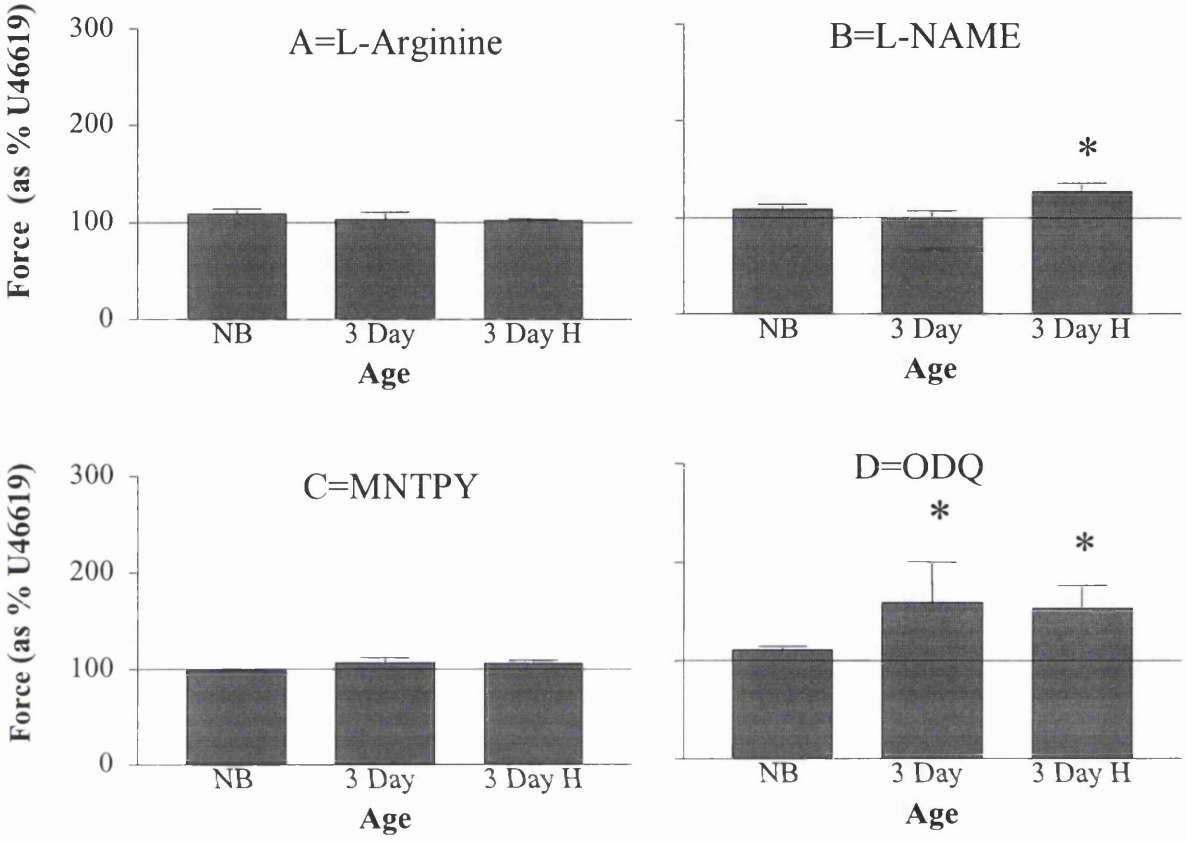

Figure 5.6 Effect of: L-arginine (1mM) L-NAME (1mM), MNTPY (10μM), or ODQ (10μM),on the endothelium independent vasoconstriction of porcine pulmonary arteries to U46619 (EC_{80} straight line) with hypertension (n=6). Comparison of responses in the newborn, 3 day old and 3 day old hypertensive (3 Day H) pigs. A: L-arginine, B: L-NAME, C: MNTP, D: ODQ. * $p<0.05$ U46619 EC_{80} significantly different from control . One sample t-test. Data expressed as mean (% of U46619 contraction after U46619 (100%) contraction)±s.e.m.

5.2.11 Relaxant responses in arteries from hypoxia induced pulmonary hypertensive pigs

In a fashion similar to that observed in experiments from chapter 4, the relaxant responses to ACh in the pulmonary hypertensive piglet were greatly diminished.

5.2.12 *Effects of L-Arginine, L-NAME, MNTPY, or ODQ on relaxant responses of porcine pulmonary arteries from pulmonary hypertensive pigs, induced by ACh.*

In a similar fashion to control vessels (untreated) the pulmonary arteries from the 3 day old pulmonary hypertensive pig did not relax to ACh in the presence of L-arginine or MNTPY. Negligible relaxation was observed to ACh in the presence of ODQ and L-NAME (Figure 5.7, Table 5.2). Following removal of the endothelium relaxation to ACh was absent.

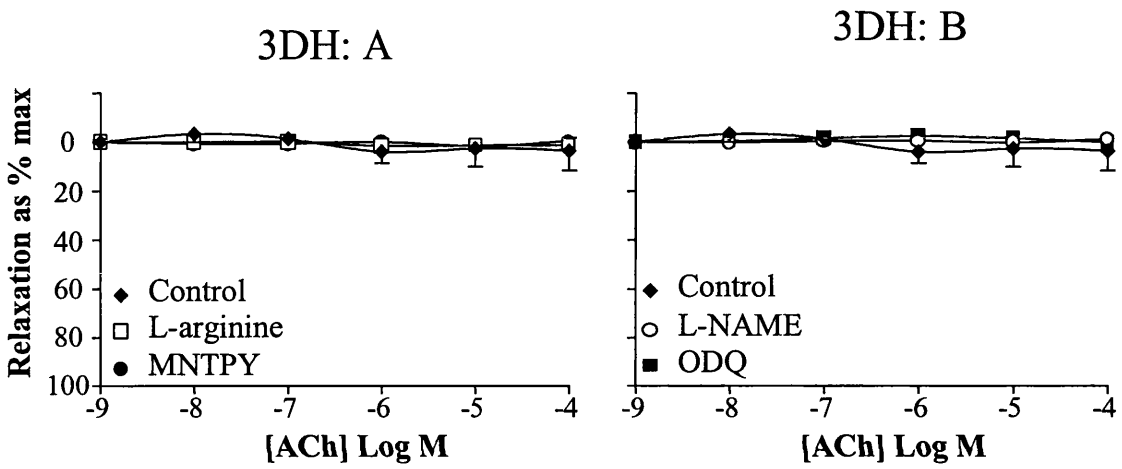

Figure 5.7 The change in porcine pulmonary arterial tone induced by ACh in the presence of various drugs in the 3 day old hypertensive pig (3DH) (n=6). The effect of A: Control L-arginine (1mM) L-NAME (1mM), MNTPY (10μM), or ODQ (10μM),.p=NS.

	Arterial Responses			
	AGE	**Newborn**	**3 day**	**3 day hypertensive**
Control	**[ACh] Log M to cause E_{max}**	-4.	-4	-4
	Relaxation (E_{max}) as % papaverine	9.9±5.1	26.2±12.1	3.82±4.5
L-arginine	**[ACh] Log M to cause E_{max}**	-4	-4	-6
	Relaxation (E_{max}) as % papaverine	11.6±6.1	24.2±9.2	1.5±1.7
L-NAME	**[ACh] Log M to cause E_{max}**	-4	-5	-5
	Relaxation (E_{max}) as % papaverine	3.9±4.5	11±7.9	0.1±1.3
MNTPY	**[ACh] Log M to cause E_{max}**	-4	-4	-5
	Relaxation (E_{max}) as % papaverine	6.1±6.3	37.7±11.3	1.4±1.0
ODQ	**[ACh] Log M to cause E_{max}**	-4	-5	~
	Relaxation (E_{max}) as % papaverine	12.5±12.6	8.7±8.3	0.0±0.0

Table 5.2 The effect of L-arginine, L-NAME, MNTPY, and ODQ on porcine pulmonary venous relaxation to ACh after precontraction with U46619 (EC_{80}) with age(n=6).Values expressed as mean ±s.e.m. p=NS

5.3 Discussion

In the pulmonary vasculature, elements of the NO/cGMP pathway alter with development (Tulloh et al., 1997; Abman et al., 1990; Steinhorn et al., 1995). The extent of the alterations of these elements with development and the subsequent changes that may occur in disease states such as PPHN remain unknown. By using agents to manipulate some of the key agents in this pathway, the development of parts of the NO/cGMP pathway with age could be deduced. In addition, by observing changes that occurred between the 3 day old pulmonary hypertensive animals and their controls, some insight could be gained as to how changes between birth and 3 days of age come about.

5.3.1 *L-arginine*

L-arginine did not affect the tone induced by U46619 in arteries from any age group prepared with or without the endothelium. Studies in adult bovine arterial rings have also shown that the addition of L-arginine exogenously produces no alteration in tone following precontraction with phenylephrine or U46619 (Gold et al., 1989). However, in the quiescent lungs of fetal lambs, L-arginine has been shown to cause vasodilation (McQueston et al., 1993). Thus differences between the responses observed might be species and age related. Relaxation to ACh, following the addition of L-arginine was not enhanced or diminished in any of the age groups. Similarly, the addition of L-arginine has previously been observed to have no effect on the vasodilation to ACh in the pulmonary arteries from fetal lamb and adult guinea pig (McQueston et al., 1993; Aisaka et al., 1989). Thus L-arginine does not appear to be rate limiting for the formation of biologically active NO in pulmonary arteries at any developmental age. Therefore the lack of vasodilation to ACh in the newborn and fetal pigs is not due to a deficiency of L-arginine. In turn this means that NOS inhibitors, such as ADMA, are unlikely to be present as the effects of such inhibitors are reversed following competition for NOS with L-arginine (Azuma et al., 1995).

5.3.2 *L-NAME*

With age, in the presence of the NOS inhibitor, pulmonary arterial resting tone tended to increase. The augmentation in contractile responses to U46619 following L-NAME addition represented the contribution of NO to arterial tone, referred to in this

study as the "basal" production of NO. In most of the age groups studied L-NAME increased arterial tone. Previous experiments have shown that in pulmonary arteries, agonist induced contraction to phenylephrine and noradrenaline is potentiated by L-NAME at a variety of postnatal age groups (Levy et al., 1995; Morecroft et al., 1998). In this chapter the increase in arterial tone observed was equivalent between all the age groups except the newborn where contraction tended to be greater.

Interestingly, from this data the "basal" release of NO was observed in the fetal arteries following inhibition by L-NAME, indicating NOS activity. However, in studies from chapter 3, whole lung assays showed that NOS activity was absent in a large proportion of lung samples from the fetal age group. This anomaly may highlight the differences between the study of whole lung samples and work in pulmonary vessels, or a difference between fully stimulated and unstimulated NOS.

The greatest increase in tone following the addition of L-NAME was at birth. In the lamb a high release of NO is required at birth for the transition from the fetal to the newborn pulmonary circulation (Tiktinsky et al., 1992; Abman et al., 1990). The basal release of NO, observed from the data in this chapter, corresponds with this event. This increase in NO release may reflect the increase in NOS activity found at this age group in chapter 3, observed to be a combination of either eNOS/ iNOS, or Ca dependent/ independent eNOS. Following endothelial removal, the observed increase in tone elicited by L-NAME was removed, in addition to the differences between the age groups highlighting the endothelial dependence of NO and thus NOS activity. This suggests that the basal production of NO in most age groups is from eNOS.

In the presence of L-NAME arterial responses to ACh tended to diminish at all ages. Although ACh did not induce good relaxation in the newborn and fetal age groups, the small relaxations observed were absent in the presence of L-NAME. This indicated the presence of a small but detectable release of NO. In a similar fashion, in newborn lambs and in the perinatal rabbit, relaxant responses to ACh were observed to be completely inhibited using the L-arginine analogue L-NNA (Steinhorn et al., 1993; Morecroft et al., 1998). In the newborn, the lack of stimulated endothelium dependent relaxation to ACh shown in data from chapter 4, in spite of high levels of basally released NO and NOS activity, could be the result of using ACh as the relaxant agonist. However, studies in the newborn porcine pulmonary artery have shown a diminished relaxant response to the Ca ionophore A23187 (Tulloh et al. 1997), indicating a decreased stimulation of NOS at this age. In fact, the change in Ca dependence of the

NOS isoform in chapter 3 may mean that the stimulation of NOS does not depend on an increase in intracellular Ca concentration, as induced by ACh.

After birth, L-NAME incompletely inhibited the relaxations to ACh at every age. Similarly in the adult human pulmonary artery following L-NAME addition, only partial attenuation of the response to ACh has been observed (Lawrence et al., 1998). Moreover, in the pulmonary vascular bed of the adult cat, whilst precontraction to U46619 was observed following addition of L-NAME, the relaxation to a bolus of ACh has been shown not to be inhibited, (Lippton et al., 1992). These occurrences have been suggested to be due to the lack of specificity for the inhibitor at these ages, which may be the case here. In addition, they may also be indicative of the release of other endothelially derived relaxing factors such as PGI_2 and EDHF, the production of which has been shown to be stimulated by ACh, (Chen &Suzuki, 1989; Zellers & Vanhoutte, 1991).

5.3.3 *MNTPY*

Thus it seems that pulmonary arteries release NO from the endothelium either basally or after stimulation with ACh. Following the addition of MNTPY, the contractile responses to U46619 were not altered. In addition to the lack of effect on tone, MNTPY did not augment relaxations to ACh at any age. In a similar fashion the addition of SOD to the small pulmonary arteries of the adult pig does not affect relaxation induced by ACh (Liu et al., 1998). This suggests that superoxide under these conditions produced either within the tissue or the organ bath does not influence the biological activity of NO. Nevertheless, an increased amount of superoxide is produced in arteries at times of cyclical stress (Ryan et al., 1995), such as occurs at birth. Thus in the pig endothelial levels of SOD may be high enough to quench any superoxide activity. This may not be the case for all species since in the rabbit, exogenous SOD decreases phenylephrine-induced contraction of pulmonary arteries from newborn animals (Morecroft &McLean, 1998).

5.3.4 *ODQ*

ODQ is a sGC inhibitor that actively competes with NO in the smooth muscle cell, irreversibly binding sGC (Schrammel et al., 1996). On addition of ODQ to the porcine pulmonary arteries, contractility was enhanced to a similar level at most ages, with the lowest responses in the newborn and 14 day old pig. Following the removal of

the endothelium, the pattern of the contractile tone to ODQ with age was accentuated, perhaps indicating the smooth muscle dependence of these responses and furthermore the normal dampening down of this contraction by an endothelium derived relaxing factor. ODQ was also tended to diminish the relaxant responses to ACh more in the younger age groups up to 3 days of age, than in arteries from the 14 day old and adult pigs. Indeed in rat lung stimulated sGC has been found to be more active in the perinatal period (1-8 days) than in the adult (Bloch et al., 1997). In the 14 day old and adult age groups, in a fashion similar to relaxant responses in the presence of L-NAME, the relaxant responses were only partially attenuated. Interestingly from the studies in this chapter, ODQ was observed to enhance the relaxation to ACh in the adult age group.

The alteration in sGC activity with age in this chapter has not been previously observed. In both neonatal (1-8 day) and adult rats, basal sGC activity is low, although the subunit expression for sGC is increased at birth, (Bloch et al., 1997). In addition, in the pig model at birth, a transient increase in basal cGMP accumulation in pulmonary arteries has been observed followed by a lower level that is maintained with age (Tulloh et al., 1997) perhaps indicative of sGC activity levels.

The contractile pattern to ODQ, at birth and at 14 days of age did not correspond with the basal release of NO. This is especially true in arteries from the newborn age group, where the low sGC activity was not reflected by a high basal release of NO. In addition, neither the arteries from the 14 day old, the adult nor the newborn pig had their responses to ACh significantly attenuated in the presence of ODQ. This lack of inhibitory response to ODQ may be created by the relaxation mediated by ACh acting independently of sGC. Arterial responses to NO in the presence of indomethacin in 6-13 day old lambs have been shown to be markedly attenuated following addition to ODQ (Gao et al., 1998). Therefore, in this chapter the ineffective sGC inhibition at 14 days of age might be due to the relaxation to ACh being created via the production of PGI_2, which uses the cAMP pathway, or EDHF, which directly hyperpolarises the smooth muscle. However, the NO/cGMP pathway does have a role in the development of the relaxant response of the arteries, as previous work by Tulloh and co-workers have shown an increase in the cGMP accumulation stimulated by ACh in porcine arteries at 17 days of age from birth (Tulloh et al., 1997). The rationale for the effect of ODQ at 14 days of age might also be applied to the newborn vessels, which also showed little sGC inhibition, or to the enhanced relaxant responses

to ACh observed in the arteries from the adult pig. In addition, the enhancement of arterial relaxation in the adult age group following sGC inhibition suggests that sGC inhibition, and its removal from the pathway, allows NO to stimulate smooth muscle relaxation, either beyond cGMP in the cGMP pathway (Clementi et al., 1995; Stoyanovsky et al., 1997), contributing to a greater relaxation.

5.3.5 **Hypoxia induced pulmonary hypertension**.

In PPHN, NO production is dysfunctional (Feletou et al.,1995). In adult patients with pulmonary hypertension, inhalation of NO is observed to lower pulmonary pressures (Pepke-Zaba et al., 1991). Moreover in rats exposed to chronic hypoxia, sustained NO inhalation inhibits the development of pulmonary hypertension and the associated structural changes (Kouyoumdjian et al., 1994). Work in chapter 4 showed the diminished relaxant response to ACh in hypoxia induced pulmonary hypertensive piglets. In hypoxic patients with chronic obstructive lung disease, chronic hypoxia has been shown to diminish endothelium dependent ACh but not SNP responses in isolated pulmonary arteries (Dinh-Xuan et al., 1991), thus affecting the stimulated production of NO.

5.3.6 *L-arginine*

L-arginine did not affect the contraction induced by U46619 in arteries from 3 day old hypoxia induced pulmonary hypertensive pigs, in a fashion similar to the age matched controls. Moreover the arterial relaxant responses to ACh in these vessels was not enhanced in the presence of L-arginine. In contrast, decreased arginine availability has been observed in newborn humans with PPHN (Castillo et al., 1995). In isolated hypoxic rat lung, others have found that where baseline levels of L-arginine are lower than normoxic rat lung, the addition of L-arginine has been shown to restore the vasodilator response to ACh, whilst not altering the pressor responses to U46619 (Eddahibi et al., 1992). In fact, in porcine pulmonary artery endothelial cells, hypoxia inhibits L-arginine synthesis (Su et al., 1995), transport and uptake. Thus, the hypoxic state induced in the piglets from birth to 3 days could similarly limit L-arginine uptake in the arteries ex-vivo. Such a reduction in uptake mechanism would explain the lack of effect of exogenous L-arginine observed in this chapter. In addition the lack of effect of L-arginine on the arterial response to U46619 or to ACh, rules out the presence of a putative inhibitor such as ADMA, which is increased in hypertensive children

(Goonasekera et al., 1997). This is because L-arginine would reverse the competitive inhibition of NOS (Azuma et al., 1995).

5.3.7 *L-NAME*

The data from this chapter showed that in the pulmonary hypertensive pig, tonic responses to U46619 increased following the addition of L-NAME, similar in fashion to those observations made using age matched controls at 3 days of age. This suggested that NO continued to contribute to basal tone in the arteries. Interestingly, in contrast to the 3 day old control, the L-NAME induced contraction remained following removal of the endothelium, suggesting that active NOS was present in the pulmonary arterial smooth muscle from hypoxia induced pulmonary hypertensive piglets. This basal production of NO could be from either isoform as both iNOS and eNOS have been found in pulmonary vascular smooth muscle (Sherman et al. 1999; Zehetgruber et al., 1993). Corresponding with the NOS activity of lung homogenate from this age group in chapter 3, the proportion of Ca independent NOS increased. In fact immunostaining in the hypoxia induced hypertensive pig lung has shown decreased levels of endothelially derived eNOS in pulmonary arteries (Hislop et al., 1995). Thus the low level of relaxation observed in the 3 day old hypoxia induced pulmonary hypertensive pig may be a result of the decrease in the proportion of Ca dependent eNOS and a subsequent increase in the amount of Ca independent NOS activity (i.e. iNOS). Nevertheless the effect of hypoxia induced pulmonary hypertension on the release of NO from pulmonary arteries is not altered basally, and is only observed to diminish when NO release is stimulated using ACh.

5.3.8 *MNTPY*

MNTPY did not affect the arterial responses to either U46619 or ACh in vessels from the 3 day old pulmonary hypertensive pig. In a similar fashion SOD in the hypoxia induced hypertensive rat lung did not affect the vascular responses to either U46619 or ACh (Eddahibi et al., 1992). Moreover in conjunction with systemic hypertension, SOD did not affect the basal arterial release of NO (Garcia et al., 1995) as assessed by measuring human forearm blood flow. However, systemically hypertensive rats do have an increased free radical activity in their arterial walls (Sharma et al., 1992), and in spontaneously hypertensive rats a fall in blood pressure is observed after administration of SOD (Nakazono et al., 1991). Thus hypertension does induce the production of

superoxide, however, in the pulmonary circulation of the pig, this may not be evident perhaps due to a large endogenous production of SOD.

5.3.9 *ODQ*

The trend in arterial contractile responses of this model of pulmonary hypertension to U46619 following the addition of ODQ was to increase. In addition the small relaxant responses to ACh diminished. Thus sGC activity, although low, was inhibitable in the hypertensive lung, and subsequently active through either stimulated or basal production of NO. In addition, basal arterial cGMP accumulation, reflective to some extent of sGC activity, has been previously shown in this pig model to be unaltered with hypertension (Tulloh et al., 1997). Thus hypertension may not be affecting this section of the pathway in the smooth muscle responses of the arteries.

In summary, there was a differentiation in the NO/cGMP pathway between age groups. L-arginine did not behave in a rate limiting fashion, affecting neither the basal or stimulated release of NO at any age. Release of NO at a basal rate from arteries was produced to an equivalent amount at all ages except at birth. In the newborn, NO was observed to contribute more to tone than any other age group. This corresponded to an increased NOS activity at this age (chapter 3). Superoxides, which inactivate NO, did not contribute to any of the age related changes in contractility or relaxation studied. However, sGC activity did alter in an age related pattern although this did not reflect the basal or stimulated release of NO, particularly in the newborn age group. At 14 days of age, little inhibition of sGC activity was observed, and in the adult the relaxant responses to ACh were enhanced suggesting that relaxation in these older age groups was not as dependent on the activity of sGC as the younger age groups.

In arteries, the production of NO basally in hypoxia induced pulmonary hypertension was unaltered from age matched controls. In addition, there appeared to be no deficiency of L-arginine, or the presence of endogenous inhibitors, superoxides or a low sGC activity. Following addition of L-NAME, the increase in tonic contraction that occurred was perhaps due to NOS inhibition in the smooth muscle. This NOS located in the smooth muscle, may be associated with the increase in Ca independent NOS activity observed with pulmonary hypertension in chapter 3. The stimulated release of NO using ACh was not present with pulmonary hypertension, and L-arginine, superoxides and sGC activity did not have a role in this. These experiments have highlighted the fact that

the basal release of NO is maintained in hypoxia induced pulmonary hypertension from birth and it is the stimulated release of NO which appears dysfunctional at this age. This may be related to either the change in isoform, the location of the isoform, or that the dysfunction occurs at some other point in the pathway.

Chapter 6. Modulation of contractile and relaxant responses in the fetal period.

6.1. Rationale

In chapter 4, pulmonary vascular reactivity was described relative to age. Those studies showed a trend for smaller contractile and relaxant responses to occur in the fetal age groups than postnatally. This may have been due to the different structure of the vessels at this time or to a diminished vascular reactivity in the endothelium or smooth muscle in association with alterations in the NO/cGMP pathway. In chapter 5 it was shown that a basal release of NO contributed to fetal arterial tone, but when stimulated by ACh, very little relaxation was observed.

The data from chapter 3 showed that in the fetal lung despite the presence of the NOS enzyme, activity was not stimulated. The lack of NO production in the fetal lung was not due to a lack of substrate or co-factors, or due to the rate limitation of endogenous SOD or L-arginine in the arteries. Thus, this chapter sought to investigate the cause of the small responses of the fetal pulmonary arteries. It was thought that the maternal circulation may have contributed to responses observed in the fetal tissue in vitro, as normal fetal growth and development depend upon the continuous supply of amino acids from maternal blood utilising placental amino acid transport systems (Moe, 1995). Putative inhibitory factors derived from the maternal circulation such as the amino acid ADMA are present in pregnancy (Fickling et al., 1993). Therefore circulating factors could be present in either maternal or fetal blood that regulate vascular responses at birth. Thus one of the aims of this chapter was to assess the effects of serum from fetal pigs on the vascular responses of vessels from fetal pigs and one postnatal age group.

In addition, the differences between the fetal and postnatal vascular reactivity may be induced by the onset of breathing. NO is involved in this process as the endothelial production of NO increases with oxygenation, ventilation and the associated pulmonary vascular shear stresses that occur (North et al., 1996; Black et al., 1997). However, studies in chapter 3 showed that ventilation did not increase the low NOS activity in fetal lung. Thus the different vasoactive responses observed between the newborn and fetal age groups may have also created by the method of delivery of the piglet. Activity of the fetal vasculature alters to certain agents, depending on whether

delivery is via Caesarean or vaginal delivery (Hakkinen et al., 1992; Haning et al., 1978). In addition, the induction of labour also increases levels of circulating oestrogen, which acutely activates NOS (Lantin-Hermoso et al., 1997).

Thus in this chapter the effect of breathing in piglets exposed to normal labour and delivery, or born by Caesarean section was investigated in association with the reactivity of the arteries and veins.

6.2 Results

6.2.1 **Organ culture of blood vessels.**

An initial set of experiments described the effects of organ culture for 24 hours on arterial vasoactivity with age. These experiments determined the maintenance of vasoactive responses in the different age groups following 24hr in culture. Vascular rings were dissected, and for each age, one ring was randomly taken for immediate use in the organ bath (control) and another used for culture with serum. After 24 hours in culture, contractile responses to KCl (125 mM) and U46619 ($1x10^{-11}$-$3x10^{-6}$ Log M), and relaxant responses to ACh ($1x10^{-9}$ - $3x10^{-4}$ Log M), were undertaken (Table 6.1).

Fresh arterial tissue was observed to follow a similar contractile pattern with age to that observed in chapter 4.

6.2.2 *Arterial contractile responses to U46619 and KCl following culture for 24 hours in medium containing fetal calf serum*

Following incubation for 24 hours, the contractility to U46619 (E_{max}) and KCl in all age groups tended to become diminished except in the fetal and 14 day old age groups, (Table 6.1). The reduction in contraction (E_{max}) to U46619 reached statistical significant in the adult age group ($p<0.05$, un-paired t-test, Table 6.1).

	n	Tension induced by KCl(mg)		E_{max} responses to U46619		Log EC_{50} for U46619	
AGE		**Control Artery**	**24 Hr in FCS**	**Control Artery**	**24 Hr in FCS**	**Control Artery**	**24 Hr in FCS**
Fetus	4	237±23.9†	212.5±23.9†	487.5±65†	412.5±46†	-6.4±0.1	-6.4±0.2
Newborn	5	444±86.9†	279.8±78.6†	637.6±151.8†	410±112.2†	-6.6±0.3	-6.2±0.03
1 day	4	333.5±65.5†	287.7±77.4†	520.5±119†	317.8±61†	-6.0±0.7	-6.0±0.2
3 day	6	499.7±81†	296.5±50†	629.8±81.3†	421.2±89†	-7.4±0.1	-5.9±0.2
6 day	5	342.2±104.6	239.9±44.5†	510±135†	296±78.8†	-6.2±0.2	-5.9±0.2
14 day	6	564.7±79.7†	683±150.4†	695.8±104.9†	919.5±211.5	-6.5±0.2	-6.8±0.1
Adult	4	3119.5±376.	2212.5±860	3875±772.	1112.5±471.8*	-6.4±0.4	-6.3±0.1

Table 6.1 Comparison of porcine pulmonary arterial responses to KCl (125mM) and U46619 (E_{max}) after incubation for 24 hours in fetal calf serum (FCS). (Data are expressed as mean ± s.e.m). † $p<0.05$ E_{max} significantly different to adult, one-way ANOVA * $p<0.05$ E_{max} to agonist significantly different between incubated artery and control from same age group, un-paired t-test.

6.2.3 *Arterial relaxant responses to ACh following incubation for 24 hours in fetal calf serum*

Following 24 hours of organ culture, the relaxant response to ACh in arteries diminished in all the age groups, except at 14 days of age, at which age relaxation tended to improve. A statistically significant reduction in maximum relaxant response (E_{max}) was observed in the fetus, at 3 days of age and in the adult ($p<0.05$, paired t-test) where following incubation no relaxation to ACh was observed in the fetal and adult age groups (Table 6.2).

		Control		24 hr FCS	
AGE	**n**	**[ACh] Log M to cause E_{max}**	**Relaxation as % papaverine**	**[ACh] Log M to cause E_{max}**	**Relaxation as % papaverine**
Fetus	4	-5.5	14±7	~	0*
Newborn	5	-4	18±10	-7	1±1
1 day	4	-5	27±13	-5	11±8
3 day	6	-4	19±3	-4	7±4*
6 day	5	-5	19±8	-5	9±4
14 day	6	-5	24±6	-6	32±11
Adult	4	-4	18±11	~	0±0*

Table 6.2 Comparison of porcine pulmonary arterial responses to ACh (E_{max}) with age after incubation for 24 hours in fetal calf serum (FCS). (Data are expressed as mean (% relaxation to papaverine)±sem). * $p<0.05$ E_{max} significantly different to control, paired t-test.

6.2.4 *The effect of sera from fetal and 14 day old pigs on contractile responses of pulmonary arteries from fetal pigs*

Arteries and serum from the 14 day old age group were used for comparison with the fetal age group, as this was the only age group without diminished vascular responses following incubation for 24 hrs in FCS. In fetal arteries the contractile responses induced by U46619 or KCl were not altered by 24 hr incubation with FCS or fetal porcine serum (FPS). Following incubation of these vessels in serum from 14 day old pigs (14 PS), low contractile responses to KCl and U46619 were observed. However these were not significantly different from responses observed following incubation in FPS (Figure 6.1, Table 6.3).

6.2.5 *The effect of sera from fetal and 14 day old pigs on contractile responses of pulmonary arteries from 14 day old pigs*

In arteries taken from the 14 day old pig, the contraction induced by U46619 (Figure 6.1) or KCl (Table 6.3) was not modified following 24 hr incubation with FCS, FPS or 14 PS.

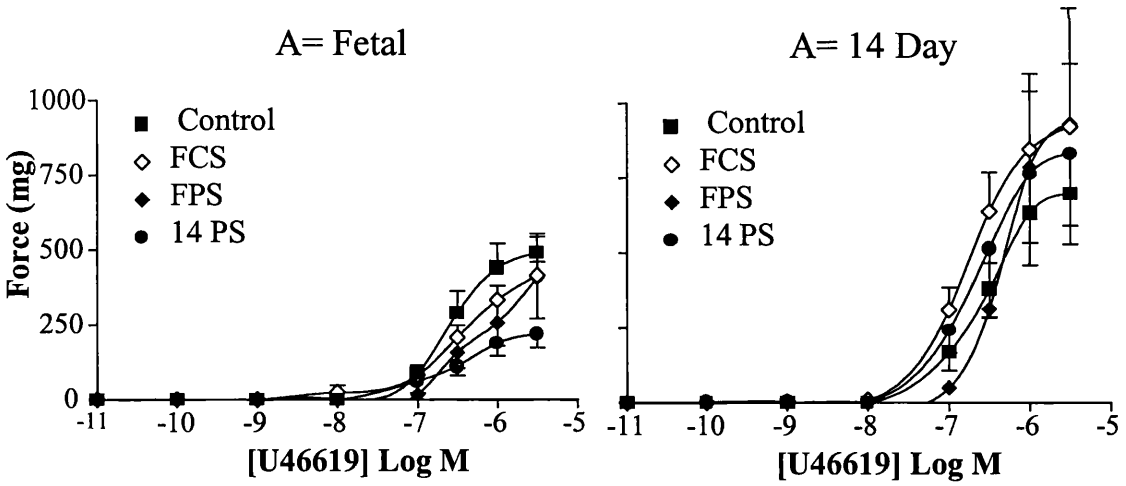

Figure 6.1 Arterial concentration response curves to U46619 in control (fresh) tissue and tissue after incubation for 24 hours in fetal calf serum (FCS), fetal porcine serum(FPS) or serum from 14 day old piglets(14PS). In the following age groups; A:fetal piglets (n=4) ,B: 14 day old piglets (n=6) (Data represented as mean(mg) ±sem).

		24 hr incubation		
		FCS	**FPS**	**14 PS**
	Age			
KCl (mg)	**Fetus**	212.5±23.9	218.7±51.4	137.5±23.9
	14 Day	683±150.4	653±221	666±253
E_{max} (mg)	**Fetus**	412±46	406±136	219±45
	14 Day	919.5±211.6	929.6±386.8	829±302
Log EC_{50}	**Fetus**	-6.38±0.16	-5.97±0.16	-6.5±0.2
	14 Day	-6.84±0.2	-6.14±0.08	-6.66±0.3

FCS= Fetal Calf Serum **FPS**= Fetal Porcine Serum **14 PS**= Serum from 14 day old pig

Table 6.3 Comparison of arterial responses to KCl (125 Mm), and U46619 (Log EC_{50} and E_{max}) in fetal (n=4) and 14 day (n=6) piglets after incubation for 24 hours in fetal calf serum (FCS), fetal porcine serum (FPS) and serum from 14 day old animals (14 PS).
(Data are expressed as mean (mg) ± sem)

6.2.6 *The effect of sera from fetal and 14 day old pigs on relaxant responses of pulmonary arteries from fetal pigs*

As was noted in chapter 4, fresh fetal arteries did not relax significantly when stimulated with ACh. Following incubation for 24hr with FCS, FPS, or 14PS, fetal arteries did not relax at all to ACh (Figure 6.2). In each case comparable maximum relaxations to papaverine were achieved.

6.2.7 *The effect of sera from fetal and 14 day old pigs on relaxant responses of pulmonary arteries from 14 day old pigs*

The concentration dependent relaxant responses to ACh in arteries from 14 day old piglets were not significantly different between the arteries following incubation in either 14 PS or FPS.

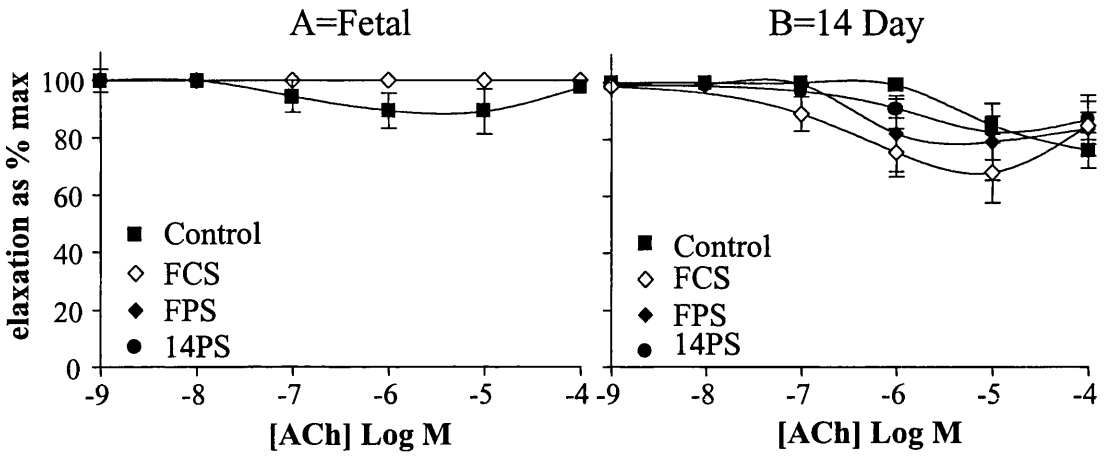

Figure 6.2 Arterial concentration response curves to ACh after incubation for 24 hours in fetal calf serum (FCS), fetal porcine serum (FPS) or serum from 14 day old piglets (14PS). A:Fetal piglets (n=4), B: 14 Day old piglets (n=6) (Data represent mean(as % maximum relaxation to papaverine) ±sem).

	Incubation for 24 hrs					
	FCS		**FPS**		**14 PS**	
	[ACh] Log M to cause E_{max}	Relaxation as % papaverine	[ACh] Log M to cause E_{max}	Relaxation as % papaverine	[ACh] Log M to cause E_{max}	Relaxation as % papaverine
AGE						
Fetus	~	0±0	~	0±0	~	0±0
14 day	-5	31.7±10.6	-5	20.8±13.6	-5	14.3±8.6

Table 6.4 Comparison of arterial responses to ACh (E_{max}) in fetal and 14 day piglets after incubation for 24 hours in fetal calf serum (FCS), fetal porcine serum (FPS) and serum from 14 day old pigs (14 PS). (Data are expressed as mean (% maximum relaxation to papaverine) ± sem).

6.2.8 **Breathing as a stimulus for developmental changes influencing vasoactivity in the fetal/newborn pulmonary circulation.**

In order to discover whether breathing was one of the mechanisms differentiating the vascular reactivity of the fetal arteries from that of the postnatal age groups, experiments were conducted using fetal pigs that had breathed and newborn pigs that had not breathed and the responsiveness of their pulmonary arteries studied.

6.2.9 *Effect of breathing on the contractile responses to U46619 and KCl in fetal porcine vasculature.*

Compared to the arterial responses of fetal pigs observed in chapter 4, the contractile response to KCl tended to increase in fetal pigs that had spontaneously breathed (5 min), although this did not reach statistical significance. Similarly, following analysis of arterial concentration response curves to U46619, contractility was significantly enhanced in fetal pigs that had breathed ($p<0.05$,two-way ANOVA, Figure 6.3,Table 6. 5).

Arterial responses to U46619 (E_{max}) or KCl did not significantly alter in breathing fetal pigs following the removal of the endothelium (Figure 6.3). However, in arteries without the endothelium, the comparison of responses between breathing and non-breathing fetal pigs (controls taken from chapter 4) showed a trend for greater contractility to both U46619 and KCl to occur in breathing fetal pigs, although this did not attain statistical significance.

The pulmonary venous responses from spontaneously breathing fetal pigs did not alter to KCl when compared to fetal controls. There was less of a difference between the contractility to U46619 and KCl in the veins from breathing and non-breathing fetal pigs than the arteries (Table 6.5).

6.2.10 *Effect of not breathing on the contractile responses to U46619 and KCl in newborn piglet arteries and veins.*

The contractile responses of pulmonary arteries from non-breathing newborn pigs, induced by either U46619 or KCl, tended to be reduced when compared to those responses from vessels of normal breathing newborn pigs (controls, taken from chapter 4), although this did not reach statistical significance. Interestingly this brought the arterial concentration response curves from these non-breathing newborn pigs closer to those of breathing fetal pigs (Figure 6.3, Table 6.5).

Endothelium removal in arteries from non-breathing newborn pigs did not alter the contractility to either KCl or U46619. Contractility in arteries without the endothelium from the non-breathing newborn pig tended to be reduced to KCl, and significantly reduced to U46619, when compared to the breathing newborn controls ($p<0.05$, un-paired t-test, two-way ANOVA, Table 6.5).

Venous contractile responses to either KCl or U46619 did not alter in the non-breathing newborn pig when compared to those from the newborn controls. Compared to the arteries, venous contractility was affected less by the onset of breathing in the newborn (Table 6.5).

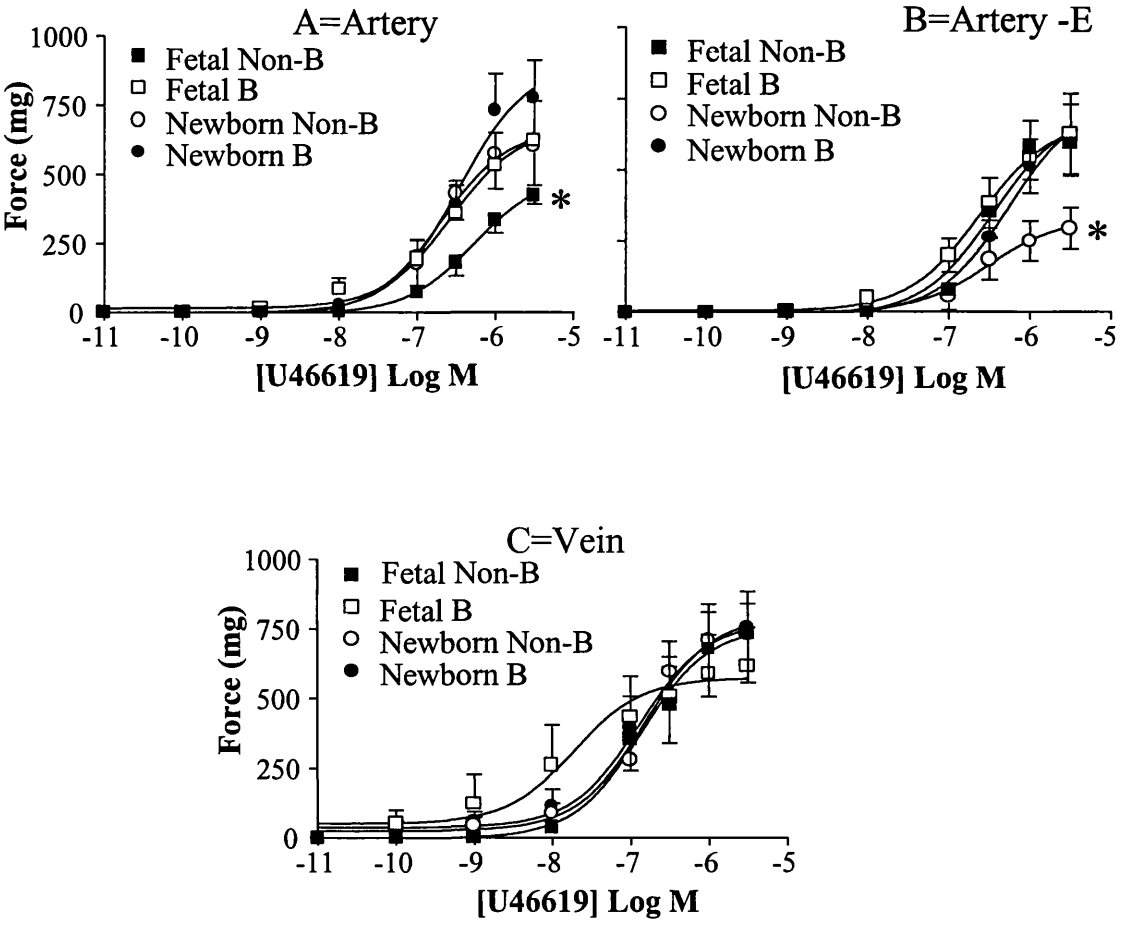

Figure 6.3 Concentration response curves to U46619 from breathing (B) and non-breathing (Non-B) fetal and newborn pigs. A: Arteries , B: Arteries without endothelium (-E), C:Veins. (Data represent mean (mg)± sem). *$p<0.05$ breathing animals significantly different from non-breathing animals within the same age group two-way ANOVA.Fetal breathing pigs (n=8); fetal non-breathing pigs (n=10); newborn non-breathing pigs (n=5); newborn breathing pigs (n=12).

	AGE	Fetal Non-breathing (n=10)	Fetal breathing (n=8)	Newborn Non-breathing (n=5)	Newborn breathing (n=12)
Arteries	KCl(mg)	250±32	417.7±106	402.7±151	519±96. 98
	E_{Max} to U46619 (mg)	424±32*	622±144	603.4±142	778.2±134
	Log EC_{50} to U46619	-6.3±0.03	-6.7±0.21	-6±1.07	-6.5±0.08
Arteries without endothelium	KCl (mg)	287±62	400.9±95.4	212.54±78.8	437±76
	E_{Max} to U46619 (mg)	593±108	623.9±144	293.9±73.9*	615.8±116.3
	Log EC_{50} to U46619	-6.42±0.07	-6.67±0.25	-6.44±0.17	-6.4±0.19
Veins	KCl (mg)	399±59	368.8±89	394.2±44.6	548±119
	E_{Max} to U46619 (mg)	730±173	615.5±139	730.6±109.6	756±129
	Log EC_{50} to U46619	-6.9±0.7	-7.47±0.39	-6.97±0.25	-6.9±0.1

Table 6.5 Comparison of arterial and venous contractile responses to KCl (mg) (125mM) and U46619, E_{max} and Log EC_{50}.In breathing and non-breathing fetal and newborn pigs, (Data are expressed as mean ± s.e.m).*$p<0.05$ breathing animals significantly different from non-breathing animals within the same age group, un-paired t-test.

6.2.11 *Effect of breathing on the relaxant responses to ACh in fetal piglet arteries.*

As described in chapter 4, ACh induced negligible relaxant responses in pulmonary arteries from fetal lungs. Similarly, pulmonary arteries from fetal pigs allowed to breathe for 5 minutes did not relax when stimulated with ACh. Removal of the endothelium produced no relaxation to ACh ($3x10^{-6}$) (data not given).

In a similar fashion to arterial responses, venous relaxation to ACh was comparable in vessels from non-breathing and breathing fetal pigs (Figure 6.4). Like the fetal controls, the responses to ACh (E_{max}) in veins from breathing fetal pigs were significantly greater than their arterial counterparts ($p<0.05$, un-paired t-test).

6.2.12 *Effect of not breathing on the relaxant responses to ACh in newborn piglet arteries.*

In a similar fashion to the fetal arteries, the arteries from the non-breathing newborn piglets, did not elicit a relaxation to ACh (Figure 6.4). Removal of the endothelium produced no relaxation to ACh ($3x10^{-6}$). ACh induced a relaxation in the veins from the non-breathing newborn pig in a fashion similar to that described in chapter 4. In non-breathing newborn pigs, venous responses to ACh (E_{max}) were significantly greater than those produced in the arteries ($p<0.05$, un-paired t-test).

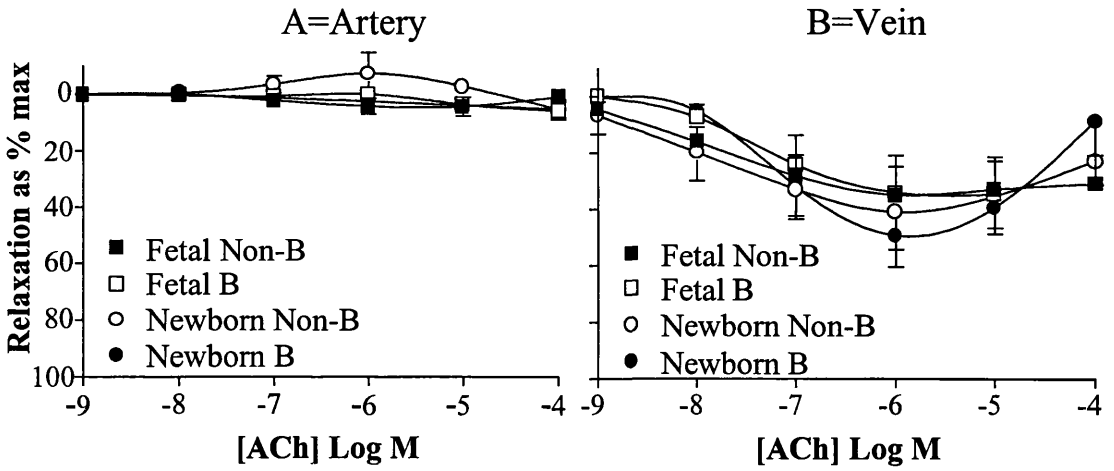

Figure 6.4 Arterial concentration response curves to ACh from breathing(B) and non-breathing(Non-B) fetal and newborn pigs. A:Arteries ,B:Veins (Data represent mean(as % maximum relaxation to papaverine) ±s.e.m).

AGE	Arterial [ACh]Log M to cause E_{max}	Arterial Relaxation as % papaverine (E_{max})	Venous [ACh]Log M to cause E_{max}	Venous Relaxation as % papaverine (E_{max})
Fetal Non-B	-5.5	4.33±3.4*	-6	34.9±10.1
Fetal B	-4	5.82±3*	-5	34.7±13.5
Newborn Non-B	-4	5.34±3.1*	-6	41.0±13.4
Newborn B	-5.5	5.3±3.7*	-6.5	49.4±11.6

Table 6. 6 Comparison of arterial and venous relaxant responses to ACh (E_{max}) in breathing (B) and non-breathing (Non-B)fetal and newborn pigs.(Data are expressed as mean ± s.e.m).*$p<0.05$ E_{max} Significantly different to veins from same age group, un-paired t-test.

6.3 Discussion.

The studies in this chapter aimed to identify the stimuli responsible for inducing the changes in vascular reactivity that occur in the pulmonary vessels at birth.

6.3.1 **Organ culture studies**

Initial experiments were undertaken to investigate the contribution that fetal porcine serum had on responsiveness of pulmonary arteries. In order to observe these effects comparatively, another older age group was studied, the vasoactive responses of which were not compromised following organ culture for 24 hours.

Thus, arteries from fetal and 14 day old age groups were compared. They were subsequently incubated for 24 hours in porcine serum from either fetal or 14 day old pigs. Studies in chapter 4 had shown that within 5 minutes of birth, newborn pulmonary arteries and veins were more responsive to the contractile agonists KCl and U46619 than fetal vessels. The improved vasoactivity (i.e. of contraction and/or relaxation), seen after birth in the perinatal period could be caused by the degradation of circulating "inhibitors". For instance, recent data has suggested the presence of the NOS inhibitor ADMA in the maternal circulation (Fickling et al., 1993) which may pass across the placenta. It was found that the low reactivity of fetal pulmonary arteries was not created by factors within the fetal serum as fetal arterial reactivity did not significantly alter following incubation in either FPS or 14 PS. In addition, the contractile and relaxant responses of arteries taken from the 14 day old pig were not significantly altered following incubation in either FPS or 14 PS. Interestingly, incubation in the serum from the 14 day old pigs did not induce fetal tissue to respond in a mature fashion and lessened the responses when compared to those from arteries that had been incubated in FPS. The reasons behind this may warrant further investigation.

The low responsiveness of fetal arteries to different contractile agents is therefore not due to circulating agents affecting vascular reactivity in an acute fashion. This corresponds with data from chapter 3, where following the semi-purification of NOS in fetal lungs, the activity of NOS did not increase relative to that found postnatally.

Thus the structural changes in the pulmonary vessels from the fetus may play a greater role in this period. In fact the more compact and rigid hypertensive structure of

the fetal arteries (Hall et al., 1987), may prevent additional vasoconstriction and subsequent vasorelaxation (Rudolph et al., 1966).

In conclusion, from this initial set of experiments, any lasting effect of fetal serum does not affect vascular reactivity in vitro. Therefore the differences that arise between the vasoactivity of fetal and postnatal age groups are most likely influenced by physical stimuli created at birth, such as breathing.

6.3.2 **Effect of breathing on the pulmonary vascular reactivity of the fetal and newborn pig**

6.3.3 *Arteries*

From the studies in chapter 4, it was shown that within 5 minutes of birth, the reactivity of the vasculature in the newborn lung was greater than that found in the fetal lung, the difference thought to be a combination of both structural and functional changes in the vasculature at this time. The induction of these changes may be initiated by the oxygenation and/or the ventilation of the lung (Teitel et al., 1990; Black et al., 1997), or following parturition. By allowing fetal pigs to spontaneously breathe the effects of increased oxygenation, ventilation and shear stresses on the pulmonary circulation were observed. In a similar fashion the effects of parturition were observed by studying the vessels of newborn pigs which had not breathed.

It was found that spontaneous breathing in fetal piglets enhanced the arterial contractile responses to U46619, compared to data from non-breathing fetal controls observed in chapter 4. In a similar fashion, Teitel and co-workers showed that the vasoactive properties of fetal ovine lungs were augmented following the induction of breathing (Teitel et al., 1990). Mechanical stimuli created by lung expansion may further stimulate the contractile proteins in the vessel wall and enhance responses to various contractile agonists. The myogenic response has previously been observed in fetal ovine pulmonary arteries in vivo (Storme et al., 1999). This active development of force in the vascular smooth muscle, which leads to the expansion of the vessels, may in turn be modulated by endothelial derived factors (Storme et al., 1999; Juncos et al., 1995). Experiments in this chapter showed that removal of the endothelium caused the differences in arterial contractility to U46619 in both breathing and non-breathing fetuses to become non-significant, suggesting that the increased contractility observed on breathing in the fetal lung was not entirely a smooth muscle related event.

This increase in arterial contractility could be due to an alignment of contractile proteins in the smooth muscle cells, stimulated by the expansion of the lung and subsequent vasodilation and increased blood flow. In the non-breathing fetuses, the level of arterial contraction elicited by U46619, which was low, was enhanced following endothelium removal. As previously suggested, because of a lack of effect on KCl responses, an inferred increase in the interaction between the thromboxane analogue and its receptors due to increased receptor sensitivity may be occurring. These occurrences were not present in breathing fetuses.

In the studies from chapters 4 and 5 it was confirmed that fetal arteries did not relax well to ACh. In this chapter, in a similar fashion, ACh did not induce vasodilation in pulmonary arteries from fetal pigs that had breathed. This lack of response in the breathing fetal pigs is not surprising as levels of pulmonary arterial relaxation induced by ACh in the newborn and fetal pigs appear analogous (chapter 4). However, the lack of relaxant response is confounding as fetal and newborn pig lungs contain equal amounts of eNOS protein to the adult (chapter 3). In chapter 5, the release of NO basally in fetal arteries was found to be lower than that in the newborn pig, in association with a lower/absent NOS activity. NO is associated with the decrease in pulmonary vascular resistance at birth. It is commonly held that oxygenation and ventilation of the lungs stimulate this increase in NO production. Following oxygenation of pulmonary artery endothelial cells from fetal sheep, NOS concentration and activity has been shown to increase (Black et al., 1997), indeed, by inhibiting NOS in fetal sheep the decrease in pulmonary vascular tone induced by oxygenation can be blocked (Tiktinsky et al., 1992). However, this chapter confirmed that breathing was not the stimulus which introduced the relaxant response to ACh, although Ca independent NOS increased to a level similar to that from the newborn lungs. It is thought that the initiation of breathing increases the shear on the endothelial cells increasing the activity of Ca independent NOS. This NOS may be either iNOS or eNOS. The Ca independent eNOS is not activated via a receptor-mediated increase in intracellular calcium, but by the phosphorylation of the NOS enzyme (Fleming et al., 1998; Dimmeler et al., 1999; Fulton et al., 1999). Therefore the lack of induced arterial relaxation following the addition of ACh in the breathing fetal and newborn pig lungs may be due to the change in isoform of NOS, which in turn may not respond to any increase in intracellular Ca when stimulated by ACh. Other explanations for the lack of relaxation to ACh in the breathing fetal and newborn pig may reside in an immaturity of ACh receptors or in the

smooth muscle cell response to NO (i.e. cGMP pathway). In addition, in the non-breathing fetus the absence of ACh induced relaxation is also likely to be created by a predominating low eNOS activity (chapter 3).

In this chapter, it was found that in the newborn pig the absence of breathing had a tendency to reduce arterial contractility. The difference in pressor response between both types of newborn artery could possibly be mediated by the stimulated myogenic response or the alignment of contractile proteins which may occur following the expansion of the lungs at the onset of breathing, as previously suggested.

The differences in the arterial pressor response observed between the lungs of fetal and newborn pigs that had not breathed was not related to the ventilation or oxygenation of the lung. The difference was therefore possibly created because, unlike the fetal pigs, the birth of the non-breathing newborn pigs involved labour and delivery via the birth canal, and not Caesarean section. Data from chapter 5 showed an increased contribution of arterial basal NO to vascular tone at birth. Associated with this was an increase in eNOS activity (chapter 3), which was not associated with breathing. The increase in activity may have therefore been created by increased levels of oestrogen, which increase eNOS in the last stages of pregnancy (Weiner et al., 1994; Goetz et al., 1994) and during labour, causing an increase in the production of NO (Lantin-Hermoso et al., 1997). Moreover, oestrogen has been shown to acutely modulate NO in a non-genomical way (MacRitchie et al., 1997) as well as by the longer term increase in the transcription of eNOS RNA and protein (Goetz et al., 1999).

Furthermore, in the fetal piglets, being delivered by Caesarean section decreases the concentrations of circulating endothelial vasoactive factors such as ET-1 and PGI_2 (Hakkinen et al., 1992; Haning et al., 1978). Thus a disruption in the endothelium-derived production of these compounds, following delivery via Caesarean section may upset modulation of vascular tone in the fetal pigs preventing further contraction of the vessel.

Despite all the putative mechanisms that might increase NO release following birth, ACh did not induce relaxation in the arteries from the non-breathing newborn pigs, much like the newborn controls. Thus, the induction of breathing in the newborn did not alter arterial relaxations to ACh.

6.3.4 *Veins*

In the veins, breathing did not significantly affect fetal or newborn contractile responses. Previous chapters have shown venous contractile responses to be the smallest in the fetal age group. Following spontaneous breathing, fetal E_{max} to U46619 had a tendency to decrease in the veins with an increase in sensitivity. This putatively infers an alteration in the number and/or availability of TP receptors following ventilation and oxygenation.

In addition, neither the immediate onset of breathing nor the birth process significantly altered the venous relaxant responses to ACh. Other studies between ovine and porcine arteries and veins have suggested that the differences between age groups to NOS stimulants and NO donors may be due to an increased NOS activity, and an increased utilisation of the smooth muscle sGC/cGMP pathway (Steinhorn et al., 1995; Steinhorn et al., 1993; Bina et al., 1998; Gao et al., 1995). However, an extensive study investigating the NO/cGMP pathway in the veins is required in this transitionary period to elucidate these possibilities.

In conclusion, the process of spontaneous ventilation in the fetus increases the contractility of the arteries (E_{max}). The improved responsiveness to U46619 may be stimulated by the increases in stretch of the smooth muscles, which occurs following the inhalation of air, and the subsequent expansion of the lungs. However, a difference in contractile responses between the fetal and newborn arteries is a combination of a multitude of stimuli applied to the arteries at birth, namely being born naturally via the birth canal and the onset of breathing. However, these same stimuli do not improve the relaxant responses to ACh. The relaxant responses in fetal arteries were not enhanced either by breathing or by the method of delivery. The venous responses to ACh, which were consistently greater than the arteries, remained equal following delivery and the onset of breathing.

In vivo this means that during the transition from the fetal to the newborn pulmonary circulation, the veins have a constant ability to vasodilate. This may prevent pulmonary oedema at birth by accommodating the increase in blood flow through the lungs, which is created by the greater number of arteries that are forced open. The enhanced constrictor responses in the arteries, and the inability to vasodilate following stimulation, may be required to moderate the initial large blood volume through the lungs at birth.

Chapter 7. General Discussion

In the lung, pulmonary vascular resistance (PVR) alters with development; in the immediate transition of the lung to the extrauterine environment, postnatally corresponding with lung growth, and in PPHN. This modulation of PVR occurs as a result of the synergistic actions of the endothelium and smooth muscle in the pulmonary vasculature. Studies in this thesis revealed that a major part of the pulmonary adaptation with age, which would ultimately affect PVR, occurred in the pulmonary arteries with the veins playing a less dynamic role.

A combination of organ bath techniques on fresh pulmonary vascular tissue and biochemical assays on whole lung samples investigated the role of NO in the developmental adaptation of the pulmonary arterial responses.

In these studies a common theme throughout the thesis was revealed. This was the diminished responsiveness of fetal lung tissue. What made these observations so striking was the improvement in responses at birth.

Studying alterations in vascular reactivity with age, the contractility of fetal arteries was observed to be the lowest of any age group studied. This was reflected in the responses to KCl and in the concentration dependent responses to U46619. This low reactivity of the fetal vasculature has been suggested to have been created by the low ratio of actin to myosin filaments which increases at birth (Belik et al., 1991). Other groups have shown that in the newborn period, the smooth muscle cells from the arteries exist in a depolarised state (Evans et al., 1998). This state would cause an influx of Ca into the smooth muscle cells via voltage operated channels, leading to an increase in contraction. This finding has been used previously to putatively explain the lack of relaxation to relaxant agents at these time points. In addition, large vasodilation may be prevented by the arrangement of the cells within the fetal vessel walls (Hall et al., 1987).

The contribution of the endothelium to pulmonary vascular reactivity has been widely studied, with many studies suggesting the importance of basally released NO in the fetal circulation in vitro and in vivo (Storme et al., 1999; Abman et al., 1990; Tiktinsky et al., 1992). Organ bath studies in this thesis confirmed the presence of basally released NO in fetal pulmonary arteries following the addition of L-NAME, however, despite the release of NO basally, little or no relaxation was observed

following the addition of ACh. This was possibly as a result of the immaturity of the muscarinic receptors found at birth (Hislop et al., 1998). In addition, the stimulation of NOS in whole lung homogenates showed that fetal lung contained very little active NOS. The lack of activity was not due to the absence of substrates or cofactors or due to the rate limitation of L-arginine, or endogenous SOD.

On closer observation of these NOS activities, it was found that only 50% of samples studied were active. Within 5 minutes of birth 100% of samples were active. Thus it appeared that some mechanism was switching on the activity of NOS at birth.

The NOS activity in the fetal lung was further investigated by exploring the possibility that the low NOS activity was caused by the presence of endogenous inhibitors, such as ADMA. However, the semi-purification of NOS did not alter the low fetal NOS activity and following the addition of L-arginine to organ baths, which reverses the effects of such NOS inhibitors, the relaxant responses remained diminished. This was further highlighted by the lack of an inhibitory effect of FPS on the vascular reactivity of arteries from 14 day old pigs, or of the enhancement of fetal vascular responses following organ culture in serum from the other more vasoactive age group (14 day). Thus the possible contribution of factors from the maternal circulation, which might have created the low fetal pulmonary vascular reactivity, were not observed.

Therefore, from the data obtained, the lack of fetal vascular relaxation following stimulation of NOS was contributed to by the low activity of NOS, and perhaps an immaturity of muscarinic receptors (Hislop et al., 1998). With the onset of breathing, the contractility of the fetal arteries increased, most likely associated with the structure of the vessel and the alignment of the smooth muscle. This is because with the increase in lung volume which stretches the lung, the smooth muscle cells are thinned out and the lumen increases in size, increasing the surface area: volume ratio (Hall et al., 1987). However, the physical expansion of the lung at birth is not the only factor contributing to the improved contractility from fetal levels. The natural delivery of the newborn piglets, and not by Caesarean section, may increase the level of humoral stimulation of the pulmonary endothelium and smooth muscle, induced either by labour or a physical force on the lungs as the piglets are born via the birth canal (Lantin-Hermoso et al., 1997; Hakkinen et al., 1992; Haning et al., 1978).

At birth an increment in NOS activity was observed. This was not stimulated by the onset of breathing, although the proportion of Ca independent NOS activity increased (i.e. iNOS or Ca independent eNOS). This increase in Ca independent NOS

was most likely stimulated by the increase in shear and strain on the pulmonary vasculature associated with the initiation of breathing. This was surprising as other studies have shown that the oxygenation and ventilation of the lung increases eNOS mRNA (Shaul et al., 1997; Black et al., 1997).

The increment in NOS activity at birth would have undoubtedly contributed to the large basal release of NO seen in the pulmonary arteries from the newborn pig when treated with L-NAME. However, it does not explain the diminished relaxant response to ACh at this age. Again, the immaturity of the muscarinic receptors at this age for ACh may be the cause of this response (Hislop et al., 1998). In addition, a low level of soluble guanylate cyclase activity was observed in the newborn, perhaps also contributing to the low responses to ACh observed.

By 1 day after birth, contractility of the piglet artery had decreased in association with the initiation of the relaxant response to ACh. This was not due to an increased activity of NOS. This pattern of arterial reactivity continued up to 3 days of age, at which age the contractile responses were low in association with enhanced relaxant responses to ACh. This decrease in contractility at 3 days of age is thought to correspond to the structural changes in the vessel wall that occur, subsequently diminishing contractile responses. The improved vasorelaxation to ACh at this age corresponded with an increase in NOS activity and content in the lung following Western blotting (chapter 3) and immunostaining (Hislop et al., 1995). In spite of a large amount of NOS and NOS activity however, the basal release of NO was found to be lower at 3 days of age than that observed at birth. In association with the increase in NOS at 3 days of age, an increase in sGC activity of the arteries from levels at birth was observed following stimulation with ODQ. Thus the enhanced relaxant responses and the diminished contractile responses at this age permit a decrease in PVR at 3 days of age, in association with the initiation of changes in arterial structure which continue throughout perinatal life (Greenwald et al., 1982; Hall et al., 1987). At 6 days of age, arterial contractile and relaxant responses appeared similar to those from the 3 day old. However, organ bath experiments in chapter 4 observed an endothelium independent relaxant response to ACh introduced at this age. This observation was associated with a slight decrease in NOS activity but not a change in NOS isoform. It may therefore be due to a smooth muscle release of PGI_2 as previously suggested (Mitchell et al., 1998).

By 14 days of age, the contractile and relaxant responses of the pulmonary artery tended to be greater than all the younger age groups studied. This may be a culmination

of the structural adaptation of the vessel walls, which are structurally stiffer (Greenwald et al. 1982), and have an increased smooth muscle cell size compared to younger age groups (Hall et al., 1987), which contributes to the augmented contractile responses observed at this age. However, the role of the maturing endothelium in the maintenance of arterial tone at this age is not clear. Investigation of the relaxant responses to ACh at this age, found that despite a NOS activity equal to that in all the other postnatal age groups, the addition of L-NAME produced very little basal release of NO. This low release of NO basally was not due to a rate limitation of L-arginine or endogenous SOD. In addition, very little alteration in tone or relaxant response to ACh was observed in the presence of sGC inhibition. This suggested that the stimulation of the endothelium by ACh did not result in the stimulation of sGC in the smooth muscle. However because relaxant responses to ACh do occur, either NO is affecting smooth muscle cell relaxation independently of sGC, or ACh is stimulating the production of other endothelium derived relaxing factors such as EDHF and PGI_2, the presence of which had been inferred in the 6 day old pig.

By adulthood, the relaxant responses in the pulmonary arteries had diminished and the contractile responses increased. The greater contractile responses were most likely due to a simple increase in smooth muscle cell layers associated with the increased growth of the vessel The decreased relaxant response may have been associated with an increased deposition of collagen and connective tissue in the arteries, leading to an increased structural stiffness (Hall et al., 1987). In addition, the lung of the adult pig was found to have decreased levels of NOS activity, although the lung NOS content was analogous to that found in the newborn and fetal porcine lung. L-arginine, superoxide and sGC did not appear to be the cause of the decreased responses to ACh at this time. Interestingly however, the ACh responses were less inhibitable by ODQ and L-NAME than in the arteries from the younger perinatal age groups. This again suggests a role for the ACh stimulated release of other endothelial derived relaxing factors, such as EDHF and PGI_2, in a similar fashion to the arterial responses at 14 days of age.

Thus, this data has demonstrated that the development of pulmonary endothelial vascular reactivity and the NO pathway are associated, although not exclusively. The development of ACh induced stimulation of other endothelium derived relaxing factors, such as PGI_2 and EDHF may be introduced from 6 days of age onwards. Experiments examining the relationship of PGI_2, EDHF and NO production with age to ACh can be

studied in the presence of indomethacin, L-NAME and K^+ channel blockers. These age related changes in vascular reactivity modulate PVR in association with structural changes that occur, which are reflected in the contractile responses of the arteries.

The relationship between structure and vascular reactivity becomes most apparent when examining the pulmonary vascular responses in hypoxia induced pulmonary hypertensive pigs. By exposing newborn or 3 day old pigs to hypoxia for 3 days, the resulting vascular reactivity may be studied, and correlations made between the structural and functional development. The trend for an increased arterial contractility following chronic hypoxia for 3 days was observed in both 3 day old and 6 day old pulmonary hypertensive pigs. This increase in contractility was most likely related to the increased myofilament density which occurs with hypoxia induced pulmonary hypertension in the pig model (Allen et al., 1986).

The lack of ACh response following hypoxia induced pulmonary hypertension from birth or 3 days of age, was not created by a lack of NOS activity. In the 3 day hypoxia induced pulmonary hypertensive pig there was no lack of NO production basally, as was shown by L-NAME. In addition, the removal of the endothelium did not remove the basal production of NO suggesting the production of NO originated from the smooth muscle. This may be associated with the increased proportion of Ca independent NOS found at this age (i.e. iNOS). Indeed, other studies have shown a decrease in the eNOS isoform in pulmonary arteries from this porcine model of hypoxia induced pulmonary hypertension (Hislop et al., 1995). A change in the proportion of the Ca dependent isoform was not observed in the 6 day old pulmonary hypertensive pig. Thus the difference in relaxant responses between the two hypoxia induced pulmonary hypertensive age groups cannot be related to exposure to chronic hypoxia alone. One differing characteristic between these two age groups is the structure of the arterial wall, which in the 3 day pulmonary hypertensive pig, resembles that of the newborn, whilst at 6 days of age, the structure of the arteries following hypoxia induced pulmonary hypertension resembles that of the age matched controls (Allen et al., 1986). The arrested development of the pulmonary artery in the 3 day old hypertensive pig may mean that the endothelially derived responses are also immature. Thus the development of muscarinic receptors may be such that relaxation to ACh is not possible. However, a lack of relaxant response to ACh in the 6 day old hypertensive pig means that chronic hypoxia was also playing a role.

In addition to the alteration of pulmonary arterial responses with age, the contribution of venous vasoreactivity was also studied. In the fetus, it was found that whilst the arteries had a low reactivity to both contractile and relaxant agonists, venous contraction and relaxation was present and of a much greater magnitude. A small insignificant increment in contractility and relaxation at birth was observed, the mechanism creating this unlikely to be due to the onset of breathing. Thus the venous relaxant responses appear to be fully developed at birth and so may significantly aid the decrease in PVR at this time. After birth the contractile and relaxant responses continued to increase in an age related fashion. In the adult age group the arterial and venous contractile responses were equivalent. This suggests that the role of the vein is more important in the perinatal period than in the adult. This increased reactivity which is maintained with age, may be required to buffer the altering arterial vascular responses with age, thus minimising any dramatic changes in PVR and preventing the onset of pulmonary vascular dysfunctions such as pulmonary oedema.

The impact of hypoxia induced pulmonary hypertension on the venous vasoreactivity was a dramatic one, diminishing both contractile and relaxant responses. The effects of chronic hypoxia from birth attenuate the development of the pulmonary arteries (Allen et al., 1986), which may be what is occurring in the veins. However further analysis of lung sections is required before the structure of the veins in this disease can be related to function.

In conclusion, in this study the changes in vascular reactivity of the lung with age have been related to the changes in structure and function that occur with pulmonary development. By doing this, it is hoped that a better understanding of the mechanisms of lung development, particularly of the NO pathway may be gained. This insight may help in the understanding of the pathological state of PPHN, as investigated in the pig following chronic exposure to hypoxia.

The data presented in this thesis, with respect to NO, showed that the NO/cGMP pathway was functional in utero, and by 24 hours of age could be humorally stimulated in the arteries with ACh. In the veins, very little differentiation was observed to ACh responses between developmental age groups. The lack of arterial relaxant responses to ACh was due to a combination of acute post-transcriptional changes, i.e. an increase in NOS activity at birth, and longer term changes such as an increase in muscarinic receptor number. By 6 days of age the presence of other ACh-induced endothelium derived relaxing factors in arteries became apparent. Therefore in the

future the COX and NOS pathways need to be studied at all developmental ages, particularly from 6 days of age onwards, in the endothelium and smooth muscle. In addition investigation into developmental alterations in the cAMP and cGMP pathways in the smooth muscle is required. The apparent arrest of arterial structural and functional development with hypoxia induced pulmonary hypertension from birth altered the relaxant responses to NO, alongside the effects of hypoxia on the endothelium and smooth muscle. However, the precise point of dysfunction within the NO pathway still needs to be located, as there is by no means a lack of basally produced NO in this model. Further study of the relationship between vessel structure and endothelial and smooth muscle production of NO is required at all ages, as data from this thesis did not clarify precisely when and in which segment of the pulmonary vascular bed NO activity/ release was switched on. This is because differentiated levels of basal and stimulated production of NO were observed, with an apparent derivation of NOS from the endothelium and smooth muscle cells at certain ages.

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