Persistent pulmonary hypertension of the newborn : a point of view from vascular pharmacology

Persistent Pulmonary Hypertension of the Newborn. A Point of View from Vascular Pharmacology

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Persistent Pulmonary Hypertension of the Newborn. A point of view from Vascular Pharmacology

PROEFSCHRIFT

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aan Ana

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Abbreviations

ACh, acetylcholine

ANP, atrial natriuretic peptide

AP-1, activator protein-1

BH_4, tetrahydrobiopterin

BPD, broncopulmonary displasia.

cAMP, cyclic adenosine-3', 5'-monophosphate

cfu, colonies forming units

cGK, cGMP-dependent kinase

cGMP, cyclic guanosine-3', 5'-monophosphate

CI, confidence interval

CLD, chronic lung disease

CO, carbon monoxide

COX, cyclooxygenase

DA, ductus arteriosus

ECE, endothelin converting enzyme

ECMO, extracorporeal membrane oxygenation

EDNO, endothelium-derived nitric oxide

EDRF, endothelium-derived relaxing factor

eNOS, endothelial nitric oxide synthase

ET, endothelin

GBS, group B Streptococcus

GTP, guanosine-5'-triphosphate

HFOV, high-frequency oscillatory ventilation

HIF, hypoxia inducible factor

HO, heme oxygenase

HPV, hypoxic pulmonary vasoconstriction

iNOS, inducible nitric oxide synthase

IL-1, interleukin-1

IP_3, inositol 1,4,5-triphosphate

L-NAME, N^{ω}-Nitro-L-arginine methyl ester (NOS inhibitor)

LPS, lipopolysaccharide

$MgSO_4$, magnesium sulfate

NA, noradrenaline

nNOS, neuronal nitric oxide synthase

NO, nitric oxide

NO_2, nitrogen dioxide

NOS, nitric oxide synthase

ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (sGC inhibitor)

OI, oxygenation index

$ONOO^-$, peroxynitrite

PAF, platelet-activating factor

PAP, pulmonary artery pressure

PDE, phosphodiesterase

PG prostaglandin

PGHS, prostaglandin endoperoxide H synthase

PGI_2, prostacyclin

PPHN, persistent pulmonary hypertension of the newborn

PVR, pulmonary vascular resistance

SAP, systemic arterial pressure

sGC, soluble guanylate cyclase

SNP, sodium nitroprusside

SOD, superoxide dismutase

SVR, systemic vascular resistance

TNF, tumor necrosis factor

TX, thromboxane

U46619, 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$ (TXA_2 analog)

VEGF, vascular endothelial growth factor

VSM, vascular smooth muscle

[X]i, X intracellular concentration

[X]o, X extracellular concentration

PART I

PART II

Chapter I. General introduction

I.1 Persistent pulmonary hypertension of the newborn (PPHN): failure of lung circulation to undergo postnatal adaptation.

During prenatal life the lungs do not participate in gas exchange and the fetus is dependent on the placenta to supply oxygen and nutrients. At this stage, the lungs receive a small proportion of venous return, to ensure pulmonary growth and development, and the rest is diverted through the foramen ovale and the ductus arteriosus to the systemic circulation. Thus, the fetal pulmonary circulation exists as a high-resistance, low-flow circuit accepting less than 10% of the combined ventricular output (Rudolph & Heymann 1968;Fineman et al., 1991; Walker, 1993; Ziegler et al., 1995a; Abman 1999; Heymann, 1999). At birth, when the lung assumes the respiratory function, the pulmonary circulation undergoes a striking transition characterized by a fall in pulmonary vascular resistance (PVR), as blood flow rapidly increases 8- to 10-fold, followed by a more gradual decline in pulmonary arterial pressure (Cassin et al., 1964; Walker, 1993; Ziegler et al., 1995a; Abman 1999; Lakshminrusimha & Steinhorn, 1999). Therefore, successful adaptation of the newborn to postnatal conditions requires a dramatic transition of the pulmonary circulation from a high resistance state in utero to a low-resistance state within minutes after birth.

Several mechanisms contribute to the normal fall in PVR at birth, including the establishment of a gas-liquid interface in the lung, increased oxygen tension, rhythmic distension of the lung and shear stress (Cassin et al., 1964; Dawes & Mott, 1962; Enhorning et al., 1966; Blanco et al., 1984; Tiktinsky & Morin, 1993; Cornfield et al., 1992; Abman, 1999). These physical stimuli act, at least partially, through the production of vasoactive products, especially the release of potent vasodilator substances, such as nitric oxide (NO) and prostacyclin (PGI_2)(Ziegler et al., 1995a; Abman 1999; Heymann, 1999).

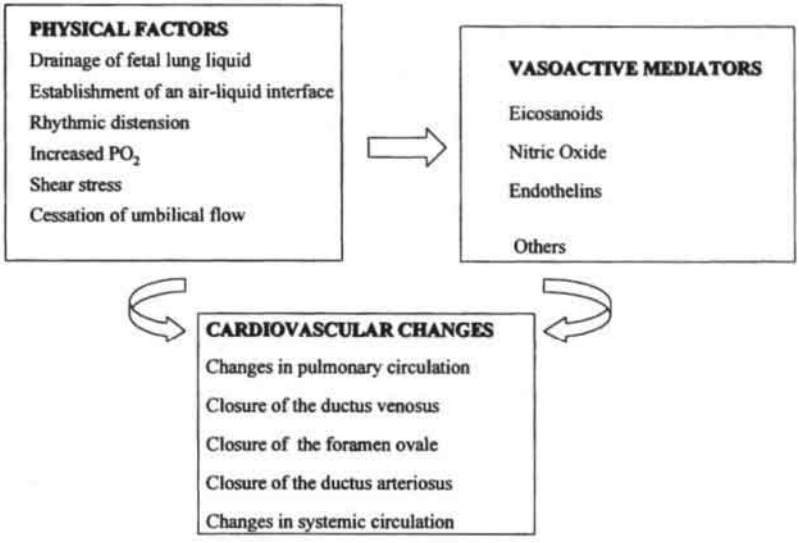

Figure 1. Factors involved in the transition between the fetal and the neonatal circulation

Failure of the pulmonary circulation to undergo a normal transition results in persistent pulmonary hypertension of the newborn (PPHN), a clinical syndrome of various neonatal cardiopulmonary disorders which are characterized by sustained elevation of PVR after birth, leading to right-to-left shunting of blood across the ductus arteriosus or foramen ovale and severe hypoxemia (Levin et al., 1976). PPHN is a pathophysiological phenomenon occurring in a heterogeneous group of diseases with a wide diversity of etiologies (Table 1). These range from transient reversible pulmonary hypertension attributable to perinatal insults to irreversible fixed structural malformations of the lung (Kinsella & Abman, 1995). Diseases associated with the syndrome of PPHN can be classified in three categories (Abman, 1999): 1) Maladaptation, in which vessels are presumably of normal structure but have abnormal vasoreactivity; 2) Excessive muscularization, in which smooth muscle cell thickness is increased and muscle extends distally to vessels that usually are nonmuscular; and 3) Underdevelopment, in which lung hypoplasia is associated with decreased number of pulmonary arteries. These categories are not watertight compartments and, in fact, the inability to effectively lower PVR during the

first moments of life in a 'maladaptative' disorder may rapidly cause aggressive hypertensive changes, leading to fixed pulmonary hypertension due to smooth muscle cell proliferation and remodeling (Roberts et al., 1997a). Altered vascular reactivity and structural remodeling appear to represent a continuum, in which, vasoconstriction induces hypertensive structural changes that further alters vascular responsiveness (Roberts et al., 1997a). Either as a primary condition or secondary to other pulmonary or extra-pulmonary diseases, PPHN is an important cause of cardiorespiratory failure and responsible for a relevant percentage of morbidity in the neonatal intensive care units (Davidson et al., 1999).

Current therapies for PPHN are aimed at lowering pulmonary vascular resistance with the use of hyperoxia, hypocarbic or metabolic alkalosis and theoretical selective vasodilators; increasing pulmonary compliance with surfactant; using lung recruitment strategies such as high frequency ventilation; and optimizing systemic tension with inotropes and volume expanders (Kennaugh et al., 1997; Goldman et al., 1996; Mok et al., 1999; Kinsella & Abman, 1999). Current therapies are often limited by adverse effects or incomplete responsiveness (Roberts et al., 1997a). Patients who fail conventional therapy often require treatment with extracorporeal membrane oxygenation (ECMO). Although ECMO has improved survival in refractory PPHN, it remains labor-intensive, is costly, has multiple side effects, and may be associated with long-term neurological sequelae (Kennaugh et al., 1997; Roberts et al., 1997a). Safer, more effective therapies for PPHN will be possible only when they can be directed toward the specific defects producing this condition. In this sense, the remarkable basic scientific discovery that the simple gas molecule NO was endogenously released by endothelial cells, producing paracrine vasodilatory effects in the adjacent vascular smooth muscle, enormously boosted the search for a specific treatment for PPHN. Administered by inhalation, characterized by a short half-life and the absence of measurable systemic effects, the use of inhaled NO as an adjunct to conventional PPHN therapy is, presently, widespread (Davidson et al., 1999; Kinsella, 1999; Mok et al., 1999).

Table 1. Factors associated with PPHN (modified from Roberts et al., 1997a)

Pulmonary	Hematological
Meconium aspiration	Polycythemia
Amniotic fluid aspiration	Thrombocytopenia
Blood aspiration	Thrombotic endocarditis
Hyaline membrane disease	Acute hemorrhage
Surfactant deficiency	
Transient tachypnea	Gastrointestinal
Diaphragmatic hernia	Omphalocele
Cystic adenomatoid malformation	Gut perforation
Pulmonary hypoplasia	Gastroschisis
Pneumonia	
Thromboemboli	Intrauterine insults
Peripheral pulmonary vascular occlusion	Chronic hypoxia
	Postmaturity
Alveolar capillary dysplasia	Premature ductal closure
Pulmonary hemorrhage	Maternal hypoxia, hemorrhage, hypotension
Phrenic nerve agenesis	
	Drug-induced
Sepsis	Aspirin
Group B *Streptococcus*	Indomethacin
Escherichia coli	Naproxen
Listeria monocytogenes	Hydantoin
Haemophilus influenzae	Parenteral lipids
Others	Amitriptyline
	Lithium
Metabolic	Terbutaline
Hypoglycemia	Tobacco smoke
Hypocalcemia	
Acidosis	Others
Hypoxia	Idiopathic
	Systemic hypertension
	Shock
	Pulmonary artery distension

I.2 Outline of the thesis

Because PPHN represents the failure of postnatal adaptation of the lung circulation at birth, understanding the basic mechanisms of normal functional and structural development of the pulmonary circulation *in utero*, and the mechanisms that contribute to transitional pulmonary vasodilation, may provide insights into the syndrome of PPHN and its treatment (Abman, 1999). From this perspective, the present thesis focuses on some aspects of the pathophysiology and treatment of PPHN. In chapters II, III, and IV the state of the art of the problem is reviewed and in chapters V to XII some original contributions are presented.

Specifically, we review, in **chapter II,** some of the mediators (NO, eicosanoids, endothelin-1, CO) most widely involved in the control of pulmonary vascular tone, as well as in the circulatory transition and, consequently, in PPHN, the failure of this process. The role of oxygen in the above-mentioned processes is also analyzed.

Chapter III review sepsis-induced changes in vascular reactivity and its role in the pathophysiology of both pulmonary hypertension, and systemic hypotension that accompany neonatal generalized infection.

Chapter IV is focused on the treatment of PPHN, describing the difficulties for finding a selective pulmonary vasodilator and use of inhaled NO in this respect.

In **Chapter V** (Am J Physiol. 1997; 272:L1013-20) we analyze the alterations that chronic intrauterine pulmonary hypertension produces on lung endothelial NO synthase (eNOS). The model of chronic intrauterine pulmonary hypertension caused by ductus arteriosus compression in the fetal lamb, closely mimics the hemodynamic and pathological changes observed in fatal clinical PPHN. We measured eNOS content and activity in this experimental model, to determine the possible involvement of an impairment of the NO relaxant pathway in PPHN.

Chapter VI (Biol Neonate 1997; 72:62-70) is focused on the pulmonary vascular response to hypoxia. We investigated the response to hypoxia in isolated intrapulmonary arteries and veins from newborn piglets, evaluating the role of NO and eicosanoids on this response. Furthermore, we compared the effects of hypoxia in pulmonary vessels with systemic (coronary and mesenteric) arteries.

Chapters VII (Br J Pharmacol. 1995; 115:261-6), **VIII** (J Vasc Res. 1996; 33:249-57), and **IX** (Pediatr Res. 1996; 40:827-33) include experimental evidences of sepsis-induced changes in pulmonary and systemic vascular contractility. We analyzed the effects of incubation of piglet pulmonary, and mesenteric arteries with heat inactivated group B *Streptococcus*, and *Escherichia coli* lipopolysaccharide, on the vascular responses to several vasoconstrictor agonists, including those involved in the pathophysiology of sepsis-induced PPHN.

In **chapter X** (Br J Pharmacol. 1997; 121:1323-33) we studied the interactions between vasoconstrictor and vasodilator agents that are released during sepsis, playing a role in PPHN, and systemic vascular disturbances. Specifically, we analyzed the interactions of noradrenaline, the TXA_2-mimetic U46619 and ET-1 with the relaxation induced via cyclic GMP. In addition, we studied the mechanisms involved in NO/cyclic GMP-induced relaxation in isolated intrapulmonary arteries.

Since lowering pulmonary artery pressure while maintaining systemic vascular resistance and good cardiac output is crucial for newborns with PPHN, the ideal drug for their treatment should be a vasodilator with selectivity for pulmonary over systemic vessels. In **chapters XI** (Eur J Pharmacol. 1996; 314:91-8), and **XII** (Pediatr Res. 1996; 39:1107-12) we compared the relaxant effects of some of the proposed, and even clinically used, selective pulmonary vasodilators (acetylcholine, sodium nitroprusside, ATP, PGE_1, tolazoline, nifedipine, and magnesium sulfate) in isolated piglet pulmonary and mesenteric arteries.

Finally, in **chapter XIII** (Pediatr Res. 2000; 48:546-553) the pulmonary vascular

Chapter II. Factors involved in the prenatal-postnatal pulmonary circulatory transition and in the pathophysiology of PPHN.

II.1 Nitric oxide

The discovery that the biological actions of endothelium-derived relaxing factor (Furchgott and Zawadzki, 1980) are due to the endogenous release of NO (Palmer et al., 1987; Ignarro et al., 1987; Khan & Furchgott, 1987) revealed the existence of an ubiquitous biochemical pathway (Moncada et al., 1989; Moncada et al., 1997). NO is a unique messenger molecule involved in the regulation of diverse physiological processes including smooth muscle contractility, platelet reactivity, central and peripheral neurotransmission, and the cytotoxic actions of immune cells (Moncada et al., 1997). Therefore, NO is crucial for many physiological functions, and inappropriate release of this mediator has been linked to a number of pathologies (Hibbs & Moncada, 1999).

NO is formed endogenously by a family of enzymes known as NO synthases (NOS). Three distinct isoforms of NOS have been identified (Moncada et al., 1997). Molecular cloning has shown these to share 50–60% homology. There is a constitutive form, neuronal (nNOS or NOS I), whose activity is regulated by Ca^{2+} and calmodulin, and which is found in neural tissue, both centrally and peripherally. A second, Ca^{2+}/calmodulin-requiring, constitutive enzyme is present in vascular endothelial cells (eNOS or NOS III). A third, Ca^{2+}-independent inducible isoform (iNOS or NOS II) can be isolated from a variety of cells following induction with inflammatory mediators and bacterial products. The association of the three NOS isoenzymes with the endothelium (eNOS), neurons (nNOS) and inducibility (iNOS) is an oversimplification (Moncada et al., 1997). For example, eNOS is located not only in the vascular endothelial cells but also in platelets (Radomski et al., 1990) and in certain neuronal populations in the brain (Dinerman et al., 1994), whereas nNOS has been found in the epithelium of the bronchi and trachea (Kobzik et al., 1993), as well as in skeletal muscle (Kobzik et al., 1994). In addition, the constitutive eNOS can be induced in certain situations such as during chronic exercise (Sessa et al., 1994) or during pregnancy, when both eNOS and iNOS are induced (Weiner et al., 1994). In

contrast iNOS appears to be present constitutively in some tissues, including human bronchial epithelium (Kobzik et al., 1993), rat kidney (Mohaupt et al., 1994) and ovine pulmonary fetal tissues (Rairigh et al., 1998

NO is generated by NOS via a five-electron oxidation of a terminal guanidinium nitrogen on L-arginine (Palmer & Moncada, 1989). The reaction is both oxygen- and NADPH-dependent and yields L-citrulline in addition to NO, in a 1:1 stoichiometry (Bush et al., 1992). This process occurs in at least two distinct steps. The initial reaction involves N-hydroxylation of the guanidinium nitrogen to form N-hydroxy-L-arginine, which is the only intermediate that has been identified (Wallace & Fukuto, 1991; Pufahl et al., 1992). In spite of extensive research, the precise mechanism by which NOS catalyzes the oxidation of L-arginine to NO remains unclear (Hibbs & Moncada, 1999). It appears that many aspects of NOS biochemistry relate directly to the actions of cytochrome P-450. NADPH acts as the source of electrons for oxygen activation and substrate oxidation, and flavin adenine dinucleotide and flavin mononucleotide shuttle electrons from NADPH to the iron heme (Hibbs & Moncada, 1999). The heme moiety of NOS resembles cytochrome P-450, supporting the thesis that the heme component of NOS represents the catalytic center, responsible for binding and reducing molecular oxygen and subsequent oxidation of substrate. In contrast to cytochrome P-450, NOS also requires tetrahydrobiopterin (BH_4) for maximal activity (Kwon et al., 1989). NOS isoforms are subject to a negative feedback control loop mediated by NO (Rogers & Ignarro, 1992; Assreuy et al., 1993), presumably via NO ligation to the heme moiety. Moreover, BH_4 is capable of preventing and reversing this feedback pathway, and although the explanation for this is unclear, this may be one role for BH_4 as a cofactor (Griscavage et al., 1994).

Mechanisms of Action of NO

Most of the physiological actions of NO are brought about by its activation of the soluble guanylate cyclase (sGC) (Murad et al., 1990; Ignarro, 1997; Hobbs 1997). Binding of NO to the heme moiety of this enzyme causes a conformational change that increases the enzyme activity approximately 400-fold and results in the enzymatic conversion of guanosine-

5'-triphosphate (GTP) to the intracellular second messenger cyclic guanosine-3',5'-monophosphate (cGMP). The activation of sGC by NO appears to be a complex process. First, NO binds to the iron in the haem group of the enzyme forming a hexacoordinate complex which then converts to a pentacoordinate nitrosyl-haem complex by one of two routes (Fig 2). For approximately 25% of the haem, this conversion occurs rapidly, whereas for the remaining hexacoordinate nitrosyl complex this process is considerably slower, and appears dependent upon the interaction of NO with an unidentified, non-haem site on the protein (Stone & Marletta, 1994; Deinum et al., 1996; Hobbs, 1997). The relevance of the formation of the pentacoordinate complex for the activation of sGC is illustrated by carbon monoxide (CO). CO also forms a complex with the haem moiety of sGC, but unlike NO, only the six-coordinate complex is formed (Stone & Marletta, 1995), resulting in a low activation of purified sGC when compared with that attained by NO (Hobbs, 1997).

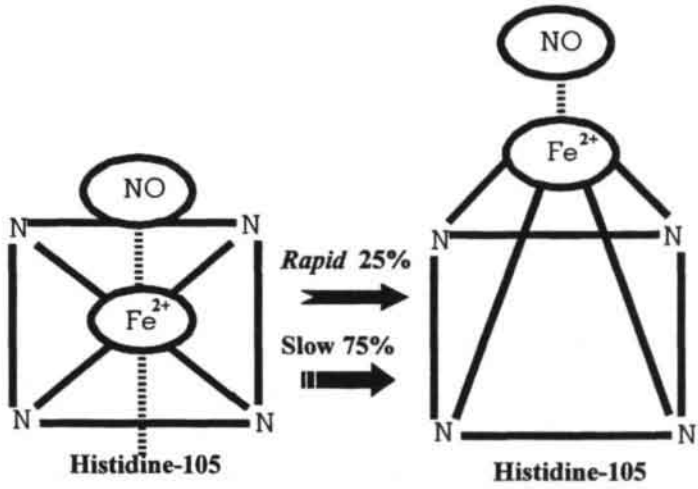

Figure 2. Activation of soluble guanylate cyclase by NO

Agents such as NO, nitrovasodilators, and ANP which raise intracellular levels of cGMP via activation of either the soluble or particulate isoform of GC, are well established to cause relaxation of vascular smooth muscle (Hobbs & Ignarro, 1997). Formation of cGMP by particulate and soluble GC is a common signal transduction pathway utilized by a diverse family of biological messengers. Consequently, to fulfill this role cGMP is capable of modulating a number of cellular functions. In contrast to the cAMP second-messenger system where the activation of a specific protein kinase is responsible for a predominant number, if not all, of the physiological effects, cGMP regulates a variety of enzymes and proteins, including cGMP-gated ion channels, cGMP regulated PDEs and cGMP-dependent protein kinases (Beavo & Reifsnyder, 1990; Kaupp, 1991; Lincoln et al, 1988; Hobbs & Ignarro, 1997). Two major isoforms of vertebrate cGMP-dependent kinase have been recognized, the cytosolic type I (cGKI) and the membrane bound type II (cGKII) (Hobbs & Ignarro, 1997).

Despite extensive research into the cyclic nucleotide receptors mediating this effect, the precise mechanisms by which cGMP induces vascular smooth muscle relaxation remain still unclear. However, there is general agreement that an important part of the process involves interaction and modulation of Ca^{2+} homeostasis by several mechanisms (Hobbs & Ignarro, 1997). First, cGMP may stimulate Ca^{2+} extrusion by activation of the Ca^{2+}ATPase on the plasma membrane (Popescu et al., 1985a, 1985b). It is though that cGK regulates this Ca^{2+} pump by phosphorylation of an intermediate protein, possibly a phosphatidylinositol kinase (Vrolix et al., 1988). A second mechanism by which cGMP may lower $[Ca^{2+}]_i$ is to enhance uptake into the endoplasmic reticulum, via regulation of a Ca^{2+}ATPase similar to that found on the plasma membrane (Twort & Van Breemen, 1988). Third, Na^+-Ca^{2+} exchange through the plasma membrane may also represent a site of cGK-mediated modulation of Ca^{2+} homeostasis (Hobbs & Ignarro, 1997). Fourth, cGMP may have a dual effect on Ca^{2+} channel activity. In addition to directly suppressing the movement of ions through L-type Ca^{2+} channels, cGMP also opposes the inward movement of Ca^{2+} via enhancement of the delayed outward K^+ current (Bkaily et al, 1988). Additionally, an inhibitory effect of cGMP on receptor operated Ca^{2+} channels has also been reported (Hobbs & Ignarro, 1997). Finally, the stimulation of phospholipase C by a variety of hormones and neurotransmitters to yield the second messenger

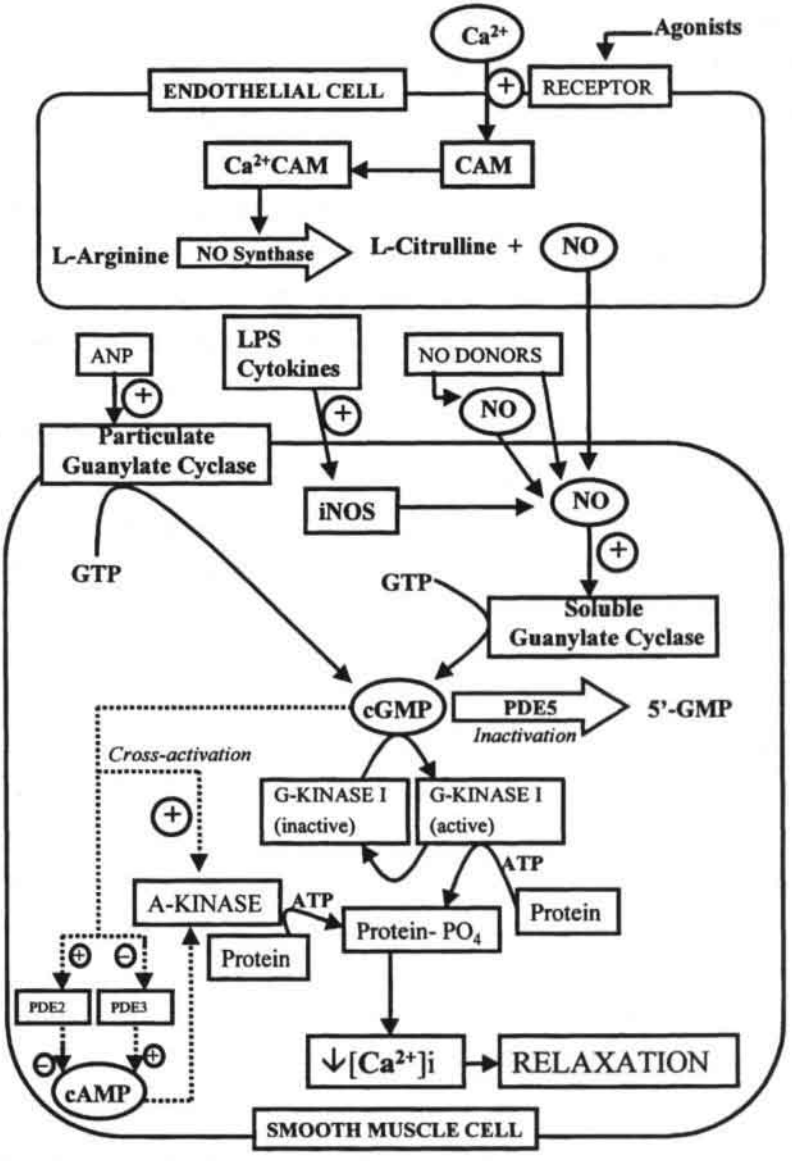

Figure 3. Mechanisms of action of NO

inositol 1,4,5-triphosphate (IP_3) and diacylglycerol is a well-established mechanism triggering vascular smooth muscle contraction (Hobbs & Ignarro, 1997). Modification of this pathway and prevention of Ca^{2+} release from the endoplasmic reticulum appears yet another method by which cGMP can regulate Ca^{2+} homeostasis (Hobbs & Ignarro, 1997).

Physiological responses to both cGMP and cAMP are governed, at least in part, by cyclic nucleotide phosphodiesterases (PDEs) that specifically hydrolyze them to biologically inert 5'-nucleotides, and thereby turn off the signal they are conveying (Beavo 1995, Polson & Strada, 1996). Seven PDE families have been described based on different kinetic properties, cyclic nucleotide preferences, regulatory mechanisms, and sensitivities to pharmacological inhibitors (Beavo, 1995; Polson & Strada, 1996). Recently, it was determined that within each major gene family, there were multiple PDE isoforms and splice variants, each exhibiting different characteristics and tissue distribution (Beavo, 1995; Polson & Strada, 1996). PDE1 (calcium/calmodulin-dependent PDE), PDE2 (cGMP-stimulated PDE), PDE3 (cGMP-inhibited PDE), PDE4 (cAMP-specific PDE), and PDE5 (cGMP-specific PDE) were identified in vascular smooth muscle (Lugnier et al, 1986; Miyahara et al, 1995; Saeki & Saito, 1993). PDE5 has a strong preference for cGMP as substrate, a high affinity noncatalityc binding site for cGMP, and accounts for the majority of cGMP hydrolysis in vascular smooth muscle. Therefore, PDE5 plays a major role in vasoregulation by lowering cGMP levels in VSM. Dipyridamole, zaprinast, and sildenafil are potent inhibitors of PDE5 (Rosman et al., 1997; Ziegler et al., 1995b; Hanson et al., 1998). On the other hand, PDE2 and PDE3 are both cAMP-selective, but they have significant relevance to the NO/cGMP transduction system since the rate of cAMP hydrolysis by PDE_2 and PDE_3 is stimulated and inhibited, respectively, by cGMP (Lindgren et al., 1991; Hobbs & Ignarro, 1997). As such, these PDEs represent a mechanism by which cGMP can mediate part of its effects (Fig 3).

Independently of its activation of sGC, exposure to NO inhibits the activity of a number of enzymes, such as aconitase and complexes I, II and IV (Nathan & Hibbs, 1991; Clementi et al., 1998). In addition, DNA synthesis can be impaired by the inhibitory action of NO on ribonucleotide reductase. Such actions render NO cytotoxic or cytostatic for invading

microorganisms and sometimes for the NO-generating cells. These actions explain, at least in part, the pathophysiological actions of NO. At this point, it is not clear to what extent these actions are due to NO itself or result from the combination of NO and other molecules, predominantly superoxide. Indeed the interaction between NO and superoxide leads to the generation of peroxynitrite (ONOO-), which is a powerful oxidant (Beckman et al., 1990; Rodi et al., 1991). Peroxynitrite induces toxicity through nitrosation and/or nitration of amino acids such as tyrosine and cysteine on various proteins. Such modifications alter protein function and consequently disrupt cellular activity. The measurement of 3-nitrotyrosine formation is being widely used as an indicator of ONOO- generation in tissues (Hibbs & Moncada, 1999).

NO and the perinatal lung

The NO/cGMP pathway has been identified as playing a critical role in the control of pulmonary fetal vascular tone and during the normal postnatal circulatory transition (Abman et al., 1990; 1991; Cornfield et al., 1992; Shaul et al., 1992; Tiktinsky & Morin, 1993; McQueston et al., 1993). In fact, despite its high PVR, the normal fetal pulmonary circulation continuously releases NO under basal conditions, as demonstrated by the hypertensive effects of intrapulmonary infusions of NOS blockers (Abman et al., 1990; Moore et al., 1992). This was originally revealed in studies using nonspecific NOS antagonists. In more recent experiments the unique contributions of iNOS and nNOS to vascular regulation was addressed. In fetal lambs, the intrapulmonary infusion of three selective iNOS inhibitors caused 69-82% increases in pulmonary vascular resistance (Rairigh et al., 1998). Similarly, it has recently been reported that the infusion of a nNOS-specific inhibitor increased pulmonary vascular resistance in the fetal lamb (Rairigh et al., 2000). These observations suggest that both iNOS and nNOS contribute to the modulation of vascular tone in the developing fetal lung. Therefore, the three isoforms of NOS are present in the developing lung and seem to play a role in the control of fetal pulmonary circulation. eNOS is predominantly present in vascular endothelial cells early in gestation in sheep, rat, and human lung, and appears to be developmentally regulated (Abman et al., 1991; Halbower et al., 1994; North et al., 1994; Parker et al., 2000). Estrogen upregulated eNOS gene expression in fetal pulmonary artery

endothelial cells through the activation of estrogen receptor (MacRitchie et al., 1997). nNOS has been identified in lung neuronal, epithelial, and endothelial cells, and its expression increases with advancing fetal age in the rat (North et al., 1994; Xue et al., 1996; Kobzik et al., 1993). iNOS has been identified in the endothelium, epithelium, and macrophages in human and fetal rat lung by immunostaining (Xue et al., 1996; Kobzik et al., 1993).

The NO/cGMP cascade plays several important physiological roles in regulation of the fetal pulmonary circulation. These include: 1) the above-mentioned modulation of basal PVR in the fetus; 2) mediation of the vasodilator response to specific physiological and pharmacological stimuli; and 3) Reduction of the strong myogenic tone in the normal fetal lung (Abman, 1999). Thus, NOS inhibition selectively blocks pulmonary vasodilation to stimuli such as acetylcholine, oxygen, and shear stress in the normal fetus (McQueston et al., 1993). However, in comparison with newborn and adult pulmonary arterial rings, rings from fetal lambs showed little relaxation to endothelium-dependent agonists, including acetylcholine, adenosine diphosphate and the calcium ionophore A23187 (Abman et al., 1991). No age-differences were observed in the response to the NO-donor sodium nitroprusside (Abman et al., 1991), suggesting that fetal pulmonary arteries display a limited capacity to release NO but are fully capable to respond to it. Additional studies have demonstrated that the response of the ovine fetal lung to acethylcoline and oxygen are absent or decreased prior to 78% of gestation, and increases toward term (Morin et al., 1988a; Lewis et al., 1976). In contrast, inhaled NO is a potent fetal pulmonary vasodilator as early as 0.75 gestation (Kinsella et al., 1994a). Thus, it appears that the ability of developing pulmonary vascular smooth muscle to respond to exogenous NO precedes maturation of the endothelium's ability to release NO. More recent studies suggested that NO release plays a role in modulating high intrinsic or myogenic tone in the fetal pulmonary circulation. The vascular myogenic response refers to the acute reaction of a blood vessel to a change in transmural pressure (Davis & Hill, 1999). This response is critically important for the development of resting vascular tone, upon which other control mechanisms exert vasodilator and vasoconstrictor influences. *In vitro* studies demonstrated the presence of a myogenic response in sheep pulmonary arteries (Belik et al., 1995) and greater myogenic activity in fetal pulmonary arteries than neonatal or adult arteries (Belik et al., 1994). More

recent studies of intact fetal lambs have demonstrated that high myogenic tone operates normally in the fetus and contributes to maintaining high PVR in utero (Storme et al., 1999). NOS inhibition further unmasks this potent myogenic response (Storme et al., 1999).

In late gestation lambs, the immediate and dramatic fall in PVR produced by cesarean section and ventilation with 100% oxygen is markedly attenuated if delivery and ventilation take place following the infusion of NOS inhibitors (Abman et al., 1990). Studies have also been performed to define which birth-related stimuli stimulate NO at birth. Vasodilation during hypoxic ventilation was attenuated by NOS inhibition, as was the fall in pulmonary artery pressure during administration of 100% oxygen (Cornfield et al., 1992; Tiktinsky et al., 1993). Similarly, NOS inhibition blocked the marked decrease in PVR during acute mechanical increases in flow or shear stress related to brief compression of the ductus arteriosus (Cornfield et al., 1992). These finding suggest that ventilation, increased oxygenation, and shear stress are independently capable of stimulating NOS activity at birth. Moreover, rhythmic distension of the lung and increased oxygenation, both concomitantly and independently, produced induction of eNOS gene expression in lung vessels (Black et al., 1997).

NO and PPHN

Altered NOS activity during pulmonary or systemic hypertension has been reported in several vascular beds. In spite of the unique responses of the developing lung, many lessons from the alterations of vascular reactivity and structure in chronic pulmonary hypertension carry over into the perinatal lung. Loss of EDNO-dependent vasodilation has been demonstrated in isolated lungs from chronically hypoxic rats (Adnot et al., 1991) and in human conduit pulmonary arteries obtained at lung transplant from patients with chronic hypoxic pulmonary hypertension (Dinh Xuan et al., 1991). In contrast, other authors demonstrate that EDNO is upregulated in experimental chronic hypoxic pulmonary hypertension, as a possible compensatory mechanism (Hampl et al., 1993; Isaacson et al., 1994). Steudel et al. (1997) investigated the pulmonary vascular phenotype of mice with targeted disruption of the eNOS gene, finding increased pulmonary vascular resistance, but only minimal hypoxic pulmonary

hypertension, and no evidence of pulmonary vascular remodeling. Yet, this group later found enhanced chronic hypoxic pulmonary hypertension in eNOS-null mice (Steudel et al., 1998). Using the same model, Fagan et al (1999) demonstrated that in the pulmonary circulation, the loss of endothelium-derived NO led to reversible vasoconstriction, with evidence of distal extension of pulmonary artery. However, information from knockout models should be cautiously interpreted, in view of possible upregulation of compensatory pathways (Mashimo & Goyal, 1999; Steudel et al., 2000). Whether eNOS is ever deficient in the pulmonary circulation of adult patients with pulmonary hypertension remains controversial. Giaid and Saleh (1995), using histochemical and immunohistochemical assays, in situ hybridization, and Northen blot analyses, demonstrated that patients with primary or secondary pulmonary hypertension have reduced eNOS mRNA and immunoreactivity. Furthermore, they showed a significant inverse correlation between the expression of eNOS and the severity of vascular morphological changes, and the increase in PVR.

Intrauterine events appear to be related to the development of PPHN (Abman & Stevens, 1997). Exposure to pulmonary hypertension or hypoxia *in utero* may initially alter vasoreactivity prior to the development of hypertensive vascular remodeling (Allen et al., 1986; Abman et al., 1989; Abman & Stevens, 1997). Thus, the duration and severity of exposure to a prenatal adverse stimulus may determine the initial severity of pulmonary vascular disease in the early postnatal period. The possible effect of hypoxia is extensively commented upon another part of this introduction (see II.4). The following information is focused on the role of chronic intrauterine pulmonary hypertension as pathogenic factor for the development of PPHN. Levin et al. (1978), demonstrated that intrauterine artery ligation of the ovine fetus caused systemic hypertension, which in the setting of a patent ductus arteriosus, transmitted high pressure directly to the pulmonary circulation and produced increased wall thickness of small pulmonary arteries. Pharmacological closure of the ductus arteriosus by indomethacin produced similar effects in fetal lambs (Levin et al., 1979). Based on these facts, Abman et al. (1989), characterized an animal model of perinatal pulmonary hypertension due to partial compression of the ductus. They observed that ductus compression, through the placement of an inflatable vascular occluder, elevated pulmonary artery pressure without altering PO_2. The pulmonary blood flow was only

transiently increased (Abman & Accurso, 1989) but fetal PVR progressively increased over several days, resulting in a situation of high pressure without high flow. After cesarean-section delivery, PVR remained markedly elevated despite ventilation with 100% O_2, leading to right-to-left shunting across the ductus arteriosus and foramen ovale and marked hypoxia. Additionally, they described, at autopsy, striking thickening of small pulmonary arteries and right ventricular hypertrophy (Abman et al., 1989). Concomitant studies from Morin (1989) and Wild et al (1989) also demonstrated that ligation of the ductus led to similar structural changes and sustained elevation of PVR. Therefore, the model of chronic compression or ligation of the ductus arteriosus mimics the hemodynamic and pathological features of PPHN and has become an useful tool for the study of the pathophysiology and the treatment of this clinical entity (Abman & Stevens 1997; Steinhorn et al., 1997a; Abman, 1999).

Alterations in every step of the NO/cGMP cascade have been described in the experimental model of PPHN produced by chronic intrauterine ductus compression (Fig 4). Initially, it was observed that there was an impaired response to pharmacological and physiological stimuli acting through the activation of eNOS such as acetylcholine (McQueston et al., 1995), the calcium ionophore A23187 (Steinhorn et al., 1995a), oxygen (Abman et al., 1989, Zayek et al., 1995) and shear stress (Abman et al., 1989), but relative sparing of responses to endothelium -independent agonists, like atrial natriuretic peptide (McQueston et al., 1995) and inhaled NO (Zayek et al., 1995). These findings led to the hypothesis of an impairment of eNOS, and later research demonstrated a decrease in eNOS enzymatic activity, protein content, and mRNA (Shaul et al., 1997; present thesis). Additionally, a dysregulation of the normal content of the α_1, α_2 and β_1 subunits of soluble guanylate cyclase (Tzao et al., 1998; 1999) as well as an increase in PDE5 activity (Hanson et al., 1998) have been described in this experimental model. Therefore, after chronic intrauterine pulmonary hypertension there is a reduced production of NO in response to pharmacological or physiological stimuli, due to a reduction in eNOS. This NO is acting over an impaired sGC and, as a consequence of that, less cGMP is produced. This cGMP is more rapidly degraded to inactive 5'GMP due to an increased PDE5 activity (Figure 4). It is difficult, however, to determine whether the impairment of the NO/cGMP pathway is the cause or the consequence of the pulmonary hypertension. If deficient basal EDNO synthesis or activity

was a cause of pulmonary hypertension in DA-compressed animals, then chronic pharmacological depletion of EDNO should reproduce the hemodynamic effects of DA compression. Infusion of low doses of a NOS blocker for 10 days before delivery does not increase basal PVR, but it markedly blunts its decrease and the increase in flow at birth (Fineman et al., 1994). This effect is not different from the one of acute infusion of NOS blockers (Abman et al., 1990) and suggests that impairment in NOS activity could not be responsible for the increase in PVR during chronic intrauterine pulmonary hypertension, but could play a determinant role in the failure in postnatal adaptation and the lack of response to the vasodilatory birth-related stimuli observed in these animals.

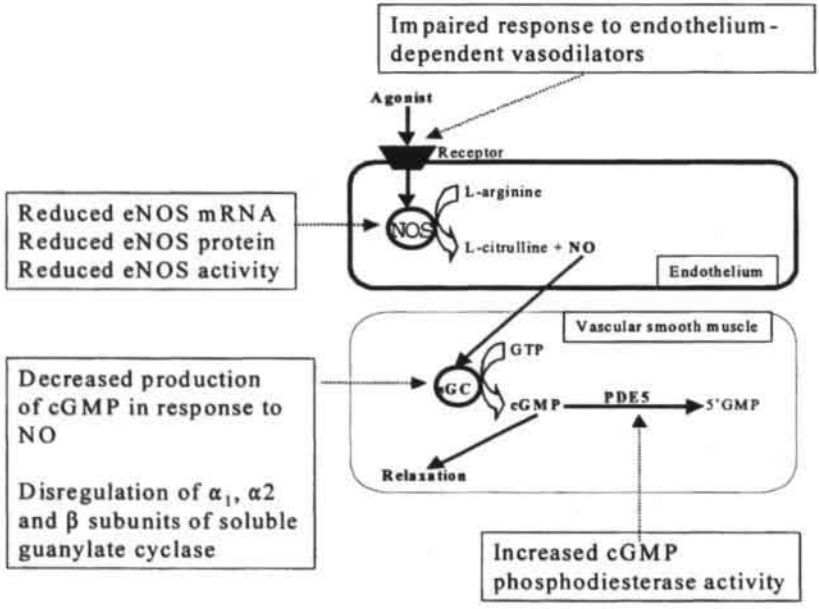

Figure 5. Alterations of the NO/cGMP pathway described in the experimental model of PPHN produced by chronic intrauterine ductus compression.

Direct evidences of an impairment of the NO/cGMP pathway in patients with PPHN are difficult to obtain. However, Dollberg et al. (1995) have shown that the urinary metabolites of NO, i.e. nitrite and nitrate, were reduced in PPHN patients and Christou et al. (1997) that the plasma concentrations of cGMP were significantly lower compared to healthy newborns. Castillo et al. (1995), demonstrated a reduced nitrate urinary excretion and plasma arginine utilization during the acute vasoconstrictive state of PPHN compared to the convalescence period. Evidence based on measurement of urinary metabolites of NO is often confounded by variations in dietary intake of nitrate (Wang et al., 1997), but the newborns included in the above mentioned studies received a homogeneous diet. Finally, umbilical vein endothelial cells cultured from infants with PPHN showed a decrease in eNOS gene expression (Villanueva et al., 1998). However, this occurred only in newborns who suffered intrapartum stress and not in PPHN without a history of perinatal asphyxia.

II.2 Endothelin-1

Endothelin-1 (ET-1), a 21-amino acid peptide, was identified in 1988, as a potent vasoconstrictor and pressor substance, in the supernatant of cultured porcine aortic endothelial cells (Yanagisawa et al., 1988). ET-1 is present in many mammalian species, including humans. Two additional human ET isopeptides, ET-2 and ET-3 (Inoue et al., 1989) are encoded by separate genes. These isoforms of ET show a high degree of amino acid sequence identity. ET-1 is initially synthesized as a prepropeptide. Human preproET-1 is a 212-amino acid protein proteolytically cleaved to form a 38-amino acid residue intermediate peptide, termed big ET-1 (Kido et al., 1997). Big ET-1 is subsequently cleaved to ET-1 by another endopeptidase, termed ET converting enzyme (ECE), which appears to be specific for ET (Yanagisawa et al., 1988). The conversion of big ET-1 to ET-1 is essential for biological activity, because the pressor action of big ET-1 is almost completely abolished by inhibition of ECE (Matsumura et al., 1990; Gardiner et al., 1991). ECE expressed on endothelial cells may differ to ECE expressed on vascular smooth muscle cells. Whilst endothelial ECE has been found to have specificity for big ET-1 (Plumptom et al., 1996; Davenport et al., 1998), smooth muscle ECE is capable of processing big ET-2 and big ET-3 (Tsukahara et al., 1993; Maguire et al., 1997; Davenport et al., 1998). The physiological role of this and other novel putative converting enzymes on regulation of ET remains to be determined (Russell & Davenport, 1999).

Although vascular endothelial cells are the major source of ET-1, the genes that encode the three ET isopeptides are expressed in a wide variety of cell types, including cardiac myocytes, vascular smooth muscle, renal tubular epithelium, glomerular mesangium, glia, the pituitary, macrophages, mast cells, etc, which suggests that the peptides may participate in complex regulatory mechanisms in various organs (Inoue et al., 1989; Sakurai et al., 1991). In blood vessels, ETs act by at least three different receptor subtypes, the ETA receptor, which is localized on vascular smooth muscle cells and mediates vasoconstriction, and two different ETB receptor subtypes. The ETB1 receptor subtype is present in vascular endothelial cells and mediates endothelium-dependent vasodilation. The ETB2 receptor subtype is present on smooth muscle cells causing vasoconstriction (Zimmermann & Seifert, 1998). ET-1 and -2 are

more potent agonists of the ETA receptor than ET-3. For the ETB receptors the three isopeptides have almost equal potency (Miyauchi & Masaki, 1999)

Despite numerous studies of ET function, the physiological significance of ET in the cardiovascular system is unclear (Miyauchi & Masaki, 1999). Because the plasma concentration is very low, ET is not a circulating hormone, but it may be a paracrine/autocrine mediator. The ET-1 that is released from vascular beds presumably acts on the underlying smooth muscle to increase peripheral vascular resistance (Haynes et al., 1996). *In vivo* and *in vitro* studies have demonstrated that infusions of ET-1 cause progressive and sustained vasoconstriction in most vascular beds, often following a transient dilator response (Miyauchi & Masaki, 1999). The initial depressor response is due to a relaxing factor released from endothelium (NO or PGI_2) and the subsequent vasoconstriction is due to the direct action of ET-1 on smooth muscle. In isolated cardiac muscle, ET-1 exerts a potent positive inotropic action. ET-1 is also reported to induce a positive chronotropic action via ETB receptors and a negative chronotropic action via ETA receptors. In addition to controlling vascular tone and contraction of myocytes, ET-1 also participates in the modulation of growth properties of the underlying vascular smooth muscle and the hypertrophy of cardiac myocyte (Miyauchi & Masaki, 1999).

Expression of ET-1 mRNA is increased after treatment of endothelial cells with growth factors and cytokines such as thrombin (Emori et al., 1992), transforming growth factor ß (Kurihara et al., 1989), tumor necrosis factor (Marsden et al., 1992), immunoglobulin 1 (Maemura et al., 1992), and insulin, or with vasoactive substances (Marsden et al., 1991; Imai et al., 1992) such as norepinephrine, angiotensin II, vasopressin, and bradykinin. Furthermore, high shear stress decreases ET-1 mRNA expression, whereas low shear stress increases it (Shaefki et al., 1991; Malek et al., 1992; Yosizumi et al., 1989). In contrast, the expression of ET-1 mRNA is inhibited by endothelium-derived NO, PGI_2, and ANP, presumably via cGMP-mediated inhibition of phosphatidylinositide metabolism (Emori et al., 1993).

Endothelin-1 and the perinatal lung

The physiological role of ET-1 in the normal fetal lung is controversial. ET-1 is present in the perinatal lung (MacCumber et al., 1989), and is vasoactive in the fetus (Ivy et al., 1994, 1996; Chatfield et al., 1991; Jones & Abman, 1994; Tod & Cassin, 1992). Brief infusion of ET-1 causes acute pulmonary vasodilation (Lippton et al., 1991; Cassin et al., 1991). However, with prolonged infusion, pulmonary hypertension prevails (Chatfield et al., 1991; Liben et al., 1993). Some studies of exogenous infusion of ET-1 have emphasized that the major effect of ET-1 in the normal late gestation fetal lung is vasodilation, and that the majority of ET-1 receptors active in the ovine fetal lung are the ETB1 receptors (Cassin et al., 1991; Wong et al., 1993) which mediate only vasodilation (Ivy et al., 1994). In contrast, several studies suggest that the ETA receptors play an important role in mediating vasoconstriction in the late gestation ovine fetus (Wang & Coceani, 1992; Ivy et al., 1994, 1996). Infusion of exogenous ET-1, on the other hand, may not accurately describe the hemodynamic effects of endogenous production of ET-1 in the fetal lung (Ivy et al., 1997). As mentioned above, evidence suggests that ET-1 acts as a local autocrine and paracrine factor rather than a circulating hormone, since secretion of ET-1 by endothelial cells is polar and directed abluminally toward the interstitial region (Wagner et al., 1992). Intrapulmonary infusion of big-ET-1, the precursor to ET-1, causes progressive and sustained pulmonary hypertension without even transient vasodilation (Ivy et al., 1994; Jones & Abman, 1994), suggesting that stimulation of endogenous ET-1 may have very different effects than brief exogenous infusions of ET-1. Blockade of the ETA receptors causes vasodilation (Ivy et al., 1994; Wong et al., 1994), whereas selective blockade of the ETB receptors does not change basal pulmonary tone in the ovine fetus (Ivy et al., 1996a). Brief and prolonged stimulation of the ETB receptors with sarafotoxin S6c, however, causes only vasodilation in the ovine fetal lung, suggesting the presence of only ETB1 receptors (Ivy et al., 1994). In contrast, studies in newborn piglets suggest the presence of both ETB1 and ETB2 receptors in the neonatal lung (Perreault & Baribeau, 1995). Therefore, it appears that the primary role of ET-1 in the normal late-gestation fetal lung is vasoconstriction. On the other hand it has been shown that combined ETA and ETB receptor blockade with Ro 47-0203 did not change the decrease in

PVR with *in utero* oxygen ventilation, suggesting that endogenous ET-1 activity does not play a major role in the pulmonary vascular changes during the normal transitional circulation at birth (Winters et al., 1996).

Endothelin-1 and PPHN

Circulating ET-1 levels in patients with PPHN are markedly elevated, correlate with disease severity, and decline with resolution of PPHN (Rosenberg et al., 1993). In general, ET-1 levels are increased in several forms of pulmonary hypertension including primary pulmonary hypertension (Cacoub et al, 1993), the Eisenmenger syndrome (Cacoub et al., 1993), and children with pulmonary hypertension associated with congenital heart disease (Vincent et al., 1993) and bronchopulmonary dysplasia (Allen et al., 1993). In adult patients with primary pulmonary hypertension, increased expression of ET-1-like immunoreactivity and ET-1 mRNA in vascular endothelial cells have been reported suggesting that the local production of ET-1 may contribute to the altered vascular reactivity and structural changes seen in this clinical entity (Giaid et al., 1993).

High ET-1 concentrations, ET-1 mRNA expression, and ET receptor mRNA expression have been described in adult animal models of pulmonary hypertension, such as the monocrotaline (Miyauchi et al., 1993; Yorikane et al., 1993) or the chronic hypoxia models (Li et al., 1994). In the experimental model of PPHN due to chronic compression of the ductus, Ivy et al. (1996b) demonstrated a marked increase in ET-1 levels with a progressive loss of the vasodilator activity of ET-1, mediated through ETB receptors, but a persistence of the vasoconstrictor activity, mediated through ETA receptors. Moreover, they observed, in the same model, increased steady-state preproET-1 mRNA and decreased ETB-receptor mRNA without changes in ECE-1 mRNA or ETA-receptor mRNA expression, suggesting that the combination of increased ET-1 production and decreased ETB-receptor-induced vasodilation may contribute to increased vasoconstrictor tone (Ivy et al., 1998a). On the other hand, chronic intrauterine blockade of the ETA receptor with BQ 123 decreased distal muscularization of small pulmonary arteries, and right ventricular hypertrophy, and partially attenuated the failure in

circulatory transition observed at delivery in lambs subjected to chronic intrauterine ductus compression. Selective ETA receptor blockade acutely lowered PVR, in this model, after ventilation with 100% O_2 and inhaled NO, demonstrating that ETA-mediated vasoconstriction is responsible for maintaining a residual high tone, that is not reversed by potent vasodilatory stimuli (Ivy et al., 1997).

Giaid & Saleh (1995), showed, in adult patients with pulmonary hypertension, a significant inverse correlation between the decreased expression of NOS and the increased expression of ET-1, leading to the hypothesis that down-regulation of the endothelium-derived relaxing and antiproliferative factors (e.g. NO) and up-regulation of the endothelium-derived vasoconstrictor and mitogenic factors (e.g. ET-1) contribute to chronic pulmonary hypertension. Interestingly, a similar coordinated reduction of genes of the NO pathway and up-regulation of genes of the ET-1 pathway has been described in the ovine model of PPHN by ligation of the ductus (Black et al., 1998). In addition, concomitantly increased ET-1 and decreased cGMP plasma concentrations have been found in patients with PPHN (Christou et al., 1997). Interestingly, treatment with inhaled NO decreased ET-1 levels in these patients.

Finally, the ET system is highly activated during septic shock and ET-1 together with TXA_2 (see chapter III) seems to play a role in sepsis-induced pulmonary hypertension in adult animal models (Weitzberg et al., 1996; Wanecek et al., 1997). The plasma concentrations of ET-1 and the expression of ET-1 mRNA in pulmonary artery and aorta were significantly increased in rats after LPS injection. In isolated pulmonary arteries from septic rats, ET-1 induced vasoconstriction, mediated through ETA receptors, was maintained, but ET-1-induced vasodilatation, mediated through ETB receptors was impaired (Curzen et al., 1996). Very recently, Fujii et al. (2000) demonstrated that activation of ETA receptors, by rising ET-1 concentration, enhanced NO production and iNOS expression in pulmonary and systemic vessels of septic rats, suggesting an interaction between the NO and the ET-1 pathways in both sepsis-induced systemic hypotension and lung injury. In fact, in patients with septic shock the response to NOS inhibitors depends on the plasma level of ET-1, suggesting that NOS induction may mask a tonic pressor response of ET-1 in septic shock (Avontuur et al., 1999).

II.3 Eicosanoids

Eicosanoids are arachidonic acid metabolites that are generated by several cellular types including vascular endothelial cells (Coceani & Olley, 1988). Arachidonic acid may be metabolized via two main pathways. Cyclooxygenase (COX), also referred as prostaglandin endoperoxide H synthase (PGHS), leads to the formation of prostaglandins (PGs) and thromboxanes (TXs). The lipoxygenase pathway produces hydroxyeicosatetranoic acids and leukotrienes. A third pathway, involving a cytochrome P450-linked monooxygenase, may generate several epoxides, hydroxy acids and other products whose physiological importance remains to be clarified (Murphy, 1989; Smith & DeWitt 1996). Also of interest are the isoprostanes, i.e. PG-like compounds that are produced independent of the COX pathway by a non-enzymatic free-radical-induced peroxidation of arachidonic acid (Roberts & Morrow, 1997; Morrow et al., 1999). The isoprostanes can provide an exclusive and selective index for the oxidant component of several inflammatory and degenerative diseases (Roberts & Morrow, 1997). Moreover, because isoprostanes are isomeric to COX derived PG, which exert potent biological activity, they may not be simply markers of lipid peroxidation but also posses a specific activity (Roberts & Morrow, 1997). In fact 8-iso-$PGF_{2\alpha}$, one of the more abundant isoprostanes that is produced *in vivo* (Morrow et al., 1994), has been found to be a potent pulmonary, renal, and coronary vasoconstrictor (Kang et al., 1993; Roberts & Morrow, 1997; John & Valentin, 1997; Wilson et al., 1999).

PGs and TXs are synthesized from arachidonic acid by COX-1 and COX-2. Both enzymes catalyze the formation of PGH_2 from arachidonic acid, and both are inhibited by non-steroidal anti-inflammatory drugs such as aspirin. COX-1 is generally constitutively active, whereas COX-2 is induced by growth factors, cytokines, and tumor promoters (Smith & DeWitt 1996). Both COX-1 and -2 have been localized immunocytochemically to the lumen of the endoplasmic reticulum and the outer nuclear envelope (Morita et al., 1995), which implies that the site of PG synthesis is intracellular. PGs and TXs play fundamental roles in health and disease and, increasingly, as therapeutic agents. Examples of these broad roles include gastric protection and peptic ulcer formation; pregnancy, labor, delivery, abortion, luteolysis, and

menstruation; glaucoma; blood pressure control and vascular tone; intestinal fluid secretion; liver protection and damage; airway resistance and asthma; fever; and modulation of inflammatory cells (Schuster, 1999). Additionally, eicosanoids have been implicated in the regulation of pulmonary vascular tone under physiological and pathological conditions (Barnes & Liu, 1995).

Eicosanoids and the perinatal lung

It appears that the COX pathway is the predominant for arachidonic acid metabolism in the fetal and transitional circulation, with prostacyclin (PGI_2), the most influential prostanoid mediator formed (Coceani & Olley, 1988; Ziegler et al., 1995a). Several pioneering studies revealed the critical importance of PGI_2 in the developing pulmonary circulation. It has been shown that PGI_2 is produced by fetal pulmonary blood vessels *in vitro* (Terragno & Terragno, 1979), and that it causes very potent vasodilation when infused into the fetal lung *in vivo* (Cassin et al., 1981). However, *in vivo* COX inhibition has minimal effect on basal PVR and does not increase myogenic tone in the fetal lamb (Abman, 1999). Throughout gestation, a maturational increase in PGI_2 parallels the decrease in PVR in the fetal third trimester (Shaul et al., 1993a). The developmental upregulation of PGI_2 synthesis is related to a maturational increase in COX-1 gene expression (Shaul et al., 1999). Physiologic concentrations of estrogen cause direct upregulation of COX-1 gene expression in fetal pulmonary artery endothelial cells, and this is mediated by estrogen receptor activation (Brannon et al., 1998). Moreover, PGI_2 synthesis in fetal intrapulmonary arteries is acutely modulated by changes in O_2, with decreasing O_2 causing attenuated synthesis and increasing O_2 causing enhanced synthesis (Shaul et al., 1992). This process is due to an effect on COX activity, it causes marked parallel changes in the production of the second messenger cAMP, and it is specific to the pulmonary circulation (Shaul et al., 1992; 1999). In contrast, in the newborn it has been shown that lung PGI_2 production is stimulated by hypoxia and that inhibition of PGI_2 production augments hypoxic pulmonary vasoconstriction (Green & Leffler, 1984; Tyler et al., 1975).

It has been suggested that PGI_2 plays a major role in the transition of the pulmonary

circulation at birth since inhibition of PG synthesis attenuates the birth-related decline in pulmonary vascular resistance. (Davidson, 1988; Leffler, 1978; Velvis, 1991). In the late gestation fetus, PGI_2 production increases acutely with ventilation and acute oxygenation (Leffler et al., 1980; 1984a; 1984b). However, Velvis et al. (1991) demonstrated that COX inhibition abolished the drop in PVR associated with rhythmic distension of the lungs (with a hypoxic gas), but had no effect on PVR during static distension or during ventilation with oxygenation. Therefore, under normal circumstances (i.e. ventilation with oxygenation), PGI_2 production is not essential for a smooth postnatal transition (Velvis et al., 1991; Ziegler et al., 1995a).

Prostaglandins of the D and E series manifest vasodilatory activity when infused into the fetal lung (Coceani & Olley, 1988). Prostaglandins of the F series induce vasoconstriction in the fetal pulmonary vasculature (Coceani & Olley, 1988), but these PGs probably are not important, given the predominantly vasoconstrictive effect of COX inhibition in the fetus at birth (Ziegler et al., 1995a). Moreover, the effects of exogenous and endogenous $PGF_{2\alpha}$ on perinatal circulation are difficult to assess because the circulatory responses involve a complex interaction of uterine, umbilical, and ductal blood flow, and fetoplacental metabolism of $PGF_{2\alpha}$ (Leffler et al., 1979).

Lipoxygenase activity has been demonstrated in human fetal lungs as early as 12 to 18 weeks of gestation (Saeed & Mitchell 1982). Leukotriene inhibition decreases PVR in fetal lambs (Soifer et al., 1985; Heymann et al., 1988; Lebiodois et al., 1987). It was further suggested that reduced leukotriene production, at birth, contributes to the drop in PVR during postnatal transition (Ziegler et al., 1995a). However, the nonspecificity of the leukotriene antagonists used in these studies made speculations regarding leukotriene importance in the fetal circulation questionable (Ziegler et al., 1995a). In fact, it has been found that leukotriene content in fetal lamb lung tissues and fetal lamb tracheal fluid was very low (Cassin et al.,1990; Abman & Stenmark, 1992), and that no significant changes in lung leukotrienes levels during the transition in normal or hypertensive ovine fetuses were produced (Abman & Stenmark, 1992). Therefore, the specific role of leukotrienes in maintaining high PVR in the

fetus and in the transitional circulation is still unclear.

Eicosanoids and PPHN

There is evidence from experimental animal studies and human patients that alterations in eicosanoid homeostasis may be involved in the pathogenesis of PPHN (Stenmark et al., 1983; Shaul, 1999). It has been demonstrated that PGI_2 synthesis is markedly reduced in intrapulmonary arteries isolated from newborn calves with pulmonary hypertension (Badesch et al., 1989). However, increased levels of 6-keto-$PGF_{1\alpha}$ and TXB_2 and, stable metabolites of PGI_2 and TXA_2 respectively, have been observed in an experimental model of meconium aspiration-induced pulmonary hypertension (Soukka et al., 1998). Moreover, the levels of TXB_2 were significantly correlated with pulmonary artery pressures in individual infants with a primary diagnosis of meconium aspiration (Bui et al., 1992). Concentrations of TXB_2, 6-keto-$PGF_{1\alpha}$, PGD_2, PGE_2, LTB_4, and LTE_4 were elevated in infants with PPHN that required ECMO treatment. Eicosanoid concentrations decreased in all infants with a good clinical outcome after ECMO, but they remained elevated in those with a poor outcome (Dobyns et al., 1994).

PPHN has been reported in newborns of mothers who received COX inhibitors, such as indomethacin or aspirin, during pregnancy (Csaba et al., 1978; Levin et al., 1978b; Van Marter et al., 1996). However, this effect seems to be more related to the premature closure of the ductus arteriosus, and consequently with the presence of intrauterine pulmonary hypertension, than to a direct effect of COX inhibition in the pulmonary vessels (Steinhorn et al., 1995b; Abman & Stevens, 1997). Nevertheless, PGI_2 is an important modulator of vascular cell growth in the pulmonary circulation (Huttner et al., 1977) and chronic inhibition of its synthesis induces pulmonary hypertension and alterations of lung vessel morphology in adult sheep (Meyrick et al., 1985).

Finally, there is a great body of evidence about the relevant role of TXA_2 in sepsis-induced PPHN. This aspect, together with other vasoactive mediators involved in sepsis-induced altered pulmonary vasoreactivity, is extensively discussed in chapter III.

II.4 Carbon monoxide

Over the last years, there is increasing interest in the potential role of CO as a regulator of vascular tone. CO has been traditionally considered as a toxic pollutant that poisons by binding to the iron containing heme group found in hemoglobin and some enzymes, but evidence is accumulating that CO can be an endogenous regulator of various physiologic processes (Marks et al., 1991). CO appears to mimic many of the function of NO, including smooth muscle relaxation and inhibition of platelet aggregation (Brunne & Ullrich 1987), which are mainly mediated through the activation of sGC (Kharitonov et al., 1995).

CO is produced endogenously by two sources, i.e. enzymatic peroxidation of microsomal lipids and heme destruction catalyzed by heme oxygenase (HO) (Marks et al., 1991; Maines, 1997). HO is a ubiquitous protein that exists in two isoforms: HO-1, an inducible form, and HO-2, a constitutive form (Maines, 1997). HO-2 is the predominant isoform present in endothelial cells, smooth muscle cells, connective tissue, and epithelial cells (Zakhary et al., 1996). HO-1 is also present in endothelial cells (Coceani et al., 1997) and is upregulated by many stimuli including shear stress, hypoxia, hyperoxia, metals, and heat shock (Cantoni et al., 1991; Okinaga et al., 1996). In addition, putative inhibitors of HO, including zinc protoporphyrin IX and tin protoporphyrin IX, are capable of producing a pressor effect in vivo, causing a rise in systemic blood pressure and peripheral vascular resistance (Johnson et al., 1995; Caudill et al., 1998)

The ability of exogenous CO to induce vasorelaxation has long been known (Duke & Killick, 1952). CO-induced vasorelaxation has been described in many vascular beds from several species (Furchgott & Jothianandan; 1991; Brian et al., 1994; Wang et al., 1997). However it is not a universal finding and the sensitivity of the different vessels to CO is variable. A vasoregulatory role for endogenous CO produced by constitutive HO-2 has been postulated in the maintenance of sinusoidal tone in the perfused rat liver (Suematsu et al., 1995), and the vascular tone of porcine distal pulmonary arteries (Zakhary et al., 1996). Alternatively, endogenously released CO, as a consequence of HO-1 induction, participated in the regulation of vascular contractility in rat aorta (Sammut et al., 1998) and fetal lamb ductus

arteriosus (Coceani et al., 1997). An interaction between CO and nitric oxide may also significantly contribute to the fine-tuning of vascular tone (Maines, 1997; Wang, 1998). Recently, Grover et al (2000) studied the effects of inhaled CO on late-gestation fetal lambs. Inhaled CO did not alter pulmonary vascular resistance at any of the study doses ranging from 5 to 2500 ppm, and combined treatment with inhaled CO did not enhance the vasodilator response to inhaled NO. Moreover, they also report that zinc protoporphyrin IX had no effect on basal pulmonary vascular or DA tone, suggesting that endogenous CO production does not modulate basal pulmonary hemodynamics in the fetal lung (Grover et al., 2000).

II.5 Oxygen

Vascular tone is under the active influence of respiratory gases. One feature that distinguishes the pulmonary circulation from other vascular beds is its response to hypoxia. In the systemic circulation, hypoxia causes vasodilation or, less often, no change in vascular tone. In contrast, the pulmonary circulation responds to hypoxia with vigorous vasoconstriction. Hypoxic pulmonary vasoconstriction (HPV) is a physiological response whereby circulating blood is diverted away from hypoxic alveoli, thus matching perfusion and ventilation and optimizing arterial oxygenation (Barnes & Liu, 1995).

Despite extensive investigation, the mechanisms producing HPV are incompletely characterized (Barnes & Liu, 1995). It has been proposed that pulmonary vascular tone increases by alveolar hypoxia because of reduced release or activity of a vasodilator, increased release or activity of a vasoconstrictor, and/or because hypoxia directly stimulates contraction of vascular smooth muscle cells (Barnes & Liu, 1995). In the search for chemical mediators of HPV many vasoactive substances have been considered as candidates including catecholamines (Fishman 1976), histamine (Hauge 1968), angiotensin-II (Berkov 1974), prostaglandins (Weir et al 1976; Shaul et al., 1991), leukotrienes (Morganroth et al., 1984; McDonnell et al., 1990), 5-HT (Fishman, 1976), PAF (McCormack 1989a), or ATP (McCormack 1989b). However, none of these substances has proved to be essential for HPV (Barnes & Liu, 1995), although they may have a modulatory role, or might play a role in setting up the background that is necessary for HPV to occur. Endothelial factors are important modulators of vascular tone but the role of endothelium in mediating or modulating HPV has not been clearly defined. Hypoxia causes an immediate, transient contraction of isolated pulmonary and systemic arteries that is endothelium dependent and mediated by inhibition of the basal activity of endothelium-derived NO (De Mey & Vanhoutte 1983; Holden & McCall, 1984; Rubanyi et al., 1985; Johns et al., 1989; Graser & Vanhoutte, 1991; Kovitz et al., 1993;). In contrast, hypoxic constriction in the intact pulmonary circulation does not involve this mechanism (Archer et al., 1989; Brashers et al., 1988), which might suggest that the constriction is endothelium independent. In fact, hypoxia-induced contraction of cultured pulmonary vascular smooth muscle cells has been

demonstrated (Murray et al., 1990; Madden et al., 1992), suggesting that a reduction in pO_2 can exert a direct effect on vascular smooth muscle.

Several possible mechanisms have been proposed to explain how the direct action of hypoxia on vascular smooth muscle cells causes pulmonary vasoconstriction: (1) inhibition by hypoxia of several metabolic pathways including glycolisis, tricarboxylic acid cycle or oxidative phosphorylation (Rounds & McMurtry, 1981; Stanbrook & McMurtry, 1983); (2) Existence of a heme protein, such as the cytochrome P450 system, which acts as oxygen sensor (Sylvester and McGowan, 1978); (3) regulation by oxygen tension of the production of reactive oxygen species, which control transmembrane Ca^{2+} flux (Archer, 1993); or (4) inhibition by hypoxia of an oxygen-sensitive K^+ channel causing membrane depolarization and calcium entry through the voltage-dependent calcium channels (Post et al., 1992; Weir & Archer, 1995). In addition, it has been suggested that the responses of specific vessels at different stages of development (fetal, neonatal and adult) to changes in oxygen tension may be determined by the distribution of a variety of ion channels in the smooth muscle cells (Weir et al., 1997). Moreover, there are morphological and electrophysiological differences between individual pulmonary artery smooth muscle cells. For example, in some cells the hypoxia-sensitive K^+ current is predominantly carried by delayed rectifier channels and in others by calcium-sensitive K^+ channels cells (Archer et al., 1996). Delayed rectifier channels are more common in the resistance pulmonary arteries and calcium-sensitive potassium channels in the conduit arteries (Archer et al., 1996). This diversity explains, in part, the segmental differences in the response of the pulmonary circulation to hypoxia (Archer et al., 1996). In addition, age dependent differences in the ionic channels that may control HPV have been described. In adult pulmonary arterial smooth muscle cells the oxygen-sensitive K^+ channel appears to be a 4-aminopyridine-sensitive delayed rectifier channel (Reeve et al., 1998). In contrast, in fetal pulmonary arterial smooth muscle cells hypoxia produces inhibition of a calcium-sensitive K^+ channel (Reeve et al., 1998).

Although decreased production of endothelium-derived dilators or increased production of endothelium-derived constrictors does not primarily mediate HPV, they may play an important

role in initiating or amplifying smooth muscle constriction in response to hypoxia. Gaine et al. (1998) demonstrated that hypoxic pulmonary endothelial cells release a diffusible, cyclooxygenase-independent and distinct from endothelin, contractile factor that mediates hypoxic constriction in isolated pulmonary arteries. The nature of this factor and the mechanisms controlling its activity remain to be determined. On the other hand, studies in pulmonary endothelial cells, isolated pulmonary vascular rings, isolated perfused lungs, and whole animals support an important role for NO in modulating the pulmonary vascular response to oxygen (Voelkel, 1986; Nelin et al., 1996; Phelan & Faller, 1996; Johns et al., 1989; McQueston et al., 1993; Cornfield et al., 1996; Blitzer et al., 1996). Hypoxia interferes several steps of NO synthesis, release or activity. Hypoxia inhibits: (1) L-arginine uptake by pulmonary artery endothelial cells (Block et al., 1995); (2) the conversion of L-citrulline to L-arginine in pulmonary artery endothelial cells (Su & Block, 1995); (3) NOS activity by limiting the availability of oxygen, a substrate for NOS that is a dioxygenase which catalyses the reaction between molecular oxygen and L-arginine (Rengasamy & Johns, 1991); (4) endothelial ATP content (Rounds & McMurtry, 1981) which is necessary for agonist-induced production of EDNO; (5) eNOS by reducing the endothelial intracellular Ca^{2+} concentration which primarily regulates eNOS activity (Mathew et al., 1991; Stevens et al., 1994); (6) the supply of NADPH and 6-methyltetrahydropterine both essential cofactors for NOS activity (Rubanyi & Vanhoutte, 1986). Finally, (7) hypoxia could induce the production of superoxide anions by the endothelial cells that would inactivate EDNO (Rubanyi & Vanhoutte, 1986). In contrast, experimental data are compatible with the hypothesis that, unlike severe hypoxia, moderate hypoxia does not reduce (Warren et al, 1989) and may actually increase (Hampl et al., 1995) NO synthesis in pulmonary endothelial cells.

Extravascular produced NO has also been proposed to modulate HPV. The free diffusion of NO and the close apposition of airways to medium-sized pulmonary vessels, which modulate pulmonary vascular tone (Sobol et al., 1963), suggest that NO, produced in airways proximal to the alveoli, may mediate pulmonary vasodilatation. Furthermore, hemoglobin in blood vessels may serve as a natural biological sink for NO, creating a continuous concentration gradient for NO to move toward perivascular myocytes and thus regulate blood

flow (Dweik et al., 1998). According to this hypothesis, Dweik et al. (1998) showed that oxygen regulates NO levels through effects on iNOS enzyme kinetics in proportion to the inspired oxygen concentration throughout the physiological range.

Oxygen and the perinatal lung

Oxygen tension plays a critical role in the regulation of pulmonary vascular tone *in utero* and during transition at birth through direct and indirect effects on vascular function. The fetal pulmonary circulation can sense small changes in pO_2, which is at least partly responsible for maintaining high PVR in utero and decreasing PVR at birth (Abman et al., 1987; Blanco et al., 1988). The vessel wall responds to a continuum of pO_2 whereas normal fetal pO_2 is approximately 20 mmHg and changes of 5-10 mmHg markedly alters PVR (Abman et al., 1987). Mechanisms of fetal O_2 sensing may be, therefore, critical to enhance our understanding of vascular function with normal development and with vascular injury in diseases such as PPHN.

The acute response to hypoxia in the normal fetus is vigorous and is characterized by increased pulmonary and systemic arterial pressures with a fall in pulmonary blood flow (Campbell et al., 1967a; 1967b). The fetal hypoxic response is present before 0.7 term in the fetal lamb, and increases with gestational age (Lewis et al., 1976). Carotid chemoreceptors mediate the cardiovascular response to hypoxia in the fetal lamb (Blanco et al., 1984; Bartelds et al., 1993). However, acute elevation of fetal PVR is likely to be due to direct effects of lowered pO_2 on smooth muscle (Cornfield et al., 1994), and modulated by various neural and humoral mediators. Changes in oxygen tension influence the release of endothelium-derived products, such as NO, ET, prostaglandins and others, which provide short term modulation of fetal pulmonary vascular tone (Ziegler et al., 1995a). Acute fetal HPV is not blocked by alpha-adrenergic antagonists (Lewis et al., 1976). However, with severe of prolonged intrauterine hypoxia neurohumoral stimulation contributes to altered pulmonary vascular tone and reactivity (Parer et al., 1980; Abman et al., 1987).

The change in oxygen tension is one of the central stimuli of the cascade of events that regulate the transition between the prenatal and postnatal circulation (Walker, 1993). At birth, arterial pO_2 increases from fetal levels of 20 to over 50 mmHg within minutes (Abman & Stevens, 1997). Increased oxygen tension in the absence of other birth related stimuli, such as rhythmic distension of the lung or umbilical cord occlusion, produced a marked increase in fetal lung blood flow (Blanco et al., 1988; Morin et al., 1988a; Tittinsky & Morin, 1993). Reactivity of the human fetal pulmonary circulation to hyperoxygenation increases with gestational age but is absent before 26 weeks of gestation (Rasanen et al., 1998). In the ovine fetus, oxygen-induced increase in pulmonary blood flow was unaffected by COX inhibition (Morin et al., 1988b), or endothelin receptor blockade (Winters et al., 1996), but markedly reduced by NOS inhibition (Moore et al., 1992; Tittinsky & Morin, 1993; McQueston et al., 1994). Experimental studies, in which rhythmic distension of the lungs with hypoxic gases and oxygenation without ventilation have been studied sequentially, have not completely succeeded in determining which of these changes at the onset of air breathing is the more important (Walker, 1993). Dawes (1966) attributed one third of the total vasodilation to each of ventilation, oxygenation, and decreasing CO_2 levels. Other studies have attributed almost the entire response to oxygenation alone (Morin et al., 1988a) or ventilation alone (Teitel et al., 1990). Individual responses within a group of animals can vary between these two extremes (Teitel et al., 1990) and it is possible that differences in lung liquid clearance and lung compliance produce varying responses in the pulmonary circulation at birth (Walker, 1993).

Hypoxia and PPHN

Hypoxia is considered a major cause of PPHN (Haworth, 1997). In newborns who die soon after birth with PPHN precipitated by severe intrauterine or intrapartum hypoxia, without significant parenchymal lung damage, post-morten examination shows thick-walled undilated pulmonary arteries (Haworth & Reid, 1976; Haworth, 1997). However, whether chronic hypoxia alone can cause PPHN is controversial (Abman, 1999). Initial studies demonstrated that newborn rats exposed in utero to chronic hypoxia showed smooth muscle thickening in

small pulmonary arteries (Goldeberg et al., 1971). Other studies have failed to consistently find morphological evidences of hypertensive changes in fetal or neonatal pulmonary arteries after chronic intrauterine hypoxia (Murphy et al., 1986; Geggel et al., 1986). In adult rats, chronic hypoxia induced pulmonary hypertension but only slightly increased the medial area and did not alter the lumen area of pulmonary arteries with an external diameter between 30-200 μm (van Suylen et al., 1998). Physiological studies of sheep have suggested that hypoxia caused by placental embolization (Drummond & Bissonnette, 1978), maternal hypotension (Gersony et al., 1976) or partial cord compression (Soifer et al., 1987) may alter pulmonary vascular reactivity. Additionally, immediate postnatal exposure to hypoxia also produced in piglet pulmonary arteries medial hypertrophy and a marked increase in connective tissue (Allen & Haworth, 1986), as well as an altered reactivity (Tulloh et al., 1997).

The role of NO production in chronic hypoxia-induced pulmonary hypertension in adults and newborns remains unclear. In the adult rat lung, gene expression for endothelial eNOS is upregulated in the alveolar arteries after three weeks of normobaric hypoxic exposure (Shaul et al., 1995; Le Cras et al., 1996). Upregulation by hypoxia of iNOS and nNOS expression has also been reported (Xue et al., 1994; Shaul et al., 1995; Le Cras et al., 1996), but it is not an universal finding (Tyler et al., 1999). Chronic hypoxia in adult rats enhanced the vasoconstrictor response to the NOS inhibitor L-NAME, implying increased NO release (Isaacson et al., 1996). Concentrations of nitrites and nitrates, the stable metabolic products of NO, were higher in lung perfusate of chronically hypoxic rats than in control rats (Isaacson et al., 1994; Muramatsu et al., 1996; Tyler et al., 1999). In contrast, other investigators report impaired acetylcholine-induced relaxation following chronic hypoxic exposure in the adult and neonatal lung (Adnot et al., 1991; Crawley et al., 1992; Shaul et al., 1993b; Eddahibi et al., 1992; Fike & Kaplowitz, 1996; Tulloh et al., 1997). This observation is not incompatible with normal or even upregulated endothelial NOS. The excessive contraction of pulmonary arterial smooth muscle cells in a hypoxic environment may stimulate NO release (Wanstall et al., 1995). Shear stress will increase in contracted vessels and an increase in shear stress also increases NO production (Ohno et al., 1993). The balance of evidence suggests that NO production is at least normal during chronic hypoxia, but that endothelium-dependent

relaxation is impaired. This suggests that other parts of the NO effector system may be altered (Hislop et al., 1997). However, not only NO production but also eNOS content was reduced in lungs from neonatal piglets subjected to postnatal hypoxia (Fike et al., 1998). Hislop et al. (1997) showed an interesting pattern of up- down-regulation in neonatal piglets depending on the time of exposure to hypoxia. In animals made hypoxic from birth, the lung vessel eNOS immunoreactivity was decreased, but in those animals made hypoxic after 3 to 6 days, was increased. In contrast, Berkenbosch et al. (2000) showed that exposure of newborn piglets to 3 days of hypoxia did not affect eNOS activity, but exposure to 14 days reduced it. Interestingly, they observed that exposure to hypoxia also reduced the response to endothelium-independent stimulators of the NO/cGMP pathway, such as NO donors or the cGMP analog 8-bromo-cGMP, suggesting hypoxia-induced impairment of cGMP-mediated vasodilation.

Changes in the environmental oxygen tension to which cells are exposed *in vivo* result in physiological and sometimes pathological consequences that are associated with differential expression of specific genes (Faller, 1999). Hypoxia affects endothelial cellular physiology in vivo and in vitro in a number of ways, including the transcriptionally regulated expression of vasoactive substances, and matrix proteins involved in modulation of vascular tone and structure. While much of the mechanisms coupling pO_2 to changes in gene expression are still unknown, a part of this pathway is emerging. Several transcription factors have been found to be regulated by hypoxia including hypoxia inducible factor 1 (HIF-1), activator protein-1 (AP-1), the tumor suppressor p53, nuclear factor kB and the heat shock transcription factor HSF (Faller, 1999). Therefore, hypoxia results in the transcriptional induction of genes encoding vasoconstrictors and smooth muscle mitogens such as platelet derived growth factor-B, ET-1 (Kourembanas et al., 1991), vascular endothelial growth factor (VEGF) (Namiki et al., 1995) or thrombospondin-1 (Phelan et al., 1998); genes encoding matrix or remodelling molecules such as collagenase IV (Bandyopadhyay et al., 1995), and reciprocal transcriptional inhibition of vasodilatory or anti-mitogenic effectors such as NOS (Phelan et al., 1996; Faller, 1999). Short-term exposure of endothelial cells to low oxygen tension results in the synthesis of predominantly vasoconstricting effectors, while longer-term and more severe hypoxic exposure generates factors that can induce smooth muscle proliferation and remodelling (Faller, 1999).

Thus, the endothelial cell response to hypoxic stress can result in two different consequences in the surrounding tissues, depending on the duration of the exposure: short-term exposure causes physiological and reversible modulation of vascular tone and blood flow; chronic hypoxic stress results in irreversible remodelling of the vasculature and surrounding tissues, with smooth muscle proliferation and fibrosis. This dichotomy of responses to hypoxia may explain, in part, both the acute and chronic pathophysiological sequelae of diseases characterized by regional hypoxia, including pulmonary hypertension (Faller, 1999).

Chapter III. Sepsis and PPHN

The lung is a major target organ in neonatal sepsis (Ablow et al 1976). In general, the lung plays a determinant role in the sepsis in some animal species that exhibit a pulmonary bacterial clearance (e.g. sheep, pig) but not in species (e.g. dog, rat, rabbit) in which bacteria localize predominantly in the liver and spleen (Winkler, 1988). The uptake of bacteria by pulmonary intravascular macrophages and the subsequent release of inflammatory mediators are central to the pathological changes produced in animal models of sepsis-induced adult respiratory distress syndrome and sepsis-induced PPHN (Warner et al., 1987; Bowdy et al., 1990). Moreover, pulmonary hypertension is the single most reliable circulatory disturbance noted in every laboratory model of neonatal sepsis (Gibson et al., 1987; Runkle et al., 1984; Truog et al., 1986; Goldberg et al., 1986; 1988; Hammerman et al., 1988; Meadow et al., 1986; Philips et al., 1988; Schreiber et al., 1992; Covert & Schreiber, 1993; Meadow & Rudinsky, 1995). In these models sepsis produces a complex pulmonary response in which two different phases (i.e. early and late sepsis-induced pulmonary hypertension) have been delimited.

III.1 Early phase sepsis-induced pulmonary hypertension.

In most animal models, pulmonary arterial pressure begins to rise within the first 5 minutes of the sepsis protocol, eventually reaching two to three times its baseline value (Meadow & Rudinsky, 1995; Gibson et al., 1987; Runkle et al., 1984). This rise in pulmonary arterial pressure occurs despite a concurrent fall in cardiac output. As a result, PVR increases by as much as 5 times (Meadow & Rudinsky, 1995).

At least in laboratory animals, the mediator of this early phase pulmonary hypertension in newborn experimental sepsis is well known. TXA_2 has been shown to be responsible for sepsis-induced pulmonary hypertension in both piglets and lambs (Meadow & Rudinsky, 1995). Within minutes of infusion of live GBS organisms (Hammerman et al., 1988; Runkle et al., 1984; Truog et al., 1986) or heat-killed GBS organisms (Barefield et al., 1994) both plasma levels of TXA_2 and PAP rise in concert. This parallel rise in TXA_2 and PAP is blocked

by either the cyclooxygenase inhibitor indomethacin (Runkle et al., 1984), the TX synthase inhibitor dazmagrel (Truog et al., 1986, Hammerman et al, 1988), or after TX receptor blockade (Sandberg et al., 1994). Moreover, TXA_2 mimetics (e.g.U46619) induce changes in the pulmonary circulation indistinguishable from the early changes induced by GBS (Crowley et al., 1991). Finally, newborn human infants infected with GBS who demonstrate elevated PVR have been shown to have elevated levels of circulating TX metabolites (Hammerman et al., 1987). Therefore, TXA_2 appears to be both necessary and sufficient to account for the early phase pulmonary hypertension noted in models of neonatal sepsis (Meadow & Rudinsky, 1995).

III.2 Late phase sepsis-induced pulmonary hypertension.

A second TXA_2-independent rise in PVR is described to occur by about 4 hours in most animal neonatal sepsis protocols (Runkle et al., 1984; Truog et al., 1986; Meadow & Rudinsky, 1995). This phase is associated with pulmonary vascular injury (Hellerqvist et al., 1981) and generalized pulmonary edema, with consequent abnormalities of ventilation/perfusion matching and lung compliance (Truog et al., 1986; Meadow & Rudinsky, 1995). The mediators of this late phase of pulmonary insult have not been well established. A large amount of vasoactive substances is stimulated by, and contributes to, pulmonary tissue damage during sepsis (Suffredini, 1994). Attempts to inhibit some of them (e.g. TNF, IL-1) in the course of experimental sepsis have met with only partial effect on the cardiopulmonary events (Meadow & Rudinsky, 1995). The complex interrelationships between the inflammatory and vasoactive factors involved in the late onset sepsis-induced pulmonary hypertension are likely to explain the partial effect of therapeutics based on the inhibition of one isolated factor.

III.3 NO and sepsis-induced changes in vascular contractility.

NO is generated in large quantities during host defense and immunological reactions (Nathan & Hibbs, 1991;Nussler & Billiar, 1993). Such generation of NO was first observed in activated macrophages (Hibbs et al., 1988; Marletta et al., 1988; Stuehr et al., 1989), where it

contributes to their cytotoxicity against tumor cells, bacteria, viruses and other invading microorganisms. The cytostatic/cytotoxic actions of NO result from its inhibitory actions on key enzymes in the respiratory chain and in the synthesis of DNA in the target cells (Hibbs et al., 1990; Nguyen et al., 1992). NO may also interact with oxygen-derived radicals to produce other toxic substances (Hibbs, 1992) such as peroxynitrite (Beckman et al., 1990). Thus, NO plays a role in immunological host defense and is also involved in the pathogenesis of conditions such as septic shock and inflammation.

During sepsis, pathological overproduction of NO due to cytokine- or endotoxin-related induction of iNOS can lead to inappropriate vasodilation, loss of systemic vascular resistance and hypotension (Hobbs et al., 1999). The role of increased NO generation in the hypotension associated with septic shock has been demonstrated in numerous animal studies (Thiemermann & Vane 1990, Rees et al., 1990, Killbourn et al., 1990; Thiemermann, 1994). Moreover, urinary nitrate is significantly elevated in patients with infectious disease, which suggests a systemic increase in NO production (Wagner et al., 1984; Goede et al., 1995; Wong et al., 1995). An early phase of NO-induced hypotension and vascular hyporeactivity, independent of iNOS induction, has been described in experimental sepsis (Szabo et al., 1993), but a later involvement of iNOS in this process has been clearly established. Thus, in a rodent model of shock, dexamethasone and aminoguanidine prevent the drop in mean arterial blood pressure and pO_2 and the increase in plasma glutamate-pyruvate transaminase (a marker of hepatocellular injury), urea/creatinine (markers of renal dysfunction), and nitrate (a degradation product of NO) (Kengatharan et al., 1996). Nonselective inhibition of NOS also attenuates the microvascular injury associated with the pathophysiological changes that follow *Escherichia coli* LPS administration in rodents (Laszlo et al., 1995). Other inhibitors of iNOS, including 1-amino-2-hydroxy-guanidine and aminoethyl-isothiourea, increase survival of animals exposed to LPS (Wu et al., 1995; Rueten et al., 1996). Mice lacking the iNOS gene are significantly more resistant to administration of LPS than are wild-type control mice; they exhibit little or no hypotensive response and their survival rate is greater (MacMicking et al., 1995; Wei et al., 1995).

Isolated vascular rings either incubated with LPS or obtained from animals subjected to

prolonged periods of endotoxemia show a reduced contractile response to several vasoconstrictor stimuli including noradrenaline, vasopressin, angiotensin II, serotonin, histamin, and KCl (Parratt, 1973; Schaller et al., 1985; Fink et al., 1985; Wakabayashi et al., 1987; McKenna, 1990; Thiemermann, 1994). There is a great body of evidence to support that an excessive formation of NO due to induction of iNOS in vascular smooth muscle accounts for this vascular hyporeactivity (Fleming et al., 1990; Gray et al., 1990; Julou-Schaeffer et al., 1990; Rees et al., 1990; Thiemermann, 1994). Endotoxemia, incubation with LPS or with cytokines -such as TNF or IL-1 induce *de novo* biosynthesis of iNOS in vascular smooth muscle (Busse & Mulsch, 1990; Rees et al., 1990; Thiemermann, 1994). This effect is prevented by inhibition of protein biosynthesis with cycloheximide or by the presence of dexamethasone (Busse & Mulsch, 1990; Rees et al., 1990; Thiemermann, 1994). Expression of iNOS mRNA have been demonstrated in vascular tissues from endotoxemic rats but was absent in control animals (Weigert et al., 1995; Taguchi et al., 1996). NOS inhibitors –both nonselective and selective for iNOS- reversed the vascular hyporeactivity associated with sepsis or vascular incubation with LPS or cytokines (Fleming et al., 1990; Busse & Mulsch, 1990; Griffiths et al., 1995a; Joly et al., 1994; 1995).

As stated above, the hemodynamic findings of sepsis in adult patients or experimental models in adult animals can be at least partially explained by iNOS induction. However the extrapolation of these findings to septic newborns or experimental models of neonatal sepsis is controversial. Sepsis in adults is characterized by two distinct hemodynamic constellations: "warm" and "cold" shock (Meadow & Rudinsky, 1995). Warm, or hyperdynamic, shock describes a condition of increased cardiac output, reduced blood pressure, and reduced systemic vascular resistance (Breslow et al., 1987). Cold, or hypodynamic, shock describes a condition in which cardiac output is reduced, blood pressure falls and SVR is elevated markedly (Tracey et al., 1987). Because the hemodynamic disturbances noted in the warm shock phase of sepsis are consistent with excessive vasodilation, a pathological augmentation of NO production seems to be a plausible explanation (Meadow & Rudinsky, 1995). However, in contrast to descriptions of sepsis in adults, clinical descriptions of septic newborns are uniformly characterized by cold shock (Meadow & Rudinsky, 1995). Moreover, detailed descriptions of hemodynamic changes

during sepsis in neonatal animal models consistently describe reduced cardiac output and elevated systemic and PVR (Meadow & Meus, 1986; Runkle et al., 1984; Truog et al., 1986; Philips et al, 1988). This constellation of hemodynamic findings does not square with an overproduction of vasodilators. However, it has been demonstrated increased nitrite and nitrate levels in the urine of septic newborns, supporting NO as a potential mediator of the cardiovascular responses observed in these neonates (Shi et al, 1993). Moreover, methylene blue, a sGC inhibitor, has been shown to increase blood pressure in neonates with hypotension, presumably secondary to infection, unresponsive to colloids, inotropic agents and corticosteroids (Driscoll et al., 1996). In fact, in neonates with early-onset GBS sepsis, the development of hypotension is one of the most sensitive predictors of mortality (Cabal et al., 1990; Hocker et al., 1992).

III.4 Coexistence during sepsis of elevated pulmonary vascular NO production and pulmonary hypertension.

Investigators have long recognized that sepsis is characterized by maldistribution of blood flow that is different between different vascular beds (Lang et al., 1984). The most obvious changes are pulmonary hypertension coupled with systemic hypotension. This dichotomous response may be due to a different sensitivity of the pulmonary and systemic vessels to the vasoactive factors released in sepsis. These factors include, among others, the vasoconstrictors TXA_2 and ET-1 (see above), and the vasodilator NO that are produced in large amounts during systemic inflammatory response.

Exhaled NO concentration increases significantly in humans and animals with sepsis, but neither the source nor NOS isoforms responsible for this rise in pulmonary NO production are clear (Stewart et al., 1995; Hussain et al., 1996; Stitt et al., 1997; Fujii et al.,1998; Fischer et al., 1999). Fujii et al. (1998) demonstrated in pigs, a rise in exhaled NO concentration in the extrathoracic compartment (i.e. upper airways, nasal and paranasal) after 45 minutes of LPS injection. Exhaled NO in the intrathoracic compartment (i.e. bronchi,

bronchioles and alveoli) also rose significantly but after 90 minutes of endotoxin infusion. However, this rise in exhaled NO was accompanied by an increase in Ca^{2+}-dependent but not in Ca^{2+}-independent NOS activity in the lung. Moreover, LPS injection elicited no significant alterations in the pulmonary expression of iNOS in dogs (Hussain et al., 1996) and pigs (Fuji et al., 1998; Mehta et al., 1999). In contrast, protein and mRNA expression of iNOS was significantly enhanced in rat pulmonary artery (Griffiths et al., 1995b) and rat lung after LPS injection (Kobzik et al., 1993; Carraway et al., 1998; Wang et al., 1999). Moreover, increased expression of iNOS has been described in alveolar macrophages of septic patients (Kobayashi et al., 1998).

In vitro studies demonstrated that iNOS mRNA is induced in rat pulmonary artery smooth muscle and endothelium when cultured cells are stimulated by a combination of cytokines and LPS (Nakayama et al., 1992; Geiger et al., 1997). Induction of iNOS and decreased responses to pressor agents have been reported after *in vitro* incubation of pulmonary arteries from piglets (present thesis) or adult rats (Griffiths et al., 1993; Zelenkov et al., 1993) with LPS or GBS. Griffiths et al. (1995b) reported the presence of iNOS mRNA in the pulmonary artery after in vivo LPS, which was associated with hypocontractility to KCl and phenylephrine. Li & McKenna (1996) showed that vascular reactivity was significantly depressed in lungs from septic rats in comparison to sham-operated controls, and that pretreatment with the NOS inhibitor L-NAME restored the depressed vasoreactivity. In contrast, other investigators found no effect of LPS on pulmonary vascular contractility in rats (Pulido et al., 2000) sheep (Nelson et al., 1991) or pigs (Suba et al., 1992). The explanation for these differences remains unknown, but it has been pointed out the vasomotor effects of iNOS-produced NO may vary, depending on the local NO tissue concentration, the specific mechanisms producing vasoconstriction, and the type of vessel (conductance or resistance) (Stoclet et al., 1998; Pulido et al., 2000). In the pulmonary circulation, the contractile and pharmacological response of large and small arteries are not identical and the vascular reactivity depends on the agonist employed (Zellers Vanhoutte 1989; Leach et al., 1992; Kemp et al., 1997; Gao et al., 1998). Similar differences are present in the systemic circulation. Although the LPS-induced increase in vascular iNOS and subsequent marked hyporeactivity

has been well characterized in large systemic elastic arteries, other investigators (Mitchell et al., 1993; Schneider et al., 1994) failed to find significant vascular dysfunction in smaller resistance arteries, despite a measured increase in iNOS activity. Kleschyov et al. (1998) implicated the adventitia as an important source of NO, and the relatively greater amounts of adventitial cells in the large conductance vessels, versus the smaller resistance arteries, may explain these divergent findings (Pulido et al., 2000). However, iNOS protein was expressed in lung tissues from septic rats mainly in the resistance vessels (Li & McKenna, 1996). The possible role of these factors in the vascular responsiveness of pulmonary vessels from septic animals requires further investigations.

The coexistence of pulmonary hypertension and systemic hypotension in septic shock presents particular problems in elation to potential therapeutic approaches. The evidence that excessive NO synthesis within the vasculature is one of the primary mechanisms causing the hemodynamic manifestations of septic shock, including the pathological systemic vasodilation and diminished response to vasoconstrictors (see above), led to the use of NOS inhibitors to treat this condition. Studies using adult animal models of septic shock reported restoration in the balance of systemic vasomotor tone and reduction in mortality for animals treated, after the onset of shock, with NOS inhibitors (Kilbourn et al., 1990a; 1990b; Meyer et al., 1994; Rees et al., 1995). However, NOS inhibitors have been also reported to produce serious and even lethal side effects in some animal models, presumably as a result of blocking the adaptive consequences of increased NO synthesis during inflammation (Cobb, 1999). These adaptive consequences may include maintenance of microvascular blood flow, inhibition of platelet agglutination and leukocyte adhesion, nonspecific immunity, and inhibition of stress-induced endothelial cell apoptosis (Cobb & Danner, 1996; DeMeester et al., 1998; Cobb, 1999; Galley et al., 1999).

In fact, trials of NOS inhibitors in adult sepsis were stopped recently following safety reports, which demonstrated increased mortality rates in patients who received these drugs (Kilbourn, 1999). The seemingly positive effects reported, such as increased systemic vascular resistance and decreased requirements of vasoactive drugs, appeared to be offset by the

negative effects, such as decreased cardiac output, increased pulmonary hypertension and potential myocardial ischemia (Avontuur et al., 1998a; 1998b; Cobb, 1999). The cause of the progressive cardiac dysfunction produced by NO inhibition during sepsis may be multifactorial, including 1) coronary artery vasoconstriction with myocardial ischemia and reduced contractility; 2) reduced endocardial cell production of NO with potential decreased myocardial performance, and 3) increased cardiac afterload with the acute increase in PVR and SVR (Gibson et al; 1994; Cohen et al., 1998). The decrease in cardiac output, after NOS inhibition, due to increased right ventricular afterload related to a worsening in pulmonary hypertension seems to be particularly relevant in animal models of neonatal sepsis. Piglets treated with a NOS inhibitor before GBS infusion demonstrated a more rapid and profound pulmonary hypertensive response to the bacteria (Barrington et al., 2000). This suggests that GBS-induced pulmonary hypertension is usually diminished by endogenous NO production. Thus, pulmonary hypertension resulting from GBS infusion is much worse if endogenous NO production is completely inhibited (Gibson et al., 1994; Meadow et al., 1995; Barrington et al., 2000). In addition, when inhaled NO is used to reverse pulmonary hypertension in experimental sepsis, fall in cardiac output was significantly delayed and survival was improved (Klemm et al., 1995; Barrington et al., 2000).

Therefore,whether lung iNOS induction exerts a positive or a detrimental effect during sepsis is controversial. Mice deficient in the iNOS gene are more resistant to LPS induced acute lung injury than corresponding wild-type mice (Kristof et al., 1998). In contrast, Aikio et al (2000), demonstrated a diminished pulmonary iNOS expression in fulminant early-onset neonatal pneumonia, suggesting that delayed production rather than excess of pulmonary inflammatory NO was associated with severe symptoms.

Chapter IV. Treatment of PPHN. In search of a selective pulmonary vasodilator.

IV.1 Intravenous pulmonary vasodilators.

Part of the challenge in finding an effective strategy for the treatment of PPHN is that vasodilators that decrease pulmonary vascular resistance also decrease the vascular resistance of the systemic bed. Since the magnitude of the shunt depends on the difference between pulmonary artery and aortic pressure, systemic vasodilation would increase the flow across the ductus reducing the blood supply to the lung and worsening the pulmonary gas exchange. Therefore, the ideal drug for the treatment of PPHN should be a vasodilator with selectivity for pulmonary over systemic vessels (Drummond and Lock 1984; Roberts and Shaul 1993). The term selective applied to a vasodilator from the pulmonary vasculature has two meanings, each with a different pathophysiological and therapeutical implication. The term macroselectivity has been used to distinguish the effects of a vasodilator drug on the pulmonary vasculature as opposed to the systemic vasculature, as measured by the pulmonary/systemic pressure or resistance ratio (Nelin & Hoffman, 1998). The term microselective has been used to distinguish the effects of a vasodilator drug on the distribution of perfusion within the lung. Most intravenous vasodilator drugs have resulted in worsening of ventilation/perfusion matching by disruption of HPV. Thus, the property of microselectivity is important to maintain or enhance intrapulmonary gas exchange (Nelin & Hoffman, 1998).

As Truog pointed out (1998), from the original description of Gersony et al. (1969) of the syndrome of "persistent fetal circulation" -now PPHN- investigators have sought the "Holy Grail" of an agent capable of selective reduction of PVR without affecting systemic vascular resistance. Various intravenous vasodilators including the α-adrenoceptor blocker tolazoline (Stevenson et al., 1979; Ward, 1984; Gouyon and Francoise, 1992; Bos et al., 1993), PGI_2 (Bos et al., 1993), PGE_1 (Gouyon & Francoise, 1992), the calcium channel blocker nifedipine (Simonneau et al., 1981), $MgSO_4$ (Abu-Osba et al., 1992; Tolsa et al., 1995), acetylcholine (Tripp et al., 1980), the NO donor sodium nitroprusside (Benitz et al., 1985) and ATP (Fineman

et al., 1990; Konduri & Woodard, 1991) have been used to reduce the increased pulmonary vascular resistance in experimental models or in patients with PPHN (Drummond &Lock, 1984; Kulik & Lock, 1984; Gouyon & Francoise; 1992; Roberts & Shaul, 1993). However, until now there is no clinically evaluated intravenous selective pulmonary vasodilator (Finer & Barrington, 2000).

IV.2 Selective pulmonary vasodilation through inhalation of NO. From the laboratory to the clinical experience

The discovery of EDRF, and its subsequent identification as NO, a volatile gas susceptible to be administered by inhalation, dramatically changed the search of a specific pulmonary vasodilator and has led to novel therapeutic approaches in the management of PPHN. The first laboratory reports about the pulmonary selectivity of inhaled NO (Frostell et al., 1991; Fratacci et al., 1991) were confirmed in fetal and neonatal animals and in experimental models of PPHN (Kinsella et al., 1992a; Roberts et al., 1993; Zayek et al., 1993). In adult patients with pulmonary hypertension, Pepke-Zaba et al (1991) showed that inhaled NO at 40 ppm had a beneficial effect in reducing PVR with no change SVR. Two clinical reports in newborn patients appeared in 1992. One demonstrated that inhaled NO improved saturation in patients with PPHN (Roberts et al., 1992), and the other demonstrated that the clinical improvement seen with inhaled NO in patients with PPHN was sustained (Kinsella et al., 1992b). After the publication of these and others pilot studies several randomized, controlled trials were conducted to evaluate the efficacy of inhaled NO in PPHN (table 2).

The systematic review from Finner and Barrington (2000) for the Cochrane Collaboration include, until the present moment, the following trials: Barefield et al., 1996; Day et al., 1996; Neonatal Inhaled Nitric Oxide Study Group, 1997a; 1997b; Roberts et al., 1997b; Wessel et al., 1997; Kinsella et al., 1997a; and Davidson et al. 1998. This meta-analysis provides evidence about PaO_2 improvement in the inhaled NO treated infants by 46.4 mmHg (weighted mean difference) compared with controls (95% CI, 34.2, 58.5), and a significant decrease in the

oxygenation index by 10.7 (95% CI, -14.1, -7.4). The use of inhaled NO reduced the requirement for ECMO, overall, by 15% (Risk Difference -14.8%, 95% CI -23.0%, -6.6%). Thus, the number of infants needed to be treated with inhaled NO in order to expect to prevent one infant requiring ECMO would be 6.8 (95% CI 4.3, 15.2). Mortality was not reduced, relative risk for death was 1.03 (95% CI 0.62, 1.72). The combined incidence of death or need for ECMO was significantly reduced by treatment with inhaled NO, relative risk 0.72 compared to control (95% CI, 0.6, 0.87) with the majority of the improvement seen in the reduction in the need for ECMO. Therefore, according to this meta-analysis, inhaled NO appears to improve outcome in hypoxemic term and near term infants by reducing the incidence of ECMO and of the combined endpoint of death or need for ECMO. This reduction seems to be entirely a reduction in need for ECMO. The outcome of infants with diaphragmatic hernia was not improved and even, there is a suggestion that was slightly worsened. They conclude that "on the evidence presently available, it appears reasonable to use inhaled nitric oxide in a concentration of 20 ppm for term and near term infants with hypoxic respiratory failure who do not have a diaphragmatic hernia" (Finer & Barrington, 2000).

Little information is available about long-term neurodevelopmental and pulmonary follow-up of infants treated with inhaled NO. In a 1- to 2-yr follow-up study of children who received inhaled NO treatment for PPHN, neurodevelopment scores, growth rates (growth percentiles for weight, length, and occipitofrontal circumference), the frequency of airway disease, and the need for supplemental oxygen were comparable to conventionally ventilated or ECMO-treated patients (Rosenberg et al., 1997). More recently, the results of the neurodevelopmental follow-up of infants enrolled in the NINOS trial have been reported, showing that inhaled NO was not associated with an increase in neurodevelopmental, behavioral, or medical abnormalities at two years of age (Neonatal Inhaled Nitric Oxide Study Group, 2000).

Table 2. Randomized controlled trials of inhaled NO in term and near term (> 34 weeks) newborns with PPHN or hypoxic respiratory failure.

Reference	No. Pat.	Inclusion Criteria	Treatment Protocol	Outcome
Day et al., 1996	22	25 < OI < 40 Echocardiographic evidence of PPHN	20 ppm NO vs. control. If control deteriorated (OI > 40) use of NO allowed.	After 30- 60 minutes of therapy OI was 17.5 (SD 10.6) in the NO group and 32.6 (SD 13.3) in controls, Weighted Mean Difference -15.1 (95% CI -25.14,-5.06)
Barefield et al., 1996	17	PaO_2 < 100 mmHg on FIO_2 = 1 on ventilator (16 patients had echocardiographic evidence of PPHN)	NO at 20 to 40 ppm increased to 80 if PaO_2 stayed < 100. Backup use of inhaled NO allowed in case of failure of control treatment.	No differences between groups in primary outcome variables (treatment failure and meeting of ECMO criteria). After 30- 60 minutes of therapy OI was 23 (SD 18.2) in the NO group and 38 (SD 15.6) in controls. Weighted Mean Difference -15.0 (95% CI -32.76, 2.76)
Roberts et al., 1997b	58	PPHN (by echo) PaO_2 < 55 mmHg on 2 consecutive Measurements	80 ppm NO at FIO_2 0.9 vs. FIO_2 = 0.9 (control)	Responders: 53% in NO group; 7% in control group. Need for ECMO: 40% NO group; 70% control group (P=0.02). Survival: similar in NO and control groups.
Neonatal Inhaled NO Study Group (NINOS), 1997a	235	Hypoxic respiratory failure or PPHN requiring mechanical ventilation; OI > 25 (2 consecutive)	20 ppm NO, trial at 80 ppm if no response to 20, vs. control (FIO_2 = 1)	Responders: 51% 20 ppm NO; 15% control. Need for ECMO: 39% NO group; 55% control group (P=0.014). Survival: 86% NO group; 84% control
Neonatal Inhaled NO Study Group (NINOS), 1997b	53	Congenital diaphragmatic hernia (PPHN in 51 of 53 patients) OI > 25 (2 consecutive)	20 ppm NO, trial at 80 ppm if no response to 20 vs. control (FIO_2 = 1).	Responders: 48% 20 ppm NO; 19% control. Need for ECMO: 80% NO group; 54% control group (P=0.043). Survival: 52% NO group; 57% control.
Wessel et al., 1997	49	PaO_2 < 100 mm Hg during mechanical ventilation on FIO_2 = 1 Echocardiographic evidence of PPHN	80 ppm NO, reduced to 40 ppm after 1 h vs. control	Responders: 57% NO group. Improvement in PaO_2, O_2 saturation, and OI in the NO group at 15 min. and 12 h. No differences in mortality (8%), use of ECMO (33%), days on mechanical ventilation or supplemental O_2.
Kinsella et al., 1997a	205	PPHN (by echo) PaO_2 < 80 mmHg at FIO_2 = 1	NO (20, 40 ppm) vs. HFOV,crossover and combination	Overall response rate 60%. All responders survived. 72% of non responders treated with ECMO survived. Overall survival 86%

Reference	No. Pat.	Inclusion Criteria	Treatment Protocol	Outcome
Davidson et al., 1998	155	FIO_2= 1 and MAP≥10 cm H2O, PaO_2 between 40 and 100 mmHg PPHN (by echo) or preductal-postductal saturation > 10%	NO at doses of 0, 5, 20 or 80 ppm Sequential 20% decrements.	Survival: 92% NO group; 98% control (P=0.22). Need for ECMO: 22% NO group; 34% control (P=0.12). BPD: 13% NO group; 15% control.
Cornfield et al., 1999	38	OI ≥ 25 on 2 consecutive blood gases 60 minutes apart Echocardiographic evidence of PPHN	NO 2 ppm vs. control Patients (from both groups) with an OI ≥ 35 for > 1 hour treated with NO at 20 ppm	Treatment with NO at 2 ppm did not induce any detectable acute effect in oxygenation NO at 20 ppm acutely improves oxygenation in infants initially treated with 0 ppm, but not in infants previously treated with 2 ppm
Clark et al, 2000	248	Clinical or echocardiografic evidence of PPHN OI > 25. In addition, pH > 7.55 to maintain PaO_2 > 60 mmHg	20 ppm NO for max. 24 h followed by 5 ppm for max. 96 h. Control group received nitrogen	Need for ECMO: 38% NO group; 64% control (P=0.001) Survival: similar Chronic lung disease: 7 % in NO group; 20% control group (P=0.02)
Christou et al., 2000	41	Clinical or echocardiografic evidence of PPHN and hypoxemia (PaO_2 ≤100 mm Hg on FIO_2=1)	NO initiated at 40 ppm. Decreased to 20 ppm after 1 h. Dose response test daily to determine the lowest acceptable dose of NO	Need for ECMO: 14% NO group; 55% control (P=0.007) Survival: similar

Toxicity of inhaled NO

The primary observed toxic effect of NO is formation of methemoglobin (Toothill, 1967; Kinsella et al., 1993; Young & Dyar, 1996; Phillips et al., 1999). Methemoglobin is formed when NO, which has an affinity for hemoglobin nearly 1 000 000 times that of oxygen, combines with the heme group of hemoglobin. Methemoglobin formation prevents oxygen from being transported to the tissues of the body (Phillips et al., 1999). It has been reported that among adult patients receiving NO therapy, the fraction of hemoglobin converted to methemoglobin typically does not exceed 5% (Young & Dyar, 1996). Moreover, in the

neonatal multicenter study of Davidson et al. (1998) methemoglobinemia, defined as >7% methemoglobin, was seen in 13 of 37 patients administered NO at the 80-ppm level, but was not seen in the groups of 41 and 36 patients administered 5-ppm NO and 20-ppm NO, respectively.

A second potential hazard of NO is its conversion, in the presence of oxygen, to nitrogen dioxide (NO_2), a brownish gas that is a potent pulmonary irritant (Young & Dyar, 1996; Mercier et al., 1993). Because NO always exists in equilibrium with NO_2 in air, mixed NO and NO_2 are often referred to collectively as nitrogen oxides or NOx. The conversion of NO to NO_2 can be quite slow (Bouchet et al., 1993; Phillips et al., 1999): in a 20-ppm mixture of NO in air, only approximately 1- to 3-ppm NO_2 would be generated in 10 minutes. However, because the rate of NO_2 formation is proportional to the oxygen concentration and to the square of the NO concentration, NO_2 is formed more rapidly in oxygen enriched atmospheres, such as those found in a patient ventilation circuit, or at higher concentrations of NO. It may take less than one minute for 1-ppm NO_2 to be generated from a 20-ppm mixture of NO in 95% oxygen at 100% relative humidity (Bouchet et al., 1993). Effects of human exposure to various levels of NO_2 have been reported; these include increased flow resistance of the airway after 10-minute exposure at 0.7 to 2 ppm, mild irritation of the eyes, nose, and upper respiratory tract at 10 to 20 ppm, respiratory irritation and chest pain after 60-minute exposure at 25 ppm, and pulmonary edema and death after 60-minute accidental exposure at 100 ppm (Phillips et al., 1999). In neonatal patients, Davidson et al. (1998) observed NO_2 concentrations higher than 3 ppm only after treatment with 80-ppm but not with 5-ppm or 20-ppm NO.

Additionally, several concerns have been made about the exposure to NO in the caregivers of the intensive care units (Markhorst et al., 1996; Mourgeon et al., 1997). During earlier clinical NO trials, the expiratory gas was vacuum scavenged to prevent release of NO into the room air (Roberts et al., 1993). However, this practice had been discontinued and currently the expiratory limb of the ventilator is vented to the room. In spite of this fact, Phillips et al. (1999) have demonstrated that exposure of the caregivers to detectable levels of

NO and NO_2 in room air was brief, infrequent, and well below established limits.

Part of the concerns about NO toxicity relates to it being a free radical. The biological chemistry of NO as a free radical can be simplified to three main reactions (Figure 5) (Beckman & Koppenol, 1996): (1) its activation of sGC responsible for signal transduction; (2) its elimination by oxyhemoglobin to form nitrate and methemoglobin; and (3) its reaction with superoxide to form the binary peroxynitrite. NO and superoxide readily react to form peroxynitrite at nearly a diffusion-limited rate (Beckman & Koppenol, 1996). Under physiological conditions, this reaction is limited by the intracellular micromolar concentrations of superoxide dismutase (SOD) that scavenge endogenous superoxide. However, when the concentration of NO is increased (i.e. by the use of inhaled NO or by pathological conditions as sepsis) and approaches that of SOD, or in the presence of increased superoxide concentrations, or after superoxide scavengers are exhausted, significant concentrations of peroxynitrite may be produced (Beckman & Koppenol, 1996). Peroxynitrite directly causes oxidation, peroxidation, and nitration of lipids, proteins, or DNA (Szabo, 1996a; 1996b; Steudel et al., 1999). Peroxynitrite can cause cell apoptosis by DNA strand breakage, activation of poly-adenosine-diphosphate-ribosyltransferase and by inhibition of mitochondrial respiratory enzymes (Steudel et al., 1999). An important example of a reaction caused by peroxynitrite is the nitration of tyrosine. Tyrosine nitration inhibits tyrosine phosphorylation, alters the dynamics of assembly and disassembly of cytoskeletal proteins, and inhibits tyrosine hydroxylase, thereby reducing dopamine production by neurons and inhibiting cytoskeletal movements of endothelial cells (Szabo, 1996a; Steudel et al., 1999). Nitrotyrosine has been detected in lung tissue sections from adult patients with lung injury (Haddad et al., 1994a; Kooy et al., 1995). Plasma 3-nitrotyrosine content is increased during the first month of life in infants who develop bronchopulmonary dysplasia, a chronic lung disease of infancy that appears to be caused in part by oxidative stress from hyperoxia (Banks et al., 1998). This suggests that peroxynitrite-mediated oxidant stress may contribute to the development of lung injury in premature infants. In contrast, in newborns treated with inhaled NO, no evidence was found of either nitrotyrosine production or of increase in lipid peroxidation, suggesting lack of toxicity produced by peroxynitrite (Hallman et al., 1998). Additionally, no evidence was found

in these patients of NO-related increased concentration of proinflamatory or anti-inflammatory cytokines (Hallman et al., 1998). Moreover, in preterm lambs delivered at 78% of term, 5 ppm NO increased pulmonary blood flow and improved gas exchange without increasing pulmonary edema and decreased accumulation of lung neutrophils (Kinsella et al., 1997b). Lambs delivered at 90% of gestation and mechanically ventilated for 5 hours with 20 ppm NO showed no evidence of lung oxidative stress injury (Storme et al., 1998) and newborn lambs subjected to GBS sepsis and up to 60 ppm NO showed no changes in pulmonary antioxidative capacity or lipid peroxidation (Lopes-Cardozo et al., 1996). It has been demonstrated, in animal models, that inhaled NO, when administered in combination with hyperoxic gas mixtures, protected against the injurious consequences of prolonged hyperoxia, such as inflammation and apoptosis (Gutierrez et al., 1996; McElroy et al., 1997; Nelin et al., 1998; Howlett et al., 1999). However this point remains controversial. Ekekezie et al. (2000) have found, in young piglets, that the combination of NO and oxygen exposure produced an increase in lung apoptosis and that NO may prevent upregulation of SOD and catalase activity during hyperoxia, potentially increasing oxidative injury. In addition, recombinant human SOD mitigated the inflammatory changes, oxidative damage, and acute lung injury from exposure to 100 ppm NO and 90% O2 in newborn piglets (Robbins et al., 1997). Finally, hyperoxic exposure of rat pups up-regulated both iNOS and eNOS, suggesting that increased generation of endogenous NO may contribute to the pathogenesis of hyperoxia-induced lung damage (Radomski et al., 1998; Potter et al., 1999).

Another point of concern about the NO/peroxynitrite toxicity is its possible effect on the surfactant system (Robbins et al., 1995; Steudel et al., 1999). Peroxynitrite exposure impaired pulmonary surfactant function, because of peroxidation of surfactant lipids, and decreased the ability of the major hydrophilic surfactant, protein A, to aggregate lipids and act synergistically with other surfactant proteins to reduce the minimum surface tension (Haddad et al., 1994b; Hallman & Bry, 1996). These changes of surfactant protein A were associated with nitrotyrosine formation (Haddad et al., 1993). Besides peroxynitrite, methemoglobin and NO_2 deteriorate surfactant activity in vitro (Haddad et al., 1993; Hallman et al., 1996a; 1996b) Recombinant human SOD did not appear to reduce the impairment in surfactant function

produced by the combination of high doses of NO (100 ppm) and hyperoxia in newborn piglets (Robbins et al., 1995). However, no decrease in surfactant activity have been observed in PPHN patients treated with NO (Hallman et al., 1998) and even 14-ppm of inhaled NO prevented the hyperoxia-induced detrimental effects on alveolar surfactant (Issa et al., 1999).

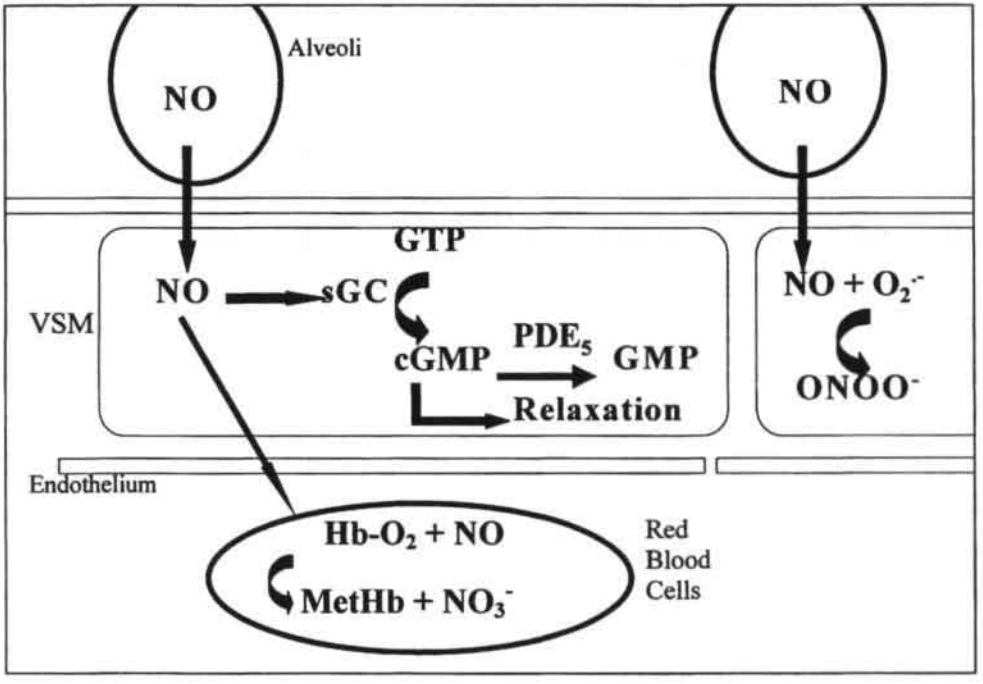

Figure 5. NO as a free radical

Lack of response to inhaled NO

Approximately 40-50% of the infants assigned to NO in randomized controlled trials failed to respond (Finer & Barrington, 2000). This is particularly notable because response was defined as a relatively modest increase in arterial oxygen tension (Truog, 1998). Several

mechanisms may explain clinical variability in responsiveness to inhaled NO therapy including: (i) the inability to deliver NO to the pulmonary circulation due to poor lung inflation that may cause intrapulmonary shunting and hypoxemia that is not reversed by vasodilators (Kinsella & Abman, 1995; Roberts et al., 1997b); (ii) the presence of myocardial dysfunction or systemic hypotension that increase the right-left shunt and elevate pulmonary wedge pressures (Kinsella & Abman, 1995; Roberts et al., 1997a); (iii) the presence of missed anatomical cardiovascular or pulmonary lesions such as total anomalous venous return, coarctation of the aorta, lung hypoplasia or alveolar capillary dysplasia (Roberts et al., 1997a; Steinhorn et al., 1997b); (iv) the inflammation of the airways due to pneumonia or aspiration of meconium that may reduce the diffusion of NO (Roberts et al., 1997b; (v) the excess of vasoconstrictive agents, such as TXA_2 or ET-1, that might limit the vasodilatory action of inhaled NO (Cristhou et al., 1997; Truog et al., 1998) ; (vi) the use of an inadequate dose. The first published experience of inhaled NO treatment in term newborns reported initial doses that ranged from 6-20 ppm (Kinsella et al., 1992b) to 80 ppm (Roberts et al., 1992). These doses were based on concentrations that had previously been found to be effective in animal experimentation (Kinsella et al., 1992a; Roberts et al., 1993). Afterwards, it has been demonstrated that acute (Finer et al., 1994, Davidson et al., 1998) as well as sustained (Davidson et al., 1998) improvement in oxygenation during treatment with inhaled NO was not different with doses ranging from 5 to 80 ppm. Moreover, the 80 ppm dose did not seem to have any advantages over the 20 and 5 ppm doses and resulted in elevated methemoglobin and NO_2 levels (Davidson et al., 1998). High doses of NO that can paradoxically worsen oxygenation due to the loss of selective pulmonary vasodilatation, producing an increased ventilation-perfusion mismatch (Kinsella & Abman, 1995). On the other hand, the use of NO at doses too low may not achieve maximal lowering of PVR. Moreover, Cornfield et al. (1999) have demonstrated that initial treatment with low dose of inhaled NO (2 ppm) may diminish the clinical response to 20 ppm and have adverse clinical sequelae.

Finally (vii), the presence of an abnormal pulmonary vascular structure and/or altered smooth muscle cell responsiveness or sensitivity can also explain the lack of action of inhaled NO. It is possible that the thickened pulmonary arteries of some infants reduce the diffusion of

NO and even continue to restrict the blood flow in spite of NO-induced relaxation (Roberts et al., 1997b; Shehata et al., 2000). Additionally, as described in animal models of PPHN, decreased sGC or increased PDE5 activities may limit the vasodilator response to NO (Steinhorn et al., 1995a; Hanson et al., 1998; Abman, 1999). Furthermore, sGC activity may be regulated via the action of an endogenous inhibitor, which has been partially purified from bovine lung and exerts an allosteric inhibition (Kim & Burstyn, 1994). It has been also suggested that genetic factors can contribute to the response of some patients to inhaled NO. Weimann et al. (1998) demonstrated, in adult patients with acute respiratory distress syndrome, a relation between the oxygenation response to inhaled NO and the ABO blood group system. Thus, the response to inhaled NO was significantly decreased in patients with genotype B who express blood group B or AB. They speculate with a possible genetic linkage between the ABO gene locus and another, as yet unknown, gene locus that might be involved in the pulmonary vascular response to NO. In contrast, in a group of newborns and children - median age 1.6 months- McFadzean et al. (1988) found no differences in the percentage of non responders to inhaled NO between patients with blood group B/AB and patients with O/A. However, they observed a trend to a earlier response in the O/A patients.

Adjuvant therapies to augment the response to inhaled NO

Based on some of the facts described above, several treatment strategies have been proposed to increase the response to inhaled NO. Considering the important role of parenchymal lung disease in many cases of PPHN, the combined use of high-frequency HFOV, with a strategy designed to recruit and sustain lung volume, plus inhaled NO was more successful than treatment with HFOV or inhaled NO alone (Kinsella et al., 1997a). Additionally, pharmacological augmentation of the response to inhaled NO with the use of PDEs inhibitors has been tested in animal models (Thusu et al., 1995; Dukarn et al., 1998; Steinhorn et al., 2000), PPHN patients (Kinsella et al., 1995; al-Alaiyan et al., 1996, Thebaud et al., 1999), and infants with pulmonary hypertension (Ziegler et al., 1998; Ivy et al., 1998b). However, the use of PDE inhibitors not selective for the pulmonary circulation raises the problem of their systemic effects similar to that described for intravenous vasodilators. Further

studies with more selective PDE5 inhibitors -such as E4021, DMPPO, and zaprinast- (Kinsella & Abman, 1998) or using these drugs by inhalation (Ichinose et al., 1998) may lead to novel clinical strategies to enhance the treatment of PPHN with inhaled NO.

On the other hand, strategies based on the augmentation of cofactors for enzymatic activity of sGC, such as the divalent cations Mg^{2+} and Mn^{2+}, might also improve the response to inhaled NO. In the presence of Mg^{2+}, stimulation of the sGC by NO is dramatically enhanced and the affinity of substrate (GTP) binding to the enzyme increased (Hobbs, 1997). Additionally, the fact that NO is a free radical and that it forms peroxynitrite with superoxide has led to the suggestion of the use of antioxidant enzymes, mainly SOD, not only as a way to reduce toxicity, but also as a method to ameliorate the response to inhaled NO. It is well known that SOD significantly increased *in vitro* relaxant activity of NO, and that SOD induces endothelium dependent vasodilatation by protecting basal NO from the destructive action of endogenously produced superoxide anions (Ohsltein & Nichols, 1989; MacKenzie et al., 1999) . The role of exogenous SOD on the relaxation induced *in vivo* by exogenous NO remains unknown, but promising preliminary results show that the use of human recombinant SOD improved the response to NO in the experimental ovine model of PPHN induced by ductus compression (Albert et al., 1999).

Inhaled NO in the premature infant

Research in human preterm infants has demonstrated that pulmonary hypertension with extracardiac right-to-left shunt can complicate the course of hyaline membrane disease and is associated with mortality, despite surfactant therapy (Walther & Benders 1992; Seppanen et al., 1993). Premature lambs with hyaline membrane disease do have elevated PVR and seem to respond to inhaled NO with an improvement in gas exchange and reduction in PVR (Kinsella et al., 1994b; Skimming et al., 1995). This improvement was not accompanied by an increase in lung edema and, in fact, inhaled NO reduced lung neutrophil accumulation, suggesting a reduction of the inflammatory process (Kinsella et al., 1994b; 1997b). Preliminary case reports

and uncontrolled studies in human premature neonates with severe hypoxemic respiratory failure supported the potential role of low-dose inhaled NO as an adjuvant therapy (Abman et al., 1993; Peliowski et al., 1995; Skimming et al., 1997). In terms of the premature infant, one of the potential risks of most concern is the prolongation in bleeding time associated with inhaled NO (Hogman et al. 1993; Samana et al., 1995). This could exacerbate intraventricular hemorrhage complications. Preliminary data from small, nonrandomized noncontrolled studies showed frequent grade 3 and 4 intraventricular bleeds and poor neurodevelopmental outcome in premature infants with severe hypoxemia treated with inhaled NO (Cheung et al., 1998). However, other factors not related to the use of inhaled NO, but related to the illness severity in these infants might be involved in the evolution of the intracraneal bleeds. On the other hand, premature children treated with inhaled NO might have a higher risk for the development of chronic lung disease because of the possible toxicity caused by NO_2 and peroxynitrite over lungs with a reduced development of antioxidant defenses (Frank, 1998; Saugstad, 1999). The findings and concerns previously described showed the need for controlled randomized trials, and three of them have been published until the present moment (Table 3)

Two of this studies (Subhedar et al., 1997; Kinsella et al., 1999) demonstrated an improvement in oxygenation with inhaled NO. Neither study, however, showed any significantly increased survival in the treatment groups. The frequency of likely adverse effects, such as intracraneal haemorrhage or chronic lung disease, was not increased in the NO group in any of these trials. The meta-analysis of Barrington and Finer (2000) only includes, until now, the study of Subhedar et al. Based on that data they conclude that there is no published information to support the use of inhaled NO in preterm infants. The inclusion in this systematic review of the results of the studies of Kinsella et al., and The Franco-Belgium group might not seem to extend the benefits of inhaled NO to outcomes other than acute improvement of oxygenation. Therefore, NO therapy for premature infants should be considered as an experimental drug and its use confined to clinical studies in which adverse effects can be monitored (Saugstad, 1999).

Table 3. Randomized controlled trials of inhaled NO in newborns < 34 weeks with respiratory failure.

Reference	No. Pat.	Inclusion Criteria	Treatment Protocol	Outcome
Subhedar et al., 1997	42	Gestational age < 32 weeks; mechanical ventilation since birth; surfactant therapy; and high risk of developing CLD (defined by a prediction score)	20 ppm NO. If response, weaning after 2 hours in steps of 5 ppm. Four groups: NO, dexamethasone, NO+dexamethasone, and control	Greater percentage decrease in OI in NO group (16,9%) than in control (no change) However, oxygenation was not well matched at baseline between the groups. No differences in the combined incidence of CLD and/or death between infants treated with NO and controls (RR 1.05, 95% CI 0.84-1.25)
Kinsella et al., 1999	80	Gestational age<34 weeks; age<7 days; severe hypoxemia (arterial/alveolar O_2 ratio<0.1 on 2 sequential blood gases) despite mechanical ventilation and surfactant (predicted mortality rate 50%)	5 ppm NO vs. control	NO improved oxygenation after 60 min (p=0·03). Survival: NO group 52%, control 47%. NO and control groups did not differ for adverse events or outcomes (intracranial haemorrhage grade 2-4: NO 28% control 33%; pulmonary haemorrhage: 13% and 9%; CLD: 60% and 80%)
The Franco-Belgium Collaborative NO Trial Group, 1999	85	Gestational age < 33 weeks; 12.5<OI< 30 on 2 consecutive measurements.	10 ppm NO vs. control. When successful NO decreased to 5 ppm and slowly tapered	No significant improvement in oxygenation with NO. Survival: NO group 73%, control 65% NO and control groups did not differ for adverse events or outcomes (intracranial haemorrhage grade 2-4 and cystic leucomalacia: NO 32%, control 27%; O_2 therapy at 28 days: 45% and 48%; O_2 therapy at 36 postconcepcional weeks: 24% and 29%)

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Chapter V. Chronic intrauterine pulmonary hypertension impairs endothelial nitric oxide synthase in the ovine fetus.

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rapid communication

Chronic intrauterine pulmonary hypertension impairs endothelial nitric oxide synthase in the ovine fetus

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Pediatric Heart-Lung Center and Departments of [2]Pediatrics and [3]Pathology, University of Colorado School of Medicine, Denver, Colorado 80218; and [1]Servicio de Neonatologia, Departmento de Pediatria, Hospital Universitario San Carlos, Madrid, Spain

Villamor, Eduardo, Timothy D. Le Cras, Marilee P. Horan, Ann C. Halbower, Rubin M. Tuder, and Steven H. Abman. Chronic intrauterine pulmonary hypertension impairs endothelial nitric oxide synthase in the ovine fetus. *Am. J. Physiol.* 272 (*Lung Cell. Mol. Physiol.* 16): L1013–L1020, 1997.—Endothelial (e) nitric oxide synthase (NOS) activity modulates pulmonary vascular tone in the normal fetus and decreases pulmonary vascular resistance (PVR) at birth. Mechanisms contributing to sustained elevations of PVR and the failure of postnatal adaptation at birth are uncertain but may include decreased eNOS activity. To test this hypothesis, we studied the effects of chronic intrauterine pulmonary hypertension on lung eNOS content and NOS activity in an ovine model of perinatal pulmonary hypertension and in normal lambs. We measured eNOS mRNA and protein content by Northern and Western blot analyses, respectively. Calcium-dependent and total NOS activities were determined by assaying the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline from lung homogenates. To determine the effects of intrauterine hypertension on lung eNOS content, fetal lung tissue was harvested 8–12 days after intrauterine closure of the ductus arteriosus (DA) performed at 125–128 days of gestation (term = 147 days). Although positive immunostaining for eNOS persisted in lung vascular endothelium, eNOS protein content was reduced by 48%, as measured by Western analysis ($P < 0.001$). Chronic hypertension reduced lung eNOS mRNA content by 30% ($P < 0.05$). Compared with age-matched controls, Ca^{2+}-dependent NOS activity was decreased after DA ligation by 75% ($P < 0.01$). We conclude that chronic intrauterine pulmonary hypertension decreases eNOS in the fetal lung. We speculate that decreased NO production contributes to failure of postnatal adaptation in this experimental model of persistent pulmonary hypertension of the newborn.

endothelium; pulmonary circulation; persistent pulmonary hypertension of the newborn

POSTNATAL SURVIVAL is dependent on successful transition of the pulmonary circulation at birth. Multiple birth-related mechanisms contribute to the normal decrease in pulmonary vascular resistance (PVR) during this transition from fetal to neonatal life (2, 9, 14, 26). These stimuli act in part by altering pulmonary vascular production of vasoactive mediators, including the endothelium-derived relaxing factor or nitric oxide (NO; see Refs. 2, 11, and 16). The endothelial cell releases NO during the conversion of L-arginine to L-citrulline by type III NO synthase (eNOS) under basal conditions and upon activation by physiological or pharmacological stimuli. NO has been shown to play a major role in control of pulmonary vascular tone in the normal fetus and newborn (2, 11, 13, 16). Despite high PVR in the fetus, endogenous NO activity modulates pulmonary vascular tone and reactivity at least as early as 0.75 term in the ovine fetus (29) and contributes to the fetal pulmonary vasodilator response to oxygen, shear stress, and other stimuli (11, 34, 46). In vivo and in vitro studies have demonstrated maturational increases in endothelium-dependent vasodilation in the fetal lung (3, 42). Because inhibition of NO production attenuates the normal fall in PVR after delivery, NO contributes to the dramatic changes in pulmonary vascular tone at birth (2, 11). Whether sustained elevation of PVR and severe hypoxemia in the sick neonate are due to an inability to sustain NO production is unknown.

Persistent pulmonary hypertension of the newborn (PPHN) is a clinical syndrome characterized by sustained elevations of PVR after birth, leading to right to left shunting of blood across the ductus arteriosus (DA) or foramen ovale and severe hypoxemia (32). Although the pathogenesis of PPHN is uncertain, experimental and clinical studies suggest that intrauterine stimuli, such as chronic hypoxia or hypertension, contribute to its etiology (4, 18, 19, 22, 33, 37). For example, clinical studies demonstrated extensive hypertensive structural abnormalities even in neonates dying during the first days of life (19). Early experimental studies suggested that prolonged hypertension in utero alters pulmonary vascular structure in fetal lambs (33). These observations led to the development of an experimental model of perinatal pulmonary hypertension that closely mimics human PPHN (4, 35, 37). In this model, chronic DA closure increases fetal PVR (4), alters pulmonary vasoreactivity (4, 35), and leads to sustained elevation of PVR after delivery (4, 37). Hypertensive structural remodeling of small pulmonary arteries and striking right ventricular hypertrophy further mimic changes observed in fatal clinical PPHN (4, 37).

Mechanisms underlying changes in the pulmonary circulation in this experimental model of perinatal

pulmonary hypertension are uncertain. Past physiological studies have demonstrated preferential impairment of the pulmonary vasodilator response to endothelium-dependent stimuli, including acetylcholine (35), shear stress (4), and oxygen (4), but relative sparing of endothelum-independent vasodilators, including atrial natriuretic peptide (ANP) and inhaled NO (35, 48). Because responses to acetylcholine, oxygen, and shear stress are dependent on NO release (2, 11, 46), we hypothesized that decreased eNOS activity contributes to failure of the pulmonary circulation to achieve and sustain low PVR after birth in experimental PPHN. To test this hypothesis, we measured eNOS content and activity in normal fetal lamb lungs and from lungs harvested from animals after chronic intrauterine pulmonary hypertension caused by DA ligation. We report that chronic intrauterine pulmonary hypertension decreases lung eNOS mRNA, protein, and activity and speculate that impaired NOS activity may contribute to failure of postnatal adaptation.

MATERIALS AND METHODS

Materials. Tri-Reagent was obtained from Molecular Research Center (Cincinnati, OH). Oligo(dT) affinity columns were from 5 Prime-3 Prime (Boulder, CO). The following cDNA fragments were used as probes: 4,091-bp *Eco*R I eNOS cDNA fragment (the eNOS cDNA clone was a kind gift from Dr. W. C. Sessa) and 550-bp *Eco*R I/*Hin*d III β-actin cDNA fragment (American Type Culture Collection, Rockville, MD). The anti-eNOS immunoglobulin (Ig) G_1 monoclonal antibody was kindly provided as a gift from Dr. Jennifer Pollock. Factor VIII monoclonal antibody was obtained from BioGenex Laboratories (San Ramos, CA). L-[U-^{14}C]arginine for the conversion assay was obtained from Amersham (Arlington Heights, IL). 2,4-ADP-Sepharose was purchased from Pharmacia (Piscataway, NJ). Chemicals not specifically mentioned were obtained from Sigma Chemical (St. Louis, MO).

Animals. Procedures used in these studies were previously reviewed and approved by the Animal Care and Use Committee at the University of Colorado School of Medicine. Fetal, neonatal, and adult Columbia-Rambouillet sheep (Nebekar Ranch, Lancaster, CA) were used in this study. Fetal ages ranged from 75 to 145 days (0.51–0.98 term; term is 147 days). Lung tissue was also obtained from neonatal lambs (1–6 days postnatal age) and adult sheep (ewes at 1–14 days after delivery). Ewes with time-dated pregnancies were fasted for 24 h and were sedated with pentobarbital sodium (10 g, total dose). Fetal lambs were rapidly delivered through a uterine incision after injection of pentobarbital sodium in the umbilical artery to prevent spontaneous breathing. Adult sheep were killed with intrajugular injection of high doses of pentobarbital sodium. Immediately after death, a thoracotomy was rapidly performed, and the lungs were isolated, freeze clamped, removed, and stored at −70°C until study. In some animals, the lungs were perfused with agarose (trachea) and paraformaldehyde (pulmonary artery) for immunostaining.

Experimental model of chronic intrauterine pulmonary hypertension. Surgical compression of the DA was performed using slight modifications of previously described techniques (4). Sixteen mixed-breed (Columbia-Rambouillet) pregnant ewes between 126 and 129 days gestation (term = 147 days) were fasted for at least 24 h before surgery. Ewes were sedated with intravenous pentobarbital sodium and were anesthetized with 1% tetracaine hydrochloride by lumbar injection (3 mg). Under sterile conditions, the fetal left forelimb was delivered through a uterine incision. After infiltration of the fetal skin with lidocaine, a left thoractomy was performed to expose the heart and great vessels. After gentle dissection of adherent connective tissue from the DA with cotton-tipped swabs, a saline-soaked umbilical tape was placed around the ductus and was tightened progressively. In some animals, polyvinyl catheters were placed in the main pulmonary artery between the pulmonic valve and DA (10–12 mm) through a purse string suture, using a 16-gauge intravenous placement unit (Angiocath; Travenol, Deerfield, IL). Catheters were also placed in the axillary artery and vein and were gently advanced into the aorta and superior vena cava, respectively. A catheter was placed in the amniotic cavity to measure pressure. The hysterotomy was closed, the uterus was returned to the maternal abdominal cavity, and the catheters were exteriorized via subcutaneous tunnels to an external flank pouch. The ewes recovered rapidly from surgery and were generally standing in their pens within 6 h. Food and water were provided ad libitum. After 8–12 days, animals were killed rapidly after high-dose maternal and fetal infusions of pentobarbital sodium, and lung tissues were harvested for the studies described below.

Lung NOS activity. Lung NOS activity was determined by duplicate measurements of the conversion of L-[U-^{14}C]arginine to L-[U-^{14}C]citrulline based on minor modifications of the original protocol of Salter et al. (39). Peripheral lung tissue (200 mg) was homogenized in 3 vol buffer containing 320 mM sucrose, 50 mM tris(hydroxymethyl)aminomethane (Tris)·HCl, 1 mM EDTA, 1 mM dithiothreitol, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor, and 2 μg/ml aprotinin. Lung homogenates were centrifuged at 4°C at 12,000 *g* for 20 min. Pellets were discarded, and supernatants were placed on ice until assay. The incubation buffer consisted of 50 mM KH_2PO_4, 120 μM NADPH, 1.2 mM L-citrulline, 2.25 μM L-arginine, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 3 μM FAD, 3 μM flavin mononucleotide, 3 μM BH_4, and 0.5 μCi/ml L-[U-^{14}C]arginine. L-Valine (60 mM) was also added to the buffer to minimize potential interference from arginase. Calcium-dependent activity was calculated as the difference beween the L-[U-^{14}C]citrulline produced from control samples containing incubation buffer and samples containing buffer plus 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). Activity of the calcium-independent NOS (inducible or type II NOS) was determined as the difference between samples containing 1 mM EGTA in the incubation buffer and samples containing 1 mM EGTA plus 2 mM N^G-monomethyl-L-arginine (L-NMMA). NOS activity was linear over 1 h and was inhibited completely by L-NMMA. All samples were run in duplicate.

Northern blots for eNOS mRNA. Total RNA was purified from hypertensive and control fetal lungs using Tri-Reagent and the method of Chomcynzski (10). Poly $(A)^+$ mRNA was purified by oligo(dT) affinity chromatography. Poly $(A)^+$ mRNA was quantified by measuring the absorbance at 260 nm. Twenty micrograms of poly $(A)^+$ mRNA per lung sample were analyzed using standard Northern blot and hybridization techniques with cDNA probes. cDNA probes were labeled with a [^{32}P]dCTP using random primer labeling (RTS Random Primer DNA Labeling System; GIBCO-BRL, Gaithersburg, MD; see Ref. 31). High-stringency washing conditions were followed. Bands were quantitated using a Phosphorimager and Imagequant software (Molecular Dynamics, Sunnyvale, CA). Autoradiographs were also obtained by exposure to film (Hyperfilm; Amersham, Arlington Heights, IL). A bovine eNOS cDNA probe was used to detect sheep eNOS

mRNA. Densitometric values were normalized to the signal for β-actin mRNA.

Western blots for eNOS protein. Frozen lung samples were homogenized in cold 50 mM Tris·HCl (pH 7.4) containing 0.1 mM EDTA, 0.1 mM EGTA, 1 mM KCl, 10% glycerol, 20 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.1% 2-mercaptoethanol (2-ME), 1 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, 1 μM pepstatin A, and 5 μg/ml aprotinin (homogenization buffer). Samples were centrifuged at 12,000 *g* for 25 min to remove cell debris and DNA. Soluble fractions were assayed for protein content by the Bradford (8) method, using bovine serum albumin as the standard. Protein (35 mg) was incubated with 70 mg preswollen 2′,5′-ADP-Sepharose (Pharmacia) at 4°C for 1 h and was centrifuged at 3,500 *g* at 4°C for 5 min. Soluble material was discarded, and the Sepharose was washed with the homogenization buffer with 0.5 M NaCl and then without NaCl. Buffer (300 μl) containing 60 mM Tris·HCl (pH 6.8) with 5% glycerol, 2% sodium dodecyl sulfate (SDS), and 5% 2-ME was added to the Sepharose. The mixure was boiled for 10 min and was subjected to SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins in the gel were transferred to nitrocellulose membrane. Membranes were blocked overnight with 5% nonfat dry milk in 40 mM Tris·HCl, pH 7.6, and 300 mM NaCl (TBS), washed, and incubated with the primary monoclonal antibody against eNOS at a dilution of 1:1,000 in 1% nonfat dry milk for 2 h at room temperature. The blot was washed three times and was incubated with a secondary antibody (horseradish peroxidase-conjugated donkey anti-mouse IgG). After washing, bands for eNOS were visualized by enhanced chemiluminescence (ECL kit; Amersham) and were quantitated by densitometry.

Western blot analysis for factor VIII. Western analysis to quantitate lung factor VIII content was performed in lung homogenates, with some modifications of the techniques described above. Briefly, 100 μg protein/lane were subjected to SDS-PAGE and then proteins from the gel were transferred to a nitrocellulose membrane. The blots were blocked with 5% nonfat dry milk in TBS with 0.1% Tween 20. Monoclonal factor VIII antibody (1:200 dilution) in blocking solution was applied overnight at 4°C. Secondary antibody (donkey anti-mouse Ig; Jackson Immunochemicals, West Grove, PA) was diluted to 1:10,000 in blocking solution and was incubated for 90 min at room temperature. After washing, factor VIII bands were visualized by ECL and were quantitated by densitometry. Protein from cultured pulmonary artery endothelial cells was used for positive controls.

Immunostaining for eNOS and factor VIII. At autopsy, lungs are prepared for immunostaining after agarose inflation and paraformaldehyde infusion into the left pulmonary artery, as we have reported (23, 24). The trachea was cannulated with plastic tubing for infusion of low-melt agarose (1%; SeaKem GTC; FMC Bioproducts, Rockland, ME) heated to 65°C. Agarose is infused slowly into the airway by perfusion pump (Masterflex; Cole-Parmer) to allow even inflation of the lung. The left pulmonary artery was cannulated for simultaneous perfusion with buffered saline followed by 1% paraformaldehyde in 0.1 M borate buffer (pH 9.5) at physiological pressure until the effluent from the left atrium was clear. After immersion overnight in 0.45 M sucrose in phosphate-buffered saline (PBS: 0.01 M phosphate, 0.15 M NaCl; pH 7.25–7.5) at 4°C, tissues were frozen and sectioned (10 μm thickness) on poly-L-lysine-coated glass slides for immunostaining. Tissue sections were stained by the avidin-biotin-peroxidase complex method. Endogenous peroxidase activity was blocked by immersing the slides in 0.03% hydrogen peroxide in methanol for 30 min followed by washing in PBS. After nonspecific binding was blocked by incubation with 3% normal horse serum in PBS, the sections were incubated overnight with anti-NOS ascitic fluid diluted in PBS containing 0.05% bovine serum albumin and 0.1% sodium azide. After three washings with PBS, sections were incubated with biotinylated horse anti-mouse IgG (1:100) and freshly prepared avidin-biotin complex (Vectastain) for 30 and 60 min, respectively. Peroxidase activity was detected using glucose oxidase-3,3′-diaminobenzidine with nickel enhancement. Immunohistochemistry was performed with a specific monoclonal eNOS antibody. The specificity of immunostaining was demonstrated by using simultaneous negative and positive controls on serial sections under the same fixation conditions. Negative controls consisted of isotype-matched IgG_2 α-antibody at the same dilution as the specific anti-eNOS antibody. Positive controls consisted of factor VIII polyclonal antibody (Boehringer Mannheim).

Data analysis. In all experiments, *n* represents the number of animals from which lung tissue was studied. Data are expressed as means ± SE. Statistical analysis was performed with the Statview SE software package (Abacus Concepts, Berkeley, CA). Statistical comparisons were performed by using Student's unpaired *t*-test. Significance was accepted at $P < 0.05$.

RESULTS

Chronic DA ligation increases mean pulmonary artery pressure and total pulmonary resistance, without affecting mean aortic pressure (Table 1). Despite chronic hypertension, eNOS immunoreactivity persisted in vascular endothelium in both normal and hypertensive fetal lungs (Fig. 1). As reported in previous studies (4), small pulmonary arteries from animals with chronic intrauterine pulmonary hypertension consistently had increased wall thickness compared with vessels from age-matched control animals. Intrauterine DA ligation decreased lung eNOS mRNA by 30% compared with control animals, as determined by Northern blot analysis ($P < 0.05$; Fig. 2). Chronic intrauterine pulmonary hypertension also reduced eNOS protein by 48 ± 4% ($P < 0.001$; Fig. 3). No differences in lung factor VIII content were found between control and hypertensive lungs (Fig. 4). Compared with age-matched controls, chronic pulmonary hypertension also decreased lung NOS activity (244 ± 72 vs. 623 ± 88 pmol·min^{-1}·mg^{-1}; $P < 0.001$; Fig. 5). As illustrated, the reduction in NOS activity from hypertensive lungs was due entirely to decreased Ca^{2+}-dependent activity [116 ± 66 (hyper-

Table 1. *Hemodynamic effects of DA ligation in late-gestation fetal lambs*

Group	Pulmonary Artery Pressure, mmHg	Aortic Pressure, mmHg	LPA Blood Flow, ml/min	TPR, mmHg·ml^{-1}·min
Control	42 ± 2	41 ± 1	74 ± 2	0.57 ± 0.026
DA ligation	77 ± 5*	40 ± 1	54 ± 9*	1.59 ± 0.26*

Values are expressed as means ± SE; $n = 5$ animals in each group. LPA, left pulmonary artery; TPR, total pulmonary resistance (mean pulmonary artery pressure/LPA blood flow); DA, ductus arteriosus. *$P < 0.05$ between groups.

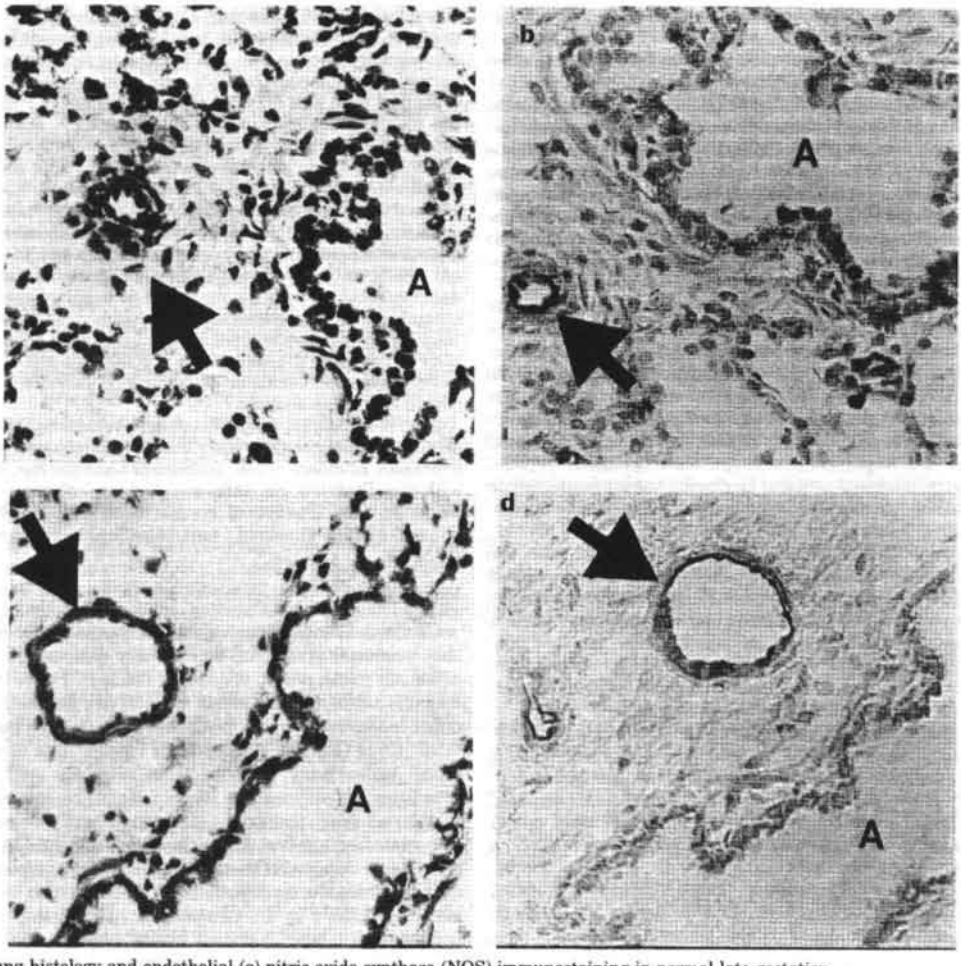

Fig. 1. Lung histology and endothelial (e) nitric oxide synthase (NOS) immunostaining in normal late-gestation lambs and after chronic intrauterine pulmonary hypertension. *a*: Hypertensive structural changes in small pulmonary arteries from lambs after ligation of ductus arteriosus in utero; *c*: example of lung histology from age-matched control; *b* and *d*: persistent immunoreactive eNOS protein in vascular endothelium of pulmonary arteries in lungs from hypertensive (*b*) and control (*d*) lambs. A, airway.

tensive) vs. 452 ± 98 (control) pmol·min^{-1}·mg^{-1}; $P <$ 0.001].

DISCUSSION

On the basis of previous studies that demonstrated the role of endogenous NO activity in the fall in PVR during the normal transition at birth (2), we hypothesized that decreased NO production in the pulmonary circulation may contribute to persistent elevation of PVR after birth in neonatal pulmonary hypertension. To test this hypothesis, we measured eNOS mRNA and protein content and NOS activity in lungs from fetal lambs after normal gestation and in an experimental model of perinatal pulmonary hypertension caused by intrauterine DA closure (4, 35, 37). Compared with age-matched fetal lambs, chronic pulmonary hypertension in utero decreased lung eNOS mRNA levels and protein content and decreased Ca^{2+}-dependent NOS activity. These findings support previous physiological studies that demonstrated preferential impairment of endothelium-dependent pulmonary vasodilation in this experimental model of perinatal pulmonary hypertension (35). We conclude that intrauterine pulmonary hypertension impairs eNOS expression, which may lead to sustained pulmonary hypertension and impaired pulmonary vasodilation at birth.

Previous studies have demonstrated that eNOS is present early in fetal life (24, 38), increases with

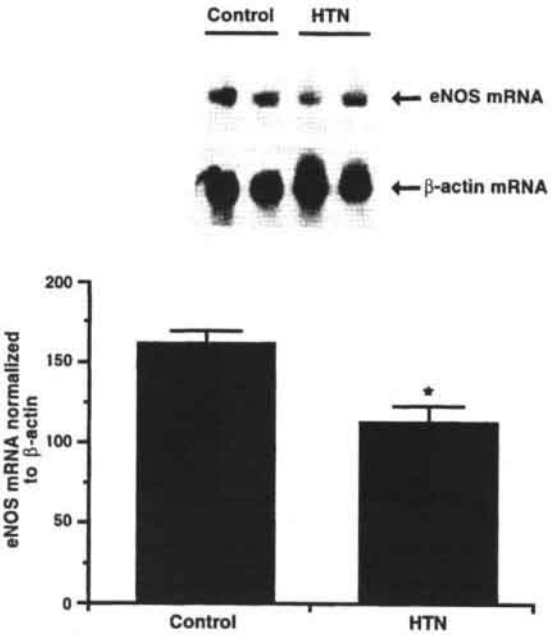

Fig. 2. Effects of chronic intrauterine pulmonary hypertension on lung eNOS mRNA expression. Representative Northern blot analyses for eNOS and β-actin mRNA from lung homogenates from control and hypertensive (HTN) lambs shown at *top* and is quantified by densitometry (*bottom*). As shown, lung eNOS mRNA as normalized to β-actin was reduced by chronic pulmonary hypertension ($n = 4$ animals within each group; $^*P < 0.05$).

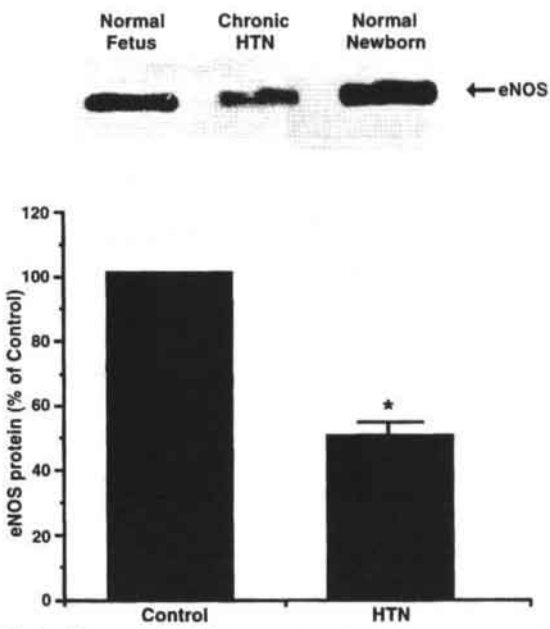

Fig. 3. Effects of chronic intrauterine pulmonary hypertension on lung eNOS protein content. *Top*: representative Western blot analysis of lung eNOS protein from normal late-gestation fetus, hypertensive fetus after chronic ductus ligation, and normal 1-day newborn. *Bottom*: summary data for quantitative densitometry shows decreased eNOS protein content after chronic ligation of ductus arteriosus ($n = 8$ animals within each group; $^*P < 0.001$).

advancing gestational age (29, 38), modulates basal PVR in the normal pulmonary circulation during late fetal life (2), and contributes to the fall in PVR at delivery (2, 11, 16). Lung eNOS mRNA and protein contents are apparently low in the early fetus and increase with advancing fetal age in the rat (38), but intense immunostaining for eNOS protein appears very early in the ovine fetus, at least as early as 0.29 gestation (24). Although eNOS is present in the early fetal lung, previous studies have demonstrated maturation-related changes in endothelium-dependent pulmonary vasodilator activity during the postnatal period (3). In contrast to conduit pulmonary artery rings from newborn and adult sheep, rings from fetal lambs showed little relaxation in response to endothelium-dependent agonists (3, 42). No age differences were observed in the response to the endothelium-indepependent agonist sodium nitroprusside, suggesting that fetal pulmonary arteries have diminished eNOS activity but are fully capable of responding to NO. Whole animal studies also suggest maturational changes in fetal pulmonary vasodilator responses to endothelium-dependent stimuli, including acetylcholine and increased fetal Po_2 (26). In contrast, endothelium-independent agonists that directly increase guanosine 3′,5′-cyclic monophosphate (cGMP) content in vascular smooth muscle, including

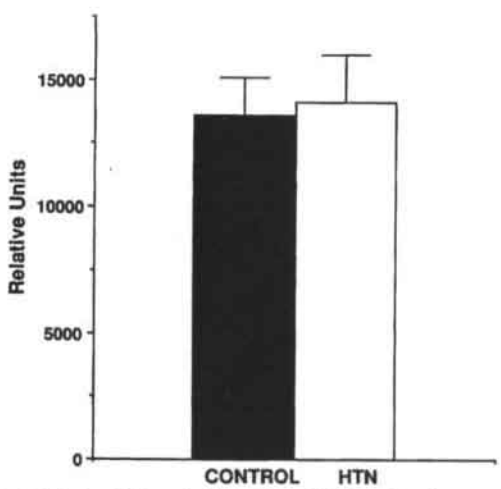

Fig. 4. Western blot analysis for factor VIII-associated antigen in lung homogenates from control and hypertensive lambs. As shown, there was no difference in factor VIII-associated antigen between groups ($n = 4$ animals within each group).

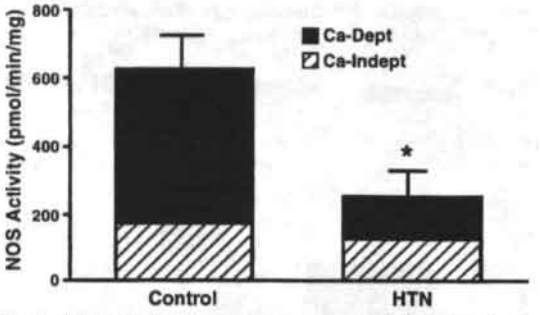

Fig. 5. Effects of chronic intrauterine pulmonary hypertension on NOS activity. As assessed by conversion of L-[U-^{14}C]arginine to L-[U-^{14}C]citrulline in lung homogenates, pulmonary hypertension markedly reduces calcium-dependent (Dept) NOS activity. Indept, independent. *$P < 0.05$.

ANP, 8-bromoguanosine 3′,5′-cyclic monophosphate, and inhaled NO, cause greater and more sustained fetal pulmonary vasodilation than endothelium-dependent agonists (1, 29).

This model of perinatal pulmonary hypertension in the ovine fetus has been used by several investigators to examine mechanisms that lead to failure of postnatal adaptation in the early period after birth (4, 6, 37). Clinical studies of neonates dying shortly after birth with severe PPHN demonstrate striking histological findings of hypertensive structural remodeling in small pulmonary arteries (19). These observations suggest that, in severe PPHN, chronic intrauterine stimuli contribute to its pathogenesis and pathophysiology. Early experimental studies showed that maternal hypoxia may cause smooth muscle thickening in small pulmonary arteries of late-gestation fetal rats, suggesting that chronic hypoxia in utero may contribute to PPHN (22). However, these observations have not been confirmed in recent studies of fetal hypoxia (18). Levin and co-workers (33) first suggested that intrauterine hypertension causes hypertensive structural lesions in the late-gestation ovine fetus. Based on these findings, investigators have demonstrated that chronic pulmonary hypertension, caused by partial or complete closure of the DA, markedly increases pulmonary artery pressure and PVR in utero (4), alters pulmonary vasoreactivity (4, 35), and causes right ventricular hypertrophy (4, 37) and hypertensive structural changes in small pulmonary arteries (4, 37). After delivery, these animals have persistent elevation of PVR despite mechanical ventilation with enriched O_2. These physiological and pathological changes mimic the characteristic clinical and histological findings of human PPHN (32), providing a useful experimental model of severe PPHN for study.

Previous studies of this experimental model have demonstrated preferential impairment of pulmonary vasodilation to acetylcholine, O_2, and shear stress but less attenuation of the dilator response to endothelium-independent agonists, including ANP (35) and inhaled NO (35, 48). As acetylcholine, O_2, and shear stress-induced pulmonary vasodilation are each partly mediated through NO release, we speculated that chronic pulmonary hypertension impairs NOS activity. This hypothesis is now supported by data from this study. After 8–12 days of intrauterine DA compression, Ca^{2+}-dependent NOS activity and eNOS mRNA and protein expression were decreased when compared with normal fetuses of the same gestational age. Therefore, decreased physiological NOS activity is due at least partially to decreased eNOS protein, which contributes to high PVR and altered vasodilator responses to endothelium-dependent stimuli in this experimental model of PPHN. Because NO decreases smooth muscle cell growth in some experimental settings (12, 17), we speculate that decreased eNOS may also contribute to hypertensive remodeling of small pulmonary arteries in utero.

Altered NOS activity has been reported in other animal models of pulmonary hypertension. Loss of endothelium-dependent vasodilaton has been demonstrated in isolated lungs from chronically hypoxic rats (5), and decreased eNOS mRNA and protein were also demonstrated from lungs of patients with pulmonary hypertension (20). In contrast, eNOS is upregulated in adult rats with pulmonary hypertension due to chronic hypoxia (27, 31). Whether decreased eNOS in this model of pulmonary hypertension is due to the type or timing of the stimulus or to differences between species is uncertain. In experimental models of pulmonary hypertension due to chronic hypoxia in adult rats and chronic DA ligation in utero, alternate mechanisms, such as increased endothelin (ET)-1 production, may also contribute to high PVR and structural changes of pulmonary arteries (21, 28). Chronic intrauterine DA compression increases lung ET-1 levels and alters ET receptor activities, which contributes to high PVR in this model (28). This reduction of eNOS with concomitant increased lung ET-1 supports the hypothesis that downregulation of NO, an endogenous vasodilator and antiproliferative factor, and upregulation of ET-1, an endogenous vasoconstrictor and mitogenic factor, contribute to chronic pulmonary hypertension (7, 30, 36). An imbalance between NO and ET-1 production could play a key role in the development of hemodynamic and morphological alterations in PPHN. Altered vasodilation may also involve other components of the NO-cGMP cascade, such as decreased soluble guanylate cyclase (44) and increased or persistent type 5 phosphodiesterase activity (25).

In summary, chronic intrauterine pulmonary hypertension caused by DA closure decreases eNOS mRNA, protein, and activity in the late-gestation fetus. These findings support the hypothesis that adverse intrauterine stimuli, such as hypertension, can cause failure of postnatal adaptation of the pulmonary circulation at birth and may contribute to the pathophysiology of PPHN.

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Chapter VI. Endothelium-derived nitric oxide-dependent response to hypoxia in piglet intrapulmonary arteries.

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Original Paper

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Endothelium-Derived Nitric Oxide-Dependent Response to Hypoxia in Piglet Intrapulmonary Arteries

Abstract

The purpose of this study was to determine the involvement of eicosanoids and nitric oxide (NO) in the response to hypoxia in isolated intrapulmonary (third branch) arteries from 10- to 17-day-old piglets. We also compared the response to hypoxia in pulmonary arteries to pulmonary veins, mesenteric arteries and coronary arteries. Hypoxia was generated in vascular rings (under resting force or precontracted with 30 m*M* KCl) by switching the gas aerating the organ chambers from one composed of 21% O_2-5% CO_2-balance N_2 (pO_2 145 ± 1.27 mm Hg) to a mixture of 5% CO_2-balance N_2 (pO_2 33.87 ± 0.24 mm Hg). In precontracted rings hypoxia produced a transient vasoconstriction (26 ± 8% of the precontraction value) reaching a peak in 3–4 min, followed by a relaxation. A similar pattern of response was observed in pulmonary veins, coronary arteries and mesenteric arteries. The contractile phase was not present in endothelium-denuded arteries or after incubation with the NO synthase inhibitor *L*-NAME (10^{-4} *M*) or the guanylate cyclase inhibitor methylene blue (10^{-5} *M*). No changes in the hypoxia-induced vasoconstriction were observed after preincubation with the NO precursor *L*-arginine (10^{-5} *M*), the cyclooxygenase inhibitor meclofenamate (10^{-5} *M*), the lipoxygenase inhibitor AA 861 (10^{-5} *M*), or the cytochrome P450 oxidase inhibitor SKF 525A (10^{-5} *M*). These findings demonstrate that the contractile response to hypoxia in the isolated intrapulmonary porcine artery is caused by the loss of the inhibitory effects of endothelium-derived NO on the vascular tone. Eicosanoids do not appear to be involved in this response. Since the response to hypoxia in isolated rings is not specific to pulmonary vessels, any correlation between this response and hypoxic pulmonary vasoconstriction should be avoided.

Key Words
Hypoxia
Nitric oxide
Eicosanoids
Endothelium
Pulmonary artery, piglet

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Introduction

Hypoxic pulmonary vasoconstriction (HPV) is an adaptive mechanism by which circulating blood is diverted to better ventilated alveoli optimizing the ventilation/perfusion matching [1, 2]. HPV is a rather unique response specific for the pulmonary vascular bed [1, 2]. During fetal life HPV is at least partially responsible for maintaining high pulmonary vascular resistance. Abolition of this HPV by elevation of arterial pO_2 has been suggested as a mechanism to produce the pulmonary vasodilation that occurs at birth [3–5]. Afterwards, the neonate is at high risk for pulmonary vascular diseases that increase pulmonary vascular resistance and in which HPV plays a determinant role [6].

Despite extensive investigation, the mechanisms producing hypoxic vasoconstriction in the pulmonary circulation are not fully understood. It has been proposed that pulmonary vascular tone increases by alveolar hypoxia due to the reduced release or activity of a vasodilator, the increased release or activity of a vasoconstrictor, and/or because hypoxia directly stimulates contraction of vascular smooth muscle cells [1].

Modulation of vascular tone is one important function of the endothelium [7]. Endothelial cells have the capacity to produce and release several constrictor and dilator substances [8]. Moreover, many agonists influence pulmonary vascular tone via the release of a secondary endothelial mediator. The role of endothelium in the pulmonary vascular response to hypoxia in isolated vessels is unclear. Contradictory results showing abolishment [9–12], as well as enhancement [13, 14], of hypoxia-induced contraction in endothelium-denuded pulmonary arteries have been reported. In addition, it is also unclear whether the endothelium is the target organ which promotes the pulmonary response to hypoxia or acts as a modulator of a response generated on the pulmonary vascular smooth muscle.

Nitric oxide (NO) and eicosanoids are important endothelium-derived vasoactive mediators which actively participate in the control of pulmonary vascular tone [1]. Contradictory results have been reported on the implication of these mediators on the pulmonary vascular response to hypoxia [15–20]. Therefore, the aim of this work was to investigate the response to hypoxia in isolated intrapulmonary arteries and veins from 2-week-old piglets and to evaluate the role of NO and eicosanoids on this response. Furthermore, we also studied the effects of hypoxia in systemic (coronary and mesenteric) arteries and pulmonary veins.

Materials and Methods

Tissue Preparation

Male neonatal piglets (10–17 days of age, 4,106 ± 242 g) were killed by exsanguination and the lungs, hearts, and mesenteric beds were rapidly immersed in cold (4 °C) Krebs solution (composition in m*M*: NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2.0, KH_2PO_4 1.2 and glucose 11) and transported immediately to the laboratory. Pulmonary arteries and veins (third branch, internal diameter 1–2 mm), mesenteric arteries (internal diameter 1–2 mm) and coronary arteries (left anterior descending, internal diameter 0.5–1 mm) were carefully dissected free of surrounding tissue and cut into rings of 2–3 mm in length under a dissection microscope. In some experiments the endothelium was removed by gently rubbing the intimal surface of the rings with a metal rod. Two L-shaped stainless-steel wires were inserted into the arterial lumen and the rings were placed in Allhin organ chambers filled with Krebs solution at 37 °C, gassed with 21% O_2-5% CO_2-74% N_2 (pO_2 145 ± 1.27 mm Hg in the organ chamber as measured by a blood gas analyzer; BGA electrolyte, Instrumentation Laboratory Inc., USA). One wire was attached to the chamber and the other to an isometric force-displacement transducer (Grass FT07) and connected to a polygraph (Grass, Model 7) as previously described [21]. The rings were initially stretched to a resting tension of 1 g (pulmo-

nary arteries, pulmonary veins, and coronary arteries) or 2 g (mesenteric arteries) and allowed to equilibrate for 60–90 min. During this period tissues were re-stretched and washed every 30 min with warm Krebs solution. After equilibration the removal of endothelium was tested by the absence of a relaxant effect of 10^{-6} *M* acetylcholine in rings precontracted with 10^{-6} *M* noradrenaline.

Experimental Protocols

In vascular rings under resting force or submaximally contracted with 30 m*M* KCl, hypoxia was induced by changing from 21% O_2-5% CO_2-74% N_2 to 95% N_2-5% CO_2 gas mixture (pO_2 33.87 ± 0.24 mm Hg). pH and pCO_2 of the experimental solution were the same with either gas mixture (7.39 ± 0.01 vs. 7.40 ± 0.01 and 29.9 ± 0.2 vs. 29.2 ± 0.2 mm Hg, respectively). Hypoxia was maintained for 1 h and then the rings were reoxygenated with the original gas mixture for 15 min.

To test whether NO, eicosanoids or ATP-dependent K^+ channels were involved in the hypoxic response in pulmonary artery rings, 10^{-4} *M* N^{ω}-nitro-*L*-arginine methyl ester (*L*-NAME, a NO synthase, NOS, inhibitor), 10^{-5} *M* methylene blue (a guanylate cyclase inhibitor), 10^{-5} *M* *L*-arginine (the NO precursor), 10^{-5} *M* meclofenamate (a cyclooxygenase inhibitor), 10^{-5} *M* 2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadynyl)-1,4-benzoquinone (AA 861, a lipoxygenase inhibitor), 10^{-5} *M* N,N-diethylaminoethyl-2,2-diphenylvalerate hydrochloride (SKF 525A, a cytochrome P450 oxidase inhibitor) or 10^{-5} *M* glybenclamide (an ATP-dependent K^+ channel antagonist) were added to the bath 30 min before the contraction induced by 30 m*M* KCl.

Drugs

The following drugs were used: (–)-noradrenaline bitartrate, acetylcholine chloride, *L*-NAME, *L*-arginine, methylene blue, glibenclamide (Sigma Chemical Co., London, UK); meclofenamate (Warner Lambert Co., USA); AA 861 (Takeda Co., Japan), or SKF 525A (Research Biochemicals Inc., USA). Drugs were dissolved in deionized distilled water, meclofenamate was dissolved in ethanol and AA 861 was dissolved in dimethylsulfoxide. Further dilutions were carried out in Krebs solution. The concentrations are expressed as the final molar concentration in the tissue chamber.

Statistical Analysis

Results are expressed as means ± SEM of measurements in the number of arteries. The contractile responses are expressed as absolute values (in milligrams) and the relaxant responses as a percentage of the precontractile tone. Statistically significant differences were calculated by means of an unpaired Student's t test. $p < 0.05$ was considered statistically significant.

Results

Isolated intrapulmonary arteries from piglets did not respond to hypoxia while at passive resting tension (fig. 1). Because a submaximal level of active tension was required for the hypoxic response to occur, muscles were precontracted with 30 m*M* KCl (832 ± 76 mg, n = 21). Under these conditions, hypoxia produced a rapid and transient increase in tension (151.4 ± 17.32 mg, n = 21). This contraction reached its maximal value in about 3–4 min, and was followed by a relaxation which was initially fast (first 5–15 min) and slow but sustained afterwards (fig. 1). At the end of the 1 h of hypoxia the mean tension was 225 ± 55 mg below the precontraction level induced by 30 m*M* KCl. Reoxygenation initially produced a small relaxation which was followed by a vigorous and sustained contraction reaching values close to non-hypoxic parallel controls (fig. 1, 2). A similar pattern of response to hypoxia was observed in pulmonary veins, coronary arteries and mesenteric arteries. Representative tracings of the response of the different vessels are shown in figure 2.

The contractile responses to hypoxia of pulmonary artery rings with or without endothelium precontracted with 30 m*M* KCl are compared in figure 3. In endothelium-denuded pulmonary arteries hypoxia-induced contraction was significantly reduced when compared to endothelium-intact arteries (44 ± 7 vs. 151 ± 17 mg, respectively, $p < 0.01$).

Incubation of pulmonary artery rings with *L*-NAME (10^{-4} *M*), a specific inhibitor

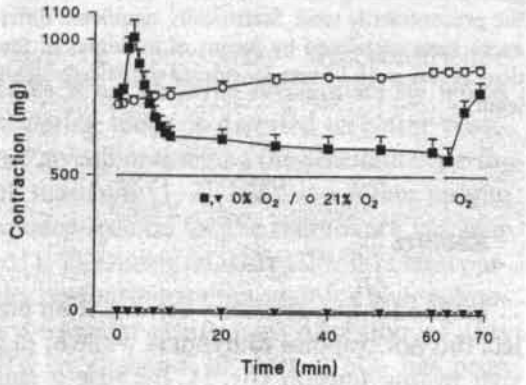
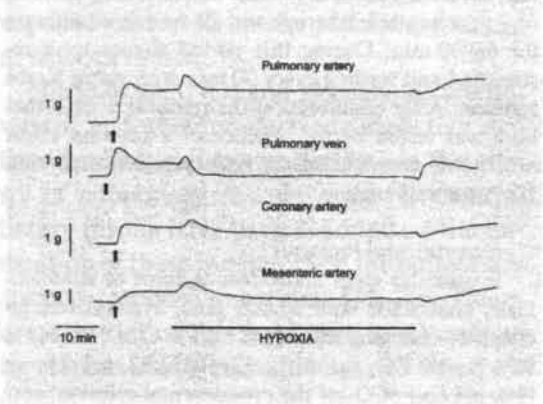


Fig. 1. Time course of the contractile response to hypoxia on piglet pulmonary artery rings at passive resting tension (▼) and after preconstriction with 30 m*M* KCl (■). Muscles were precontracted submaximally with 30 m*M* KCl and, after the plateau of the contractile response was reached, hypoxia was generated by switching the gas bubbling the organ chambers from one composed of 21% O_2-5% CO_2-balance N_2 (pO_2 145 ± 1.27 mm Hg) to a mixture of 5% CO_2-balance N_2 (pO_2 33.87 ± 0.24 mm Hg). After 1 h of hypoxia the rings were bubbled for 15 min with the original gas mixture. Rings constricted with 30 m*M* KCl but not subjected to hypoxia are also shown (○). Each symbol represents the mean ± SEM of 5–8 experiments.



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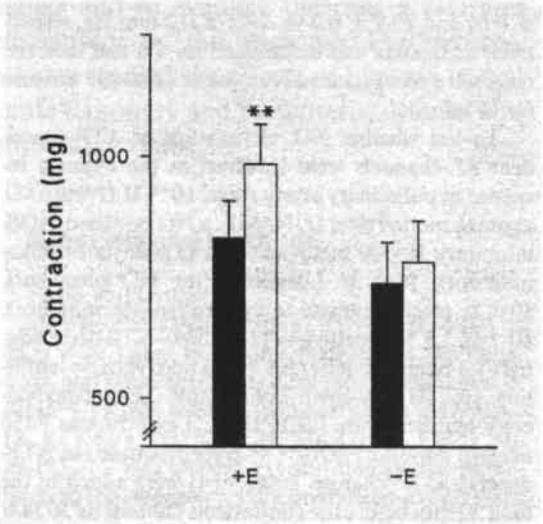

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Fig. 2. Typical tracings of the response to hypoxia in isolated rings of piglet pulmonary artery, pulmonary vein, coronary artery and mesenteric artery. The vessels were initially preconstricted with 30 m*M* KCl as indicated by the arrows and hypoxia was generated when this preconstriction reached steady state (see legend to figure 1).

Fig. 3. Role of endothelium in the contractile response to hypoxia in isolated rings of piglet intrapulmonary arteries. Contractile tension induced by 30 m*M* KCl under normoxia (■) and peak value of hypoxic constriction (□) in endothelium-intact (+E, n = 24) or endothelium-denuded (−E, n = 10) arteries. Each bar represents the mean ± SEM. ** $p < 0.01$ normoxia vs. hypoxia.

of NOS activity, or with methylene blue (10^{-5} *M*), a guanylate cyclase activity inhibitor, produced a contractile response of 263 ± 86 and 357 ± 74 mg, respectively. When 30 m*M* KCl was added, the arteries developed a final tension of 1,087 ± 96 mg (*L*-NAME + 30 m*M* KCl) and 1,105 ± 146 mg (methylene blue + 30 m*M* KCl). This response was not significantly different from the peak level of contraction that developed in response to hypoxia in the control group (984 ± 82 mg). As shown in figure 4, both *L*-NAME and methylene blue markedly reduced ($p < 0.01$) the hypoxic vasoconstriction. To exclude the possibility that the in-

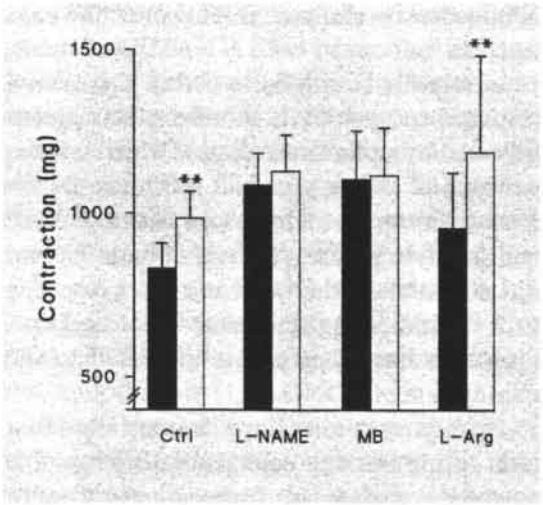

Fig. 4. Effects of *L*-NAME (10^{-4} *M*), methylene blue (MB, 10^{-5} *M*) and *L*-arginine (L-Arg, 10^{-5} *M*) on the contractile response to hypoxia in isolated endothelium intact rings of piglet intrapulmonary arteries. Drugs were added to the bath 30 min before preconstriction with 30 m*M* KCl. Contractile tension induced by 30 m*M* KCl under normoxia (■) and peak value of hypoxic constriction (□). Each bar represents the mean ± SEM of 14–24 experiments. ** $p < 0.01$ normoxia vs. hypoxia.

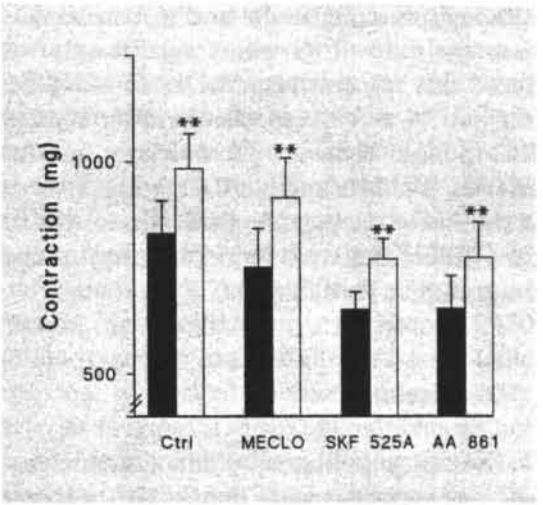

Fig. 5. Effects of meclofenamate (MECLO, 10^{-5} *M*), AA 861 (10^{-5} *M*), and SKF 525 A (10^{-5} *M*) on the contractile response to hypoxia in isolated intact endothelium rings of piglet intrapulmonary arteries. Drugs were added to the bath 30 min before the muscles were precontracted with 30 m*M* KCl. Contractile tension induced by 30 m*M* KCl under normoxia (■) and peak value of hypoxic constriction (□). Each bar represents the mean ± SEM of 11–24 experiments. ** $p < 0.01$ normoxia vs. hypoxia.

crease in the precontraction level seen with *L*-NAME or methylene blue may have limited hypoxic vasoconstriction, the hypoxic vasoconstriction was analyzed in rings in which the precontraction value was raised by the addition of 10^{-5} *M* noradrenaline plus 30 m*M* KCl. Although this combination produced a similar increase in tone (1,059 ± 98 mg, n = 8, $p < 0.05$) to that observed with 30 m*M* KCl plus *L*-NAME or 30 m*M* KCl plus methylene blue, hypoxia produced in these rings a contractile response similar to that obtained in parallel control arteries contracted by 30 m*M* KCl alone (79 ± 14 vs. 95 ± 18 mg, respectively, n = 8, $p > 0.05$).

The effects of the NO precursor *L*-arginine (10^{-5} *M*), the cyclooxygenase inhibitor meclofenamate (10^{-5} *M*), the lipoxygenase inhibitor AA 861 (10^{-5} *M*) and the cytochrome P450 oxidase inhibitor SKF 525A (10^{-5} *M*) on the hypoxic response of pulmonary arteries precontracted with 30 m*M* KCl are shown in figures 4 and 5. None of these agents significantly affected the contractile response to hypoxia in pulmonary arteries. Similarly, the ATP-dependent K^+ channel inhibitor glybenclamide (10^{-5} *M*) had no effect on the contractile response to KCl (899 ± 163 mg) or on the response to hypoxia (128 ± 35 mg, n = 7, $p > 0.05$ vs. control).

To evaluate the effects of the different pretreatments on the second vasodilator response, tension was measured at 15 min and at the end (1 h) of the exposure to hypoxia.

Although meclofenamate and glybenclamide were the only drugs which significantly reduced this relaxant response at 15 min (106 ± 6 and 94 ± 5%, respectively, of the 30 m*M* KCl-induced tone vs. 83 ± 3% in control arteries, $p < 0.01$ and $p < 0.05$, respectively), at the end of the hypoxic challenge no significant differences were found with any of the drugs studied (not shown).

Discussion

Isolated pig pulmonary and systemic vessels precontracted with 30 m*M* KCl respond to hypoxia by displaying an initial rapid increase in tension of short duration followed by a further relaxation. However, this contractile response was not observed in pulmonary arteries at resting tension. Even when the explanation for the requirement of some level of active tone is unknown, this result may indicate that hypoxic vasoconstriction is mediated by withdrawal of vasodilator tone rather than the activation of vasoconstrictor mechanisms. In addition, hypoxia-induced contraction was significantly reduced in endothelium-denuded pulmonary arteries, which suggested that hypoxia counteracts the activity of an endothelium-derived vasodilator. Moreover, the NOS inhibitor *L*-NAME or the guanylate cyclase inhibitor methylene blue raised basal tone in pulmonary arteries and markedly inhibited the hypoxic contractile response, whereas the NO precursor *L*-arginine had no effect. These results suggest that hypoxia produces an inhibition of the release and/or activity of endothelium-derived NO (EDNO). Therefore, *L*-NAME and methylene blue induced a contraction which mimics the initial contractile response to hypoxia (i.e. by removing endothelium-derived relaxing factor/NO, EDRF/NO) and further contraction cannot be achieved by hypoxia since EDRF/NO is already eliminated. However, the contraction induced by both *L*-NAME and methylene blue is sustained, whereas the overall response to hypoxia is more complex and is followed by a relaxant response which is independent of changes in NO synthesis. Moreover, in constrast to the results with *L*-NAME and methylene blue, removal of endothelium did not enhance the vasoconstrictor response to KCl, indicating that removal of endothelium also has other effects unrelated to the elimination of EDNO.

Numerous studies have demonstrated that NO, acting through activation of the soluble guanylate cyclase in smooth muscle cells, plays a major role in the regulation of pulmonary vascular tone both in fetal and postnatal life [1, 22]. NO is produced from *L*-arginine on conversion to *L*-citrulline by the enzyme NOS [23]. The formation of NO by vascular endothelial cells is catalyzed by the endothelial isoform of NOS (eNOS) [23]. Since the first classic report of the EDRF (afterwards identified as NO) [24], it is known that hypoxia inhibits agonist-induced release of NO. In pulmonary arteries from several species, including humans, it has been demonstrated that hypoxia reduced pulmonary endothelium-dependent vasodilation and diminished accumulation of cyclic GMP, suggesting a reduction in pulmonary NO activity [25, 26]. This hypoxia-induced impairment of NO activity is independent of endothelial receptors because NOS activation by calcium ionophores (which act independent of these receptors) is also reduced by hypoxia [26]. However, hypoxia does not seem to impair the ability of NO to activate soluble guanylate cyclase because it did not affect the relaxation mediated by the NO donor sodium nitroprusside [25, 26]. In agreement with the present results, other authors have demonstrated the suppression of the contractile response to hypoxia in isolated pulmonary artery rings

after NOS or soluble guanylate cyclase inhibition [11, 12, 26, 27]. These data may indicate a reduction in NO production by hypoxia. In contrast, Hampl et al. [15] demonstrated an increase in NO synthesis in pulmonary artery endothelial cells under hypoxia. To explain these contradictory data, it has been suggested that severe hypoxia (pO_2 15–30 mm Hg) impairs the release of EDNO, whereas a moderate degree of hypoxia (pO_2 40–45 mm Hg) promotes NO synthesis in the pulmonary artery endothelium [15].

Hypoxia may interfere in several steps of NO synthesis, release or activity. In fact, it has been described that hypoxia inhibits: (1) *L*-arginine uptake by pulmonary artery endothelial cells [28]; (2) the conversion of *L*-citrulline to *L*-arginine in pulmonary artery endothelial cells [29]; (3) NOS activity by limiting the availability of oxygen, a substrate for NOS that is a dioxygenase which catalyses the reaction between molecular oxygen and *L*-arginine [30]; (4) endothelial ATP content [31] which is necessary for agonist-induced production of EDNO; (5) eNOS by reducing the endothelium intracellular Ca^{2+} concentration which primarily regulates eNOS activity [32, 33]; (6) the supply of NADPH and 6-methyltetrahydropterine, both essential cofactors for NOS activity [34], and finally, (7) hypoxia could induce the production of superoxide anions by the endothelial cells that would inactivate EDNO [34].

Eicosanoids are arachidonic acid metabolites which are generated by several cellular types including vascular endothelial cells [35]. Arachidonic acid may be metabolized via two main pathways: the cyclooxygenase pathway leads to the formation of prostaglandins and thromboxane A_2, and the lipoxygenase pathway produces hydroxyeicosatetraenoic acids and leukotrienes. A third pathway, involving a cytochrome P450-linked monooxygenase, may generate several epoxides, hydroxy acids and other products whose physiological importance remains to be clarified [36]. Eicosanoids have been implicated in the regulation of pulmonary vascular tone under physiological and pathological conditions [1]. In the search for humoral mediators for HPV, leukotrienes (C_4, D_4, E_4) and prostaglandin I_2 have been proposed by several authors [17–19]. In our experiments, the inhibition of cyclooxygenase, lipoxygenase or cytochrome P450 monooxygenase did not alter the contractile response of isolated porcine intrapulmonary arteries to hypoxia. Thus, eicosanoids do not appear to be involved in mediating this initial response. In contrast, meclofenamate inhibited the initial relaxant response to hypoxia indicating a role for cyclooxygenase-derived eicosanoids in this early vasodilatation, but it had no effect on the final tone after 1 h of hypoxia. Conflicting results regarding the release of prostacyclin by hypoxia (unchanged, increased or decreased levels) have been reported [37]. Other studies have shown that glybenclamide inhibits the relaxant response to hypoxia suggesting that an opening of ATP-dependent K^+ channels is implicated in this vasorelaxation [27, 37]. Thus, the vasodilator responses to hypoxia deserve further investigation.

Even when small arteries (outer diameter <500 μm) have been identified as the location of contraction in response to alveolar hypoxia [37, 38], pulmonary artery rings of all sizes and from several species contract in response to hypoxia [2, 37]. However, it has been argued that these experiments in isolated vessels may not reflect the physiological mechanisms of HPV [1]. In fact, an in vitro hypoxic contraction has been demonstrated in several systemic arteries [12, 36, 39], whereas HPV is unique to pulmonary vessels. In the present work we demonstrate that a similar pattern of response to hypoxia is produced in pulmonary, coronary and mesenteric arteries and in

pulmonary veins from the same animals. Thus, this is not a specific response for the pulmonary vessels and correlation of this, and other studies using pulmonary vascular rings, with HPV should be avoided.

In summary, the transient hypoxic contractile response observed in isolated piglet pulmonary arteries was due to inhibition of basal EDNO production. Eicosanoids were not involved in this contractile response. Studies with isolated vessels could be useful for the knowledge of some vascular effects of hypoxia but correlation between HPV and hypoxia-induced response on isolated pulmonary arteries is unclear. Both pulmonary and systemic vessels similarly responded to hypoxia whereas HPV is a physiological response specific for the pulmonary vascular bed.

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Chapter VII. **Group B *Streptococcus* and *E. coli* LPS-induced NO-dependent hyporesponsiveness to noradrenaline in isolated intrapulmonary arteries of neonatal piglets.**
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Group B *Streptococcus* and *E. coli* LPS-induced NO-dependent hyporesponsiveness to noradrenaline in isolated intrapulmonary arteries of neonatal piglets

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1 The effects of endotoxin (*E. coli* lipopolysaccharide, LPS) and heat inactivated group B *Streptococcus* (GBS) were studied on the contractile responses to noradrenaline (NA) in isolated pulmonary arteries and on the activity of the constitutive and inducible nitric oxide synthase (NOS) in lung fragments of neonatal piglets.

2 Short-term (≤ 5 h) incubation with LPS ($1\ \mu g\ ml^{-1}$) or GBS (3×10^7 colonies forming units ml^{-1}) did not modify the vascular responsiveness to NA (10^{-8} M–10^{-4} M) in isolated intrapulmonary arteries. However, long-term incubation (20 h) with LPS or GBS produced a significant reduction in the maximal contractile responses and shifted the concentration-response curve for NA downwards.

3 Endothelium removal or the cyclo-oxygenase inhibitor meclofenamate (10^{-5} M) did not affect the GBS- and LPS-induced hyporesponsiveness to NA.

4 The presence of the nitric oxide (NO) precursor, L-arginine (10^{-3} M), 30 min prior to the contractility challenge increased the LPS- and GBS-induced pulmonary vascular hyporesponsiveness to NA. In contrast, the addition, prior to the challenge with NA, of the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M) or coincubation with dexamethasone (3×10^{-6} M), a potent inhibitor of the induction of NOS, or with the protein synthesis inhibitor cycloheximide (10^{-5} M) completely restored the reactivity to NA in LPS- and GBS-treated pulmonary arteries.

5 The incubation for 20 h of lung fragments with LPS and GBS produced a significant increase in the Ca^{2+}-independent (inducible) NOS activity determined by the conversion of radiolabelled L-arginine to citrulline, but did not modify the constitutive NOS activity. This NOS induction was abolished by coincubation with dexamethasone (3×10^{-6} M).

6 These results demonstrated that prolonged incubation with GBS and LPS causes an induction of NOS activity which results in a reduced vascular responsiveness to NA in pulmonary arteries of neonatal piglets. Thus, induction of NOS seems to be responsible for the delayed pulmonary vascular hyporesponsiveness induced by GBS (a Gram-positive) and *E. coli* (a Gram-negative), the most common causal agents of neonatal sepsis.

Keywords: Group B *Streptococcus*; lipopolysaccharide; nitric oxide synthase; pulmonary artery of piglet

Introduction

E. coli, a Gram-negative bacterium, and group B *Streptococcus* (GBS), a Gram-positive bacterium, are the most common causal agents of neonatal sepsis (Anthony, 1985; Guerina, 1991). In general, sepsis is characterized by systemic arterial hypotension, inadequate tissue perfusion and decreased responses of vascular smooth muscle to exogenous vasoconstrictors (Groeneveld *et al.*, 1988). The pulmonary system also demonstrates important abnormalities that include changes in pulmonary haemodynamics and lung mechanics, increased vascular permeability and hypoxemia, and more subtle changes in responses of both airway and the pulmonary circulation to constrictor stimuli (Brigham & Meyrick, 1986). Lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria, is the endotoxin presumed to cause injury to the lungs as well as to other organs (Thiemermann, 1994). Gram-positive bacteria do not contain LPS and a toxin common to all Gram-positive organisms has not been identified. However, intravenous infusions of GBS and other Gram-positive bacteria cause pulmonary haemodynamic and gas exchange abnormalities similar to those observed in LPS-injected animals (Rojas *et al.*, 1983; Schreiber *et al.*, 1992) suggesting a common pathway leading to these abnormalities (Auguet *et al.*, 1992).

There is evidence that enhanced release of NO plays an important role in the loss of systemic (McKenna, 1990; Julou-Schaffer *et al.*, 1990; Moncada *et al.*, 1991; for a review see Thiemermann, 1994) and pulmonary (Szabó *et al.*, 1993; Zelenkov *et al.*, 1993) vascular responsiveness that occurs in sepsis. There are at least two distinct isoforms of NO synthase (NOS) which catalyzes the formation of NO from L-arginine (Moncada *et al.*, 1991; Stuehr & Griffith, 1992). A constitutive, Ca^{2+}-dependent isoform (cNOS), is present in vascular endothelial cells. In addition, LPS and cytokines induce a Ca^{2+}-independent, isoform (iNOS) in the lung (Knowles *et al.*, 1990), macrophages (Marletta *et al.*, 1988), endothelial cells (Radomski *et al.*, 1990) and vascular smooth muscle (Busse & Mülsch, 1990). Induction of iNOS has been found in rat pulmonary arteries incubated with LPS (Zelenkov *et al.*, 1993) and in rat lung after exposure to endotoxin (Salter *et al.*, 1991; Szabó *et al.*, 1993; Thiemermann, 1994). Unfortunately, the effects of GBS on vascular reactivity and on cNOS and iNOS are still unknown. Therefore, the aim of this work was: (1) to study and compare the effects of GBS with those of LPS on the contractile responses to noradrenaline (NA) in intact and endothelium-denuded pul-

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monary arteries of neonatal piglets, and (2) to determine whether GBS- and LPS-mediated pulmonary vascular hyporesponsiveness could be related to the activation of cNOS and/or iNOS.

Methods

Tissue preparation and incubation

Male neonatal piglets (10–17 days of age, 4277 ± 343 g) obtained from the local abattoir were used in this study. Piglets were killed by exsanguination and the lungs were rapidly immersed in cold (4°C) Krebs solution containing ampicillin (10 μg ml^{-1}) and gentamicin (10 μg ml^{-1}) and transported immediately to the laboratory. The third branch of the pulmonary arteries (internal diameter 1–2 mm) was carefully dissected free of parenchyma and connective tissue and cut into rings of 2–3 mm of length. Pulmonary rings were then incubated in Krebs solution (composition in mM: NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2.0, KH_2PO_4 1.2 and glucose 11) containing ampicillin (10 μg ml^{-1}) and gentamicin (10 μg ml^{-1}). The solution was gassed with 95% O_2 and 5% CO_2 and maintained at 37°C.

The pulmonary artery rings were initially incubated in Krebs soltuion in the presence of vehicle, LPS (1 μg ml^{-1}) or heat inactivated GBS (3×10^7 colony forming units, c.f.u. ml^{-1}) for 1, 5 or 20 h. To confirm that LPS and GBS induced iNOS, in some experiments pulmonary rings were coincubated for 20 h in Krebs solution containing dexamethasone (3×10^{-6} M), an inhibitor of NOS induction, (Radomski *et al.*, 1990) or cycloheximide (10^{-5} M, an inhibitor of protein synthesis). After incubation, two L-shaped stainless-steel wires were inserted into the arterial lumen and the rings were introduced in Allhin organ chambers filled with Krebs solution. One wire was attached to the chamber and the other to an isometric force-displacement transducer (Grass FT07) and connected to a polygraph (Grass, model 7) as previously described (Pérez-Vizcaíno *et al.*, 1993). The rings were stretched to a resting tension of 0.5 g and allowed to equilibrate for 60–90 min. During this period tissues were restretched and washed every 30 min with warm Krebs solution. In some experiments the endothelium was removed by gently rubbing the intimal surface of the rings with a metal rod. The presence of functional endothelium was verified by addition of acetylcholine (ACh, 10^{-6} M) in arteries precontracted with NA. The ability of ACh to induce relaxation of unrubbed rings was taken as an indicator of the presence of functional endothelium.

Experimental protocol

In pulmonary rings previously incubated with vehicle, LPS or GBS for 1, 5 or 20 h, concentration-response curves to NA (10^{-8} M to 10^{-4} M) were constructed by increasing the organ chamber concentration by cumulative increments after a steady state response had been reached with each increment. In three groups of intact pulmonary arteries, following the incubation period of 20 h, cumulative concentration-response curves to NA were performed in the presence of N^G-nitro-L-arginine-methyl ester (L-NAME, 10^{-4} M, an inhibitor of both cNOS and iNOS, Sakuma *et al.*, 1988), L-arginine (10^{-5} M, the precursor of NO, Palmer *et al.*, 1988) or meclofenamate (10^{-5} M, an inhibitor of cyclo-oxygenase) which were added 30 min before the concentration-response curve to NA was obtained.

Assay of NOS

Lung fragments (weight 100–150 mg) were incubated in the same conditions as described for the arterial rings. After incubation, tissues were immediately stored at −80°C until studied. The frozen tissues were homogenized at 0–4°C in 5 vols of a buffer containing 320 mM sucrose, 50 mM Tris, 1 mM EDTA, 1 mM DL-dithiothreitol, 100 μg ml^{-1} phenylmethylsulfonyl fluoride, 10 μg ml^{-1} leupeptin, 100 μg ml^{-1} soybean trypsin inhibitor and 2 μg ml^{-1} aprotinin brought to pH 7.0 at 20°C with HCl. The homogenates were then centrifuged at 4°C at 12000 *g* for 20 min. The pellets were discarded and the supernatants were placed on ice until incubation the same day. NOS activity was determined by measuring in duplicate the conversion of L-[U-^{14}C]-arginine to L-[U-^{14}C]-citrulline by 10 min incubation at 37°C as described in detail by Salter *et al.* (1991). The incubation buffer contained 50 mM L-valine to minimize any interference from arginase. Ca^{2+}-dependent NOS (cNOS) activity was calculated from the difference between the L-[U-^{14}C]-citrulline produced from control samples containing incubation buffer and samples containing buffer plus 1 mM EGTA. The activity of the Ca^{2+}-independent NOS (iNOS) was determined from the difference between samples containing 1 mM EGTA in the incubation buffer and samples containing 1 mM EGTA plus 2 mM N^G-monomethyl-L-arginine (a competitive inhibitor of NOS, Palmer *et al.*, 1988).

Drugs and heat-killed GBS preparation

The following drugs were used: (−)-noradrenaline bitartrate, acetylcholine chloride, lipopolysaccharide from *E. coli* (serotype 055:B5), dexamethasone, cycloheximide, L-NAME, L-arginine (Sigma Chemical Co., London) and meclofenamate (Warner Lambert Co., U.S.A.). Meclofenamate was dissolved in absolute ethanol. The concentrations are expressed as final molar concentration in the tissue chamber. L-[U-^{14}C]-arginine was obtained from Amersham International (U.K.).

GBS type III was isolated from the blood of a neonate who developed early-onset sepsis. Bacteria were grown in Todd-Hewitt broth for 18–36 h at 37°C to late log phase and harvested by centrifugation at 5000 r.p.m. for 15 min. Bacteria were resuspended in sterile isotonic saline to a concentration determined by serial viable counts to be 1×10^9 c.f.u. ml^{-1}. Heat-killed bacteria were obtained by heating bacteria to 60°C for 60 min. GBS killing was confirmed by no growth on blood agar. Endotoxin levels in the heat-killed GBS preparation were undetectable as assayed by a standard Limulus assay kit (Sigma). Aliquots of heat-killed GBS were stored at −80°C until the study day.

Statistical analysis

Results are expressed as means ± s.e.mean of measurements in *n* arteries. Individual cumulative concentration-response curves to NA were fitted to a logistic equation. In most of the experiments the point corresponding to 10^{-4} M NA was below the maximal tension and was therefore excluded from the fitting. The drug concentration exhibiting 50% of the maximal contraction to NA was calculated for each ring and expressed as negative log molar (pD_2). The E_{max} was defined as the maximal tension induced by NA in each ring. Statistically significant differences were calculated by means of an ANOVA analysis followed by a Newman Keuls means comparison testing. $P < 0.05$ was considered statistically significant.

Results

Effects of LPS and GBS in the presence or absence of endothelium

Short-term incubation of intact pulmonary arteries with LPS (1 μg ml^{-1}) or GBS (3×10^7 c.f.u. ml^{-1}) for 1 or 5 h produced no significant effects on the concentration-response curve to NA (Table 1). However, as shown in Figure 1a, when the endothelium-intact arteries were incubated for a

longer period (20 h) with LPS (1 μg ml^{-1}) or GBS (3×10^7 c.f.u. ml^{-1}) and then transferred to the organ bath in the absence of bacterial products, the maximal contractile response to NA was reduced and thus, a downward shift of the concentration-response curve to NA was observed. This was accompanied by a small, but significant, decrease of the pD_2 values (Table 1). These results indicate that pulmonary vascular hyporesponsiveness to NA is a delayed process and suggested the induction of a biological activity. Endothelium-denuded arteries showed significantly greater maximal responses to NA as compared to endothelium-intact arteries ($P<0.01$) incubated with vehicle, LPS or GBS (Table 1). Nevertheless, mechanical removal of endothelial cells did not affect the hyporeactivity to NA observed after long-term incubation (20 h) with LPS or GBS (Figure 1b).

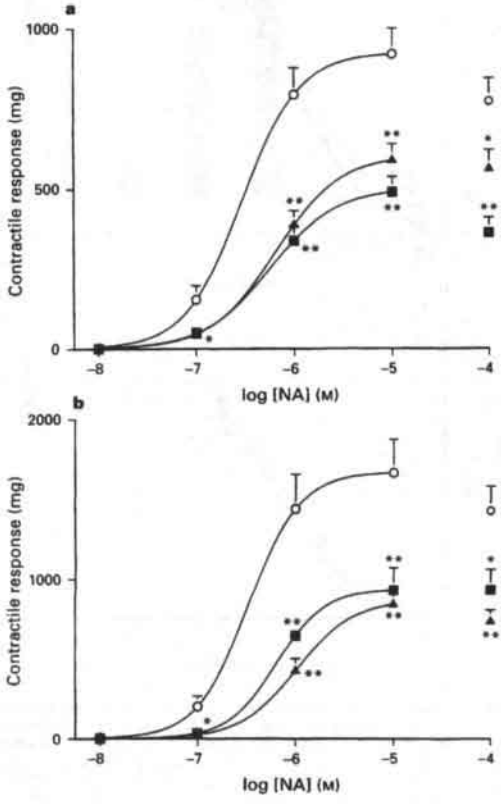

Figure 1 Concentration-response curve to noradrenaline (NA, 10^{-8}–10^{-4} M) in intrapulmonary arterial rings (a) with or (b) without endothelium, preincubated for 20 h in Krebs solution in the presence of vehicle (control, ○), *E. coli* lipopolysaccharide (LPS, 1 μg ml^{-1}, ■) or heat inactivated group B *Streptococcus* (GBS, 3×10^7 c.f.u. ml^{-1}, ▲). The concentration-response curve to NA was carried out in the absence of bacterial products. Data are expressed as means ± s.e.mean of 7–22 observations. $^*P<0.05$ and $^{**}P<0.01$ represent significant differences with respect to control group.

Effects of L-arginine, L-NAME and meclofenamate

In order to assess the role of NO production in the hyporesponsiveness to NA, a concentration-response curve to NA was performed in control, LPS- and GBS-treated arteries acutely treated with either the NO-precursor, L-arginine (10^{-5} M), or the NOS inhibitor, L-NAME (10^{-4} M). As shown in Figure 2a and Table 1, in control arteries treated with L-arginine the concentration-response curve to NA was not modified compared to that obtained in arteries in the absence of L-arginine. In contrast, in arteries incubated with LPS or GBS for 20 h, the presence of L-arginine potentiated ($P<0.01$) the hyporesponsiveness to NA. Addition of 10^{-4} M L-NAME to resting arteries produced a small contractile effect averaging 45.7 ± 6.6 mg, 44.3 ± 9.6 mg and 43.1 ± 5.9 mg, in untreated and in arteries treated with LPS and GBS, respectively. Thereafter, L-NAME induced an upward shift of the concentration-response curve to NA in the LPS- and GBS-treated arteries resulting in no differences between control, LPS- or GBS-treated arteries (Figure 2b). Therefore, L-NAME reversed the hyporesponsiveness to NA induced by LPS and GBS.

The possible role of vasodilator prostaglandins in the hyporeactivity to NA was assessed by acute treatment of the arteries with the cyclo-oxygenase inhibitor, meclofenamate. The presence of 10^{-5} M meclofenamate did not affect the hyporesponsiveness to NA in LPS- and GBS-treated arteries (Table 1).

Effect of dexamethasone and cycloheximide

In order to assess the induction of NOS by LPS and GBS, intact pulmonary arteries were simultaneously incubated with LPS and GBS and 3×10^{-6} M dexamethasone or 10^{-6} M cycloheximide for 20 h. As shown in Figure 3, both dexamethasone and cycloheximide completely restored the reactivity to NA in LPS- and GBS-treated arteries. Moreover, dexamethasone (but not cycloheximide)-treated arteries showed greater pD_2 values in control arteries (Table 1).

Table 1 Effects of *E. coli* lipopolysaccharide (LPS, 10 μg ml^{-1}) and group B *Streptococcus* (GBS, 3×10^7 c.f.u. ml^{-1}) on the parameters (E_{max} and pD_2) of the concentration-response curve to noradrenaline (NA) in the absence and presence of various inhibitors

			Control			LPS			GBS		
Drug		*t*(h)	E_{max} (mg)	pD_2	n	E_{max} (mg)	pD_2	n	E_{max} (mg)	pD_2	n
None	+E	1	1198 ± 141	6.39 ± 0.15	11	1212 ± 153**	6.47 ± 0.43	9	1157 ± 245**	6.31 ± 0.21	8
None	+E	5	1192 ± 212	6.37 ± 0.08	9	1111 ± 127**	6.66 ± 0.15*	8	876 ± 160	6.51 ± 0.19	9
None	+E	20	923 ± 80	6.52 ± 0.07	17	492 ± 47**	6.25 ± 0.10*	13	613 ± 52**	6.17 ± 0.08**	22
None	−E	20	1676 ± 208**	6.46 ± 0.08	7	946 ± 140*****	6.17 ± 0.08*	7	877 ± 68***	5.98 ± 0.10**	8
Meclofenamate	+E	20	1026 ± 98	6.49 ± 0.08	11	585 ± 55**	6.44 ± 0.06	11	450 ± 77**	6.10 ± 0.04	11
L-Arginine	+E	20	1032 ± 124	6.39 ± 0.09	8	299 ± 50***#	6.07 ± 0.08	11	341 ± 77***##	6.22 ± 0.13	9
L-NAME	+E	20	1047 ± 120	6.72 ± 0.11	10	1023 ± 99**#	6.49 ± 0.09	14	1164 ± 55**#	6.66 ± 0.08**#	12
Dexamethasone	+E	20	876 ± 63	6.89 ± 0.06*#	14	923 ± 62**#	6.72 ± 0.08*#	11	831 ± 64*#	6.82 ± 0.06**#	11
Cycloheximide	+E	20	879 ± 95	6.64 ± 0.07	7	1107 ± 103**#	6.71 ± 0.09*#	7	911 ± 82	6.66 ± 0.11	8

t = incubation period. +E = endothelium intact, −E = endothelium denuded. Meclofenamate (10^{-5} M), L-arginine (10^{-5} M) or N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M) were added to the organ bath solution 30 min before the addition of NA. Dexamethasone (3×10^{-6} M) or cycloheximide (10^{-5} M) were included during the 20 h incubation period.
$^*P<0.05$ and $^{**}P<0.01$ GBS- or LPS-treated vs control arteries. $^\#P<0.05$ and $^{\#\#}P<0.01$ vs arteries +E, 20 h, no drug. The drug concentration exhibiting 50% of the maximal contraction to NA was calculated for each ring and expressed as negative log molar (pD_2). The E_{max} was defined as the maximal tension induced by NA in each ring.

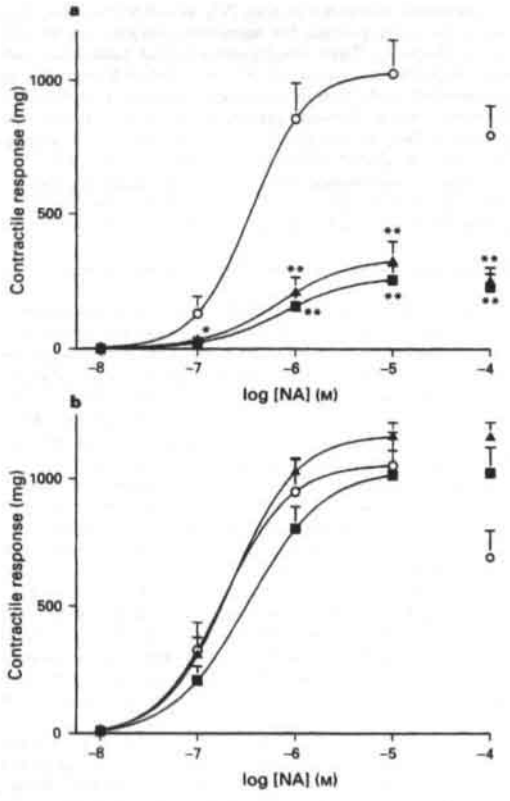

Figure 2 Effects of (a) L-arginine (10^{-3} M) or (b) N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M) on *E. coli* lipopolysaccharide (LPS)- and group B *Streptococcus* (GBS)-induced hyporeactivity to noradrenaline (NA) in intrapulmonary artery rings. Concentration-response curves to noradrenaline (NA, 10^{-8}–10^{-4} M) were performed in intrapulmonary arterial rings previously incubated for 20 h in Krebs solution in the presence of vehicle (control, ○), LPS (1 μg ml^{-1}, ■) or heat-inactivated GBS (3×10^7 c.f.u. ml^{-1}, ▲). L-Arginine (10^{-3} M) or L-NAME (10^{-4} M) was added 30 min before addition of NA. The curve was obtained in the absence of bacterial products. Data are expressed as means ± s.e.mean of 8–14 observations. $^*P<0.05$ and $^{**}P<0.01$ represent significant differences with respect to control group.

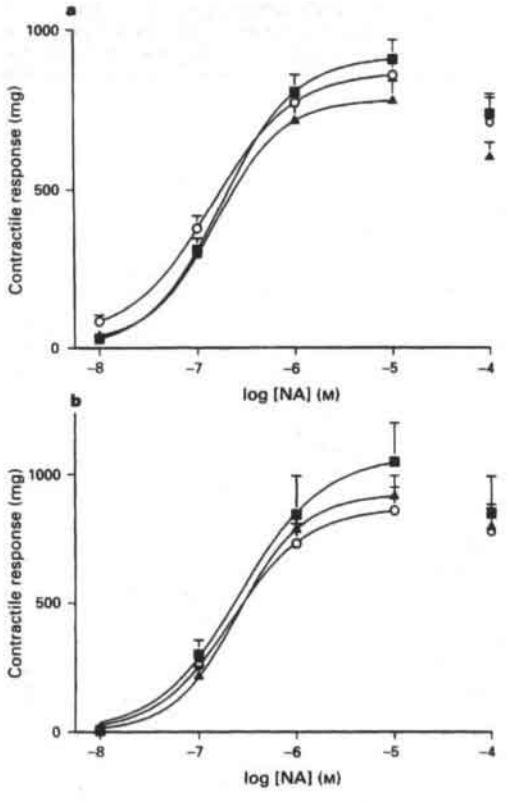

Figure 3 Effects of (a) dexamethasone (3×10^{-6} M) or (b) cycloheximide (10^{-3} M) when coincubated with *E. coli* lipopolysaccharide (LPS) and group B *Streptococcus* (GBS). Concentration-response curves to noradrenaline (NA, 10^{-8}–10^{-4} M) were performed in intrapulmonary arterial rings previously incubated for 20 h in Krebs solution in the presence of vehicle (○), LPS (1 μg ml^{-1}, ■) or heat-inactivated GBS (3×10^7 c.f.u. ml^{-1}, ▲). The curve was obtained in the absence of bacterial products. Data are expressed as means ± s.e.mean of 7–14 observations. $^*P<0.05$ and $^{**}P<0.01$ represent significant differences when compared to control group.

Effects of LPS and GBS on NOS activity

NOS activity was measured by the conversion of radiolabelled L-arginine to citrulline (Salter *et al.*, 1991). Using this method, NOS activity was almost undetectable in pulmonary arteries. Therefore, the cNOS (Ca^{2+}-dependent) and iNOS (Ca^{2+}-independent) activities were determined in lung fragments. Control values for cNOS and iNOS activities were 61.5 ± 10.7 and 36.0 ± 6.4 pmol min^{-1} mg^{-1} of tissue, respectively. As shown in Figure 4, no change in cNOS activity was detected after incubation with 1 μg ml^{-1} LPS or 3×10^7 c.f.u. ml^{-1} GBS for 20 h. In contrast, LPS and GBS significantly increased the iNOS activity ($P<0.01$). Dexamethasone (3×10^{-6} M) produced no change in cNOS activity but completely abolished the LPS- and GBS-induced increase in iNOS activity (Figure 4).

Discussion

The present results demonstrated that only after prolonged (> 5 h) incubation, did LPS and GBS reduce vascular responsiveness to NA in isolated pulmonary artery rings of neonatal pigs. After incubation, the experiments were performed in the absence of LPS or GBS which indicated that the hyporesponsiveness was a delayed process that was not due to a direct action of bacterial toxins but more likely to the induction of biological activity. The decreased response did not require the presence of an intact endothelium and was not mediated by products of cyclo-oxygenase activity since it was unaffected by endothelium removal or the cyclooxygenase inhibitor, meclofenamate, respectively. Our results strongly suggested that induction of iNOS is implicated in the GBS- and LPS-induced pulmonary vascular hyporesponsiveness to NA. To our knowledge this is the first paper showing that GBS produces hyporesponsiveness to NA and induction of iNOS activity.

There is a growing consensus that NO plays a major role in the control of pulmonary vascular smooth muscle tone (Moncada *et al.*, 1991; Dinh-Xuan, 1992; Stamler *et al.*, 1994). Thus, an impaired release of NO has been associated with an increased pulmonary vascular reactivity to constrictor stimuli and may contribute to the pathogenesis of pulmonary hypertension (Moncada *et al.*, 1991; Dinh-Xuan, 1992). In the present experiments, L-arginine potentiated the reduced vascular reactivity to NA in pulmonary arteries of

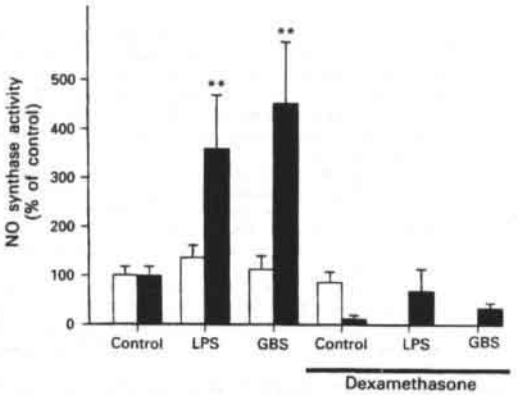

Figure 4 Ca^{2+}-dependent (constitutive, open columns) and Ca^{2+}-independent (inducible, solid columns) nitric oxide synthase (NOS) activities in lung fragments incubated for 20 h in 37°C oxygenated Krebs solution in the presence of vehicle (control), *E. coli* lipopolysaccharide (LPS, 1 μg ml^{-1}) or group B *Streptococcus* (GBS, 3×10^7 c.f.u. ml^{-1}) and in the absence or presence of dexamethasone (3×10^{-6} M). Data are expressed as percentage of the control Ca^{2+}-dependent and Ca^{2+}-independent activities, respectively (means ± s.e.mean of 5–7 observations). Control values for cNOS and iNOS activities were 61.5 ± 10.7 and 36.0 ± 6.4 pmol min^{-1} mg^{-1} of tissue, respectively. $^{**}P < 0.01$ represent significant differences when compared to control group.

newborn piglets incubated with LPS and GBS, while L-NAME completely reversed it. Moreover, dexamethasone, which inhibits the induction of NOS (Radomski *et al.*, 1990) and cycloheximide, an inhibitor of protein synthesis when coincubated with LPS or GBS for 20 h, completely reversed the reduced response to NA. All these results suggest that GBS- and LPS-induced pulmonary vascular hyporesponsiveness to NA is associated with enhanced formation of NO via the iNOS and requires the synthesis *de novo* of the enzyme in the pulmonary vasculature. However, in pulmonary arteries the NOS activity was below the limit of detection. This can be related to the fact that cNOS and iNOS activities in vascular smooth muscle are 5–50 times lower than in lung (Salter *et al.*, 1991; Mitchell *et al.*, 1993). Thus, we assessed the effects of LPS and GBS on both cNOS and iNOS in lung fragments. LPS and GBS had no effect on cNOS but induced a marked increase in iNOS activity which was abolished when lung fragments were incubated with dexamethasone. GBS- and LPS-induction of iNOS in the lung supported the functional evidence of induction of iNOS in pulmonary arteries. As previously reported in rat and rabbit lung (Salter *et al.*, 1991; Mitchell *et al.*, 1993; Szabo *et al.*, 1993), we found that iNOS activity was expressed basally in porcine lung tissue. Since basal iNOS activity was lowered by dexamethasone, it might indicate a certain endotoxin contamination of the incubation media.

GBS is able to produce sepsis only in the neonate and exceptionally in parturients and immunodepressive patients (Anthony, 1985). The mechanism of GBS-induced pulmonary injury in neonates is still a matter of controversy. Gram-positive bacteria do not contain LPS and a common toxin to all Gram-positive organisms has not been identified. It has been shown recently that lipoteichoic acid (LTA), a component of the peptidoglycan layer of the cell wall in most Gram-positive bacteria, decreased the responses to pressor agents and induced iNOS in cultured vascular smooth muscle cells, rat aorta (Auguet *et al.*, 1992) and in anaesthetized rats (De Kimpe *et al.*, 1994). Killed whole *S. aureus* also induced NOS in macrophages (Cunha *et al.*, 1993). Clinical isolates of GSB, including type III, recovered from infants with sepsis, possessed significantly higher levels of LTA in their cell wall than those isolated from asymptomatic carriers (Nealon & Mattingly, 1983). The molecular analysis of GBS LTA demonstrated a close similarity with the Group A *Streptococcus* LTA (Maurer & Mattingly, 1991). Thus, the LTA component of GBS might be responsible for the effects on vascular reactivity and iNOS induction described in the present paper. In addition, two different GBS polysaccharide toxins producing pathophysiological changes mimicking those of GBS infection in neonates have been identified, a non specific mannan polysaccharide (Hellerqvist *et al.*, 1987) and the type III specific capsular polysaccharide (Hemming *et al.*, 1984). However, the type III specific capsular polysaccharide does not seem to be required for the acute phase but it may play a role in the late phase (>2 h) of GBS-induced pulmonary haemodynamic alterations in piglets (Gibson *et al.*, 1989). In the present experiments we used heat-inactivated GSB, i.e. an unfragmented capsule, isolated from the blood of a neonate who developed early-onset sepsis. Heat-inactivated GBS has been previously shown to cause similar haemodynamic effects as the live GBS (Schrieber *et al.*, 1992). Further studies would be necessary to elucidate the specific component of GBS responsible for NOS induction.

The lung is a major target organ in neonatal sepsis (Ablow *et al.*, 1976). In general, the lung plays a determinant role in the sepsis in some animal species that exhibit a pulmonary bacterial clearance (e.g. sheep, pig) but not in species (e.g. dog, rat, rabbit) in which bacteria localize predominantly in the liver and spleen (Winkler, 1988). The uptake of bacteria by pulmonary intravascular macrophages and the subsequent release of inflammatory mediators are central to the pathological changes produced in sepsis-induced adult respiratory distress syndrome in sheep (Warner *et al.*, 1987) and in GBS-induced pulmonary hypertension in newborn piglets (Bowdy *et al.*, 1990). In these models, as in man, sepsis produces a complex pulmonary response in which two different phases have been delimited (Brigham & Meyrick, 1986). Initially there is a marked increase in pulmonary artery pressure and hypoxemia, whereas the second phase is characterized by a returning of pulmonary pressure towards baseline and an increased lung vascular permeability. This latter phase is accompanied by marked increases in guanosine 3':5'-cyclic monophosphate (cyclic GMP, Snapper *et al.*, 1983). NO-mediated vasorelaxation has been correlated with the activation of soluble guanylate cyclase which in turn increases the intracellular concentrations of cyclic GMP (Ignarro *et al.*, 1987; Moncada *et al.*, 1991). Thus, the increased production of NO following the induction of iNOS in pulmonary arteries described here could be responsible for the previously reported increased cyclic GMP production which is a main feature of the latter phase of the pulmonary response to sepsis.

In conclusion, the present results demonstrated that prolonged incubation with GBS, as previously reported with LPS, causes an induction of iNOS which results in a loss of vascular responsiveness to NA in intrapulmonary arteries of neonatal piglets. Thus, the induction of iNOS seems to be a key mediator in the delayed pulmonary vascular hyporesponsiveness characteristic of the pulmonary injury induced by either Gram-positive or Gram-negative sepsis.

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Chapter VIII. **Lack of endotoxin-induced hyporesponsiveness to U46619 in isolated neonatal porcine pulmonary but not mesenteric arteries.** (J Vasc Res. 1996; 33:249-57).

Research Paper

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Lack of Endotoxin-Induced Hyporesponsiveness to U46619 in Isolated Neonatal Porcine Pulmonary but Not Mesenteric Arteries

Key Words
Endotoxin
U46619
Nitric oxide
Pulmonary artery
Piglet

Abstract
The effects of endotoxin from *Escherichia coli* on the vasoconstrictor responses to noradrenaline (10 n*M*–100 μ*M*) and the thromboxane A_2 analog U46619 (100 p*M*–1 μ*M*) were evaluated on isolated pulmonary and mesenteric arteries from neonatal piglets. Incubation for 20 h with endotoxin (1 μg ml^{-1}) induced a decrease in the contractile responses to noradrenaline in both arteries ($p < 0.05$) which was inhibited by N^G-nitro-*L*-arginine-methyl ester (*L*-NAME, 100 μ*M*). Endotoxin-treated mesenteric arteries also showed a reduction of the maximal contractions induced by U46619 ($p < 0.05$) and this effect was inhibited by *L*-NAME. In contrast, the contractile responses to U46619 were similar in control and endotoxin-treated pulmonary arteries. In endothelium-denuded pulmonary rings, endotoxin was also unable to modify the contractile responses to U46619. In pulmonary rings, the contractions induced by U46619 (100 n*M*) were much less sensitive to sodium nitroprusside, 8-bromo-cyclic GMP or dipyridamole than those induced by 10 μ*M* noradrenaline. In conclusion, endotoxin-treated pulmonary arteries exhibited decreased responses to noradrenaline due to enhanced nitric oxide release but not to the thromboxane A_2 analog U46619. This lack of hyporesponsiveness to U46619 in pulmonary arteries may be attributed to a relative insensitivity to nitric oxide. The absence of pulmonary hyporesponsiveness to U46619 may explain why pulmonary hypertension occurs in septic shock despite Ca^{2+}-independent nitric oxide synthase induction in the lung.

Introduction

Lipopolysaccharide is the endotoxin presumed to cause most, if not all, of the hemodynamic alterations following gram-negative sepsis [1, 2]. In recent years, enhanced formation of nitric oxide following endotoxin-mediated induction of the Ca^{2+}-independent nitric oxide synthase (iNOS) has been reported in several models of endotoxic shock [2]. iNOS is induced by endotoxin in a wide variety of tissues including the spleen, liver, mesen-

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tery, lung, kidney, heart, and vascular smooth muscle [3, 4]. In systemic arteries, iNOS induction is responsible for the systemic hypotension in endotoxic shock [5]. In an attempt to counteract this severe hypotension, vasoconstrictors are endogenously released by reflex mechanisms [6] or are therapeutically administered [1]. However, sepsis is associated with a reduction in the pressor responses to vasoconstrictor agents ultimately resulting in therapy-resistant hypotension [7–9].

The lung is the major target organ in neonatal sepsis [10]. In the adult, pulmonary complication, i.e. adult respiratory distress syndrome, continue to represent an important cause of mortality in patients with postsurgical septic complications [11]. As described in the systemic vasculature, both induction of iNOS and decreased responses to noradrenaline have been reported after in vitro incubation with endotoxin or group B streptococci in neonatal piglet [12] or adult rat pulmonary arteries [13, 14]. This is paradoxical since endotoxemia, both in the adult and in the neonate, is often associated with a marked increase in pulmonary artery pressure with little changes in cardiac output or left atrial pressure [15–18]. The early pulmonary hypertension in septic shock has been reported to be due to the enhanced production of thromboxane A_2, a potent pulmonary vasoconstrictor which reaches peak levels in lung lymph when pulmonary pressure is more marked [15, 19, 20]. In fact, specific inhibitors of thromboxane synthase reduce early pulmonary hypertension in sepsis [21]. Therefore, in contrast to the systemic hypotension occurring concomitantly with increased levels of circulating vasoconstrictors, the increased levels of pulmonary vasoconstrictors, particularly thromboxane A_2, lead to pulmonary hypertension despite iNOS induction in the lung.

In an attempt to better understand the paradox of thromboxane-A_2-induced pulmonary hypertension concurrent with iNOS induction in the lung during sepsis, we investigated the effects of endotoxin on the vasoconstrictor responses to noradrenaline and the stable thromboxane A_2 analog U46619 (9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$) on isolated pulmonary and mesenteric arteries from neonatal piglets. Because anatomy and anatomical development of neonatal pig lung are similar to that of human lung, neonatal piglets have been widely used as an experimental model for sepsis-induced pulmonary hypertension of the newborn [22].

Methods

Tissue Preparation and Incubation

Male neonatal piglets (10–17 days of age, 4,277 ± 343 g) were used in this study. Piglets were killed by exsanguination and the lungs and mesenteric vascular beds were rapidly immersed in cold (4 °C) Krebs solution (composition in m*M*: NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2.0, KH_2PO_4 1.2 and glucose 11). Krebs solution was supplemented with ampicillin (10 μg ml^{-1}) and gentamicin (10 μg ml^{-1}) to avoid bacterial growth during the dissection and incubation procedures. The pulmonary arteries (third branch) or mesenteric arteries (internal diameter 1–2 mm) were carefully dissected free of surrounding tissue and cut into rings of 2–3 mm of length. Some rings were incubated in Krebs solution (containing 10 μg ml^{-1} ampicillin and 10 μg ml^{-1} gentamicin and gassed with 95% O_2 and 5% CO_2 at 37 °C) in the presence of vehicle or endotoxin (1 μg ml^{-1}) for 20 h. Two L-shaped stainless-steel wires were inserted into the arterial lumen and the rings were introduced in Allhin organ chambers filled with Krebs solution (gassed with 95% O_2 and 5% CO_2 at 37 °C). One wire was attached to the chamber and the other to an isometric force-displacement transducer (Grass FT07) connected to a polygraph (Grass, Model 7) as previously described [12]. The rings were stretched to a resting tension of 0.5 g (pulmonary rings) or 2 g (mesenteric rings) and allowed to equilibrate for 60–90 min. During this period, tissues were restretched and washed every 30 min with warm Krebs solution. In some experiments, the endothelium was removed by gently rubbing the intimal surface of the rings with a metal rod. The presence of functional endothelium was verified by addition of acetylcholine (1 μ*M*) in arteries precontracted with 1 μ*M* noradrenaline. The ability of acetylcholine to induce relaxation of unrubbed rings was taken as an indicator of the presence of functional endothelium.

Experimental Protocol

In pulmonary or mesenteric rings previously incubated with vehicle or endotoxin for 20 h, concentration-response curves to noradrenaline (10 n*M*–100 μ*M*) or U46619 (100 p*M*–1 μ*M*) were constructed by increasing the organ chamber concentration by cumulative additions after a steady-state response was reached after each increment. In some arteries the cumulative concentration-response curves to noradrenaline or U46619 were performed in the presence of N^G-nitro-*L*-arginine-methyl ester (*L*-NAME, 100 μ*M*, an inhibitor of nitric oxide synthesis) [23, 24], added 30 min before starting the concenctration-response curves.

In another set of experiments, after equilibration, fresh endothelium-denuded pulmonary rings were contracted with either 10 μ*M* noradrenaline or 100 n*M* U46619. When the contractile response to each agonist reached a stable tension, cumulative concentration-response curves to sodium nitroprusside, 8-bromo-cyclic GMP or dipyridamole were carried out by cumulative increments of drug concentration after a steady state relaxant response was reached at each increment. Concentration-response curves to sodium nitroprusside were also carried out in fresh endothelium-denuded mesenteric arteries precontracted with 1 μ*M* U46619 or 1 μ*M* noradrenaline.

Drugs

The following drugs were used: (–)-noradrenaline bitartrate, acetylcholine chloride, endotoxin (lipopolysaccharide from *E. coli*, serotype 0.55:B5), *L*-NAME, *L*-arginine, sodium nitroprusside, 8-bromo-cyclic GMP and dipyridamole (Sigma Chemical Co., London).

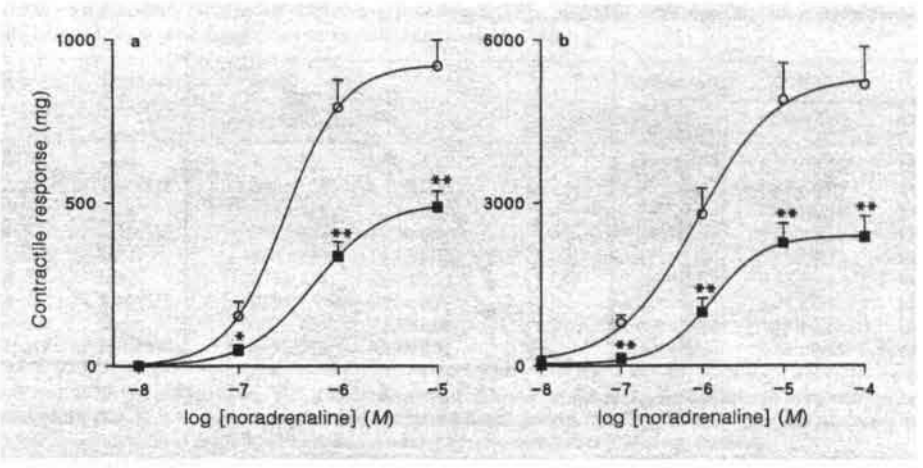

Fig. 1. Concentration-response curves to noradrenaline (10 n*M*–100 μ*M*) in pulmonary artery (**a**) or mesenteric artery (**b**) rings preincubated for 20 h in Krebs solution in the presence of vehicle (control, ○) or endotoxin (1 μg ml^{-1}, ■). The concentration-response curves to noradrenaline were carried out in the absence of endotoxin. Data are expressed as means ± SEM of 8–17 observations. Ordinate: contractile response (mg). Abscissa: log noradrenaline concentration (*M*). * $p < 0.05$ and ** $p < 0.01$ compared to control group.

U46619 was a generous gift of Upjohn Co. (Mich., USA). All drugs were dissolved in distilled deionized water (except dipyridamole in dimethyl sulfoxide) to prepare a 1 or 10 m*M* stock solution, further dilutions were made in PSS. The concentrations are expressed as final molar concentration in the tissue chamber.

Statistical Analysis

Results are expressed as means ± SEM of measurements in n arteries. Individual cumulative concentration-response curves were fitted to a logistic equation. The drug concentration exhibiting 50% of the maximal effect (E_{max}) was calculated from the fitted curve for each ring and expressed as negative log molar (pD_2). Statistically significant differences were calculated by means of an unpaired Student t test. $p < 0.05$ was considered statistically significant.

Results

Effects of Endotoxin on Noradrenaline-Induced Contractions

As shown in figure 1, when pulmonary or mesenteric arteries were incubated for 20 h with endotoxin (1 μg ml^{-1}) and then transferred to the organ bath in the absence of endotoxin, the maximal contractile response to noradrenaline (10 n*M*–100 μ*M*) was significantly reduced ($p < 0.01$). This effect was accompanied by a weak rightward shift of the curve which reached statistical significance ($p < 0.05$) in the pulmonary but not in the mesenteric artery (table 1). In order to assess the role of nitric oxide production on the hyporesponsiveness to noradrenaline, the concentration-response curve to noradrenaline was performed in untreated and in endotoxin-treated arteries in the presence of 100 μ*M* *L*-NAME at a concentration which inhibits > 80% of nitric oxide synthesis by the constitutive and inducible isoforms of nitric oxide synthase [4]. Addition of *L*-NAME to resting arteries produced a contractile effect averaging 45.7 ± 6.6 mg and 44.3 ± 9.6 mg in untreated and endotoxin-treated pulmonary arteries ($p > 0.05$), respectively, and 2,281 ± 485 mg and 2,301 ± 421 mg in untreated and endotoxin-treated mesenteric arteries ($p > 0.05$), respectively. Since this contractile effect was unexpected, we further studied it in fresh mesenteric arteries in the absence or presence of 1 m*M* *L*-arginine or in endothelium-denuded arteries (n = 9–10). Both *L*-arginine and endothelium removal greatly reduced *L*-NAME-induced contractions. Furthermore, 10 μ*M* sodium nitroprusside fully relaxed endothelium intact arteries treated with *L*-NAME near to resting values whereas it relaxed endothelium-denuded arteries below baseline (not shown). These results suggest that when NO is released from the endothelium these arteries do not show tone and only when they are denuded of endothe-

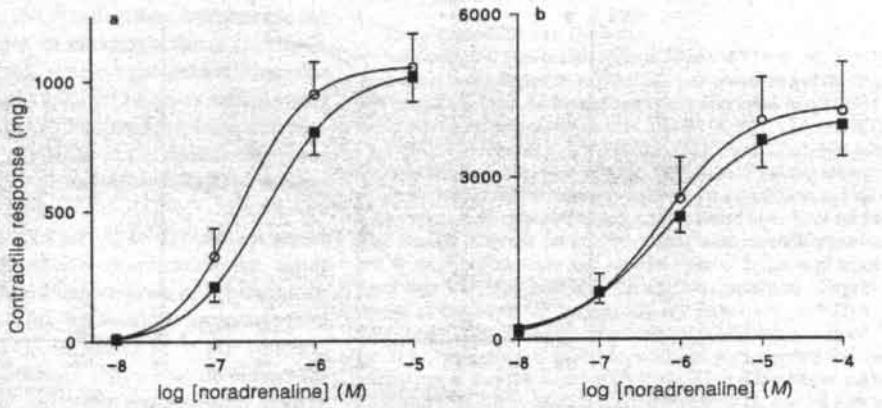

Fig. 2. Effects of *L*-NAME (100 μ*M*) on endotoxin-induced hyporeactivity to noradrenaline in pulmonary artery (**a**) or mesenteric artery (**b**) rings. Concentration-response curves to noradrenaline (10 n*M*–100 μ*M*) were performed in rings previously incubated for 20 h in Krebs solution in the presence of vehicle (control, ○) or endotoxin (1 μg ml^{-1}, ■). *L*-NAME (100 μ*M*) was added 30 min before the addition of noradrenaline. The curves were carried out in the absence of endotoxin. Data are expressed as means ± SEM of 8–14 observations. Ordinate: contractile reponse (mg). Abscissa: log noradrenaline concentration (*M*).

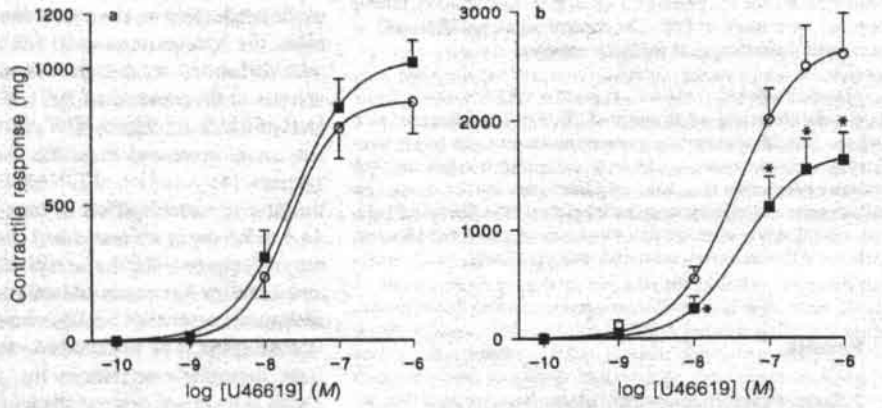

Fig. 3. Concentration-response curves to U46619 (100 p*M*–1 μ*M*) in pulmonary artery (**a**) or mesenteric artery (**b**) rings preincubated for 20 h in Krebs solution in the presence of vehicle (control, ○) or endotoxin (1 μg ml^{-1}, ■). The concentration-response curves to U46619 were carried out in the absence of endotoxin. Data are expressed as means ± SEM of 10–13 observations. Ordinate: contractile response (mg). Abscissa: log U46619 concentration (*M*). * $p < 0.05$ represent significant differences with respect to the control group.

Table 1. Effects of incubation with endotoxin (10 μg/ml^{-1}) for 20 h on the parameters (E_{max} and pD_2) of the concentration-response curves to noradrenaline and U46619 in pulmonary and mesenteric arteries (calculated from fig. 1–4)

Artery	Drug	Treatment	Control			Endotoxin		
			E_{max}(mg)	pD_2	n	E_{max} (mg)	pD_2	n
Pulmonary	NA	none	923±80	6.52±0.07	17	492±47**	6.25±0.10*	13
Pulmonary	NA	*L*-NAME	1,047±120	6.72±0.11	10	1,023±98	6.49±0.09	14
Pulmonary	U46619	none	928±116	7.51±0.06	12	988±79	7.60±0.26	13
Pulmonary	U46619	*L*-NAME	971±82	7.74±0.08	14	1,142±86	7.85±0.10	19
Pulmonary	U46619	–E	844±133	7.83±0.12	14	1,119±171	7.8±0.13	13
Mesenteric	NA	none	5,273±718	6.07±0.11	8	2,380±382**	5.88±0.1	12
Mesenteric	NA	*L*-NAME	4,288±1,184	6.22±0.06	8	4,027±611	6.23±0.1	10
Mesenteric	U46619	none	3,007±463	7.33±0.08	11	1,650±240*	7.25±0.14	10
Mesenteric	U46619	*L*-NAME	2,413±373	7.43±0.12	6	2,474±539	7.37±0.16	7

–E = endothelium-denuded. *L*-NAME (100 μ*M*) was added to the organ bath solution 30 min before the addition of noradrenaline or U46619. The concentration exhibiting 50% of the maximal contraction to noradrenaline was calculated for each ring and expressed as negative log molar (pD_2). The E_{max} was defined as the maximal tension induced by noradrenaline or U46619 in each ring.

* $p < 0.05$ and ** $p < 0.01$ endotoxin vs. control.

lium or treated with *L*-NAME do they show an intrinsic contractile tone. Furthermore, *L*-NAME increased the maximal response to noradrenaline in endotoxin-treated but not in control arteries (fig. 2). Therefore, *L*-NAME reversed the hyporesponsiveness to noradrenaline induced by endotoxin in both pulmonary and systemic arteries.

Effects of Endotoxin on U46619-Induced Contractions

As shown in figure 3a and in contrast to the results obtained with noradrenaline, the maximal contractile responses to U46619 were similar in control and in endotoxin-treated pulmonary arteries and no change in the pD_2 values was observed (table 1). The effect of 20 h incubation with endotoxin was also tested in rings in which the endothelium was removed after the incubation procedure and prior to the concentration-response curve to U46619. Under these experimental conditions, endotoxin did not modify the maximal contractile response to U46619 (table 1). In the presence of 100 μ*M* *L*-NAME, the maximal contractile responses induced by U46619 were not significantly different in endotoxin-treated compared to control arteries (fig. 4a, table 1).

Figure 3b shows that, in contrast to pulmonary arteries, endotoxin-treated mesenteric arteries showed a significant reduction of the maximal contractions induced by U46619 when compared to controls. Furthermore, pretreatment with *L*-NAME increased the maximal response to U46619 in endotoxin-treated but not in untreated mesenteric arteries (fig. 4b). Therefore, *L*-NAME reversed the hyporesponsiveness to U46619 induced by endotoxin in mesenteric arteries (table 1).

Effects of Sodium Nitroprusside, 8-Bromo-Cyclic GMP and Dipyridamole on the Contractions Induced by Noradrenaline and U46619

In endothelium-denuded pulmonary rings a single concentration of noradrenaline (10 μ*M*) or U46619 (100 n*M*) elicited contractile responses which averaged 691 ± 87 mg (n = 23) and 734 ± 40 mg (n = 27), respectively ($p > 0.05$ noradrenaline vs. U46619). Once the contraction had reached a maximum stable contraction, the addition of sodium nitroprusside (10 n*M*–100 μ*M*) resulted in a concentration-dependent relaxation. However, there were marked differences in the relaxant potency of sodium nitroprusside depending on the agonist which precontracted the artery. In pulmonary arteries (figure 5a) U46619-induced contractions were much less sensitive to sodium nitroprusside than noradrenaline-induced contractions ($pD_2 = 5.7 \pm 0.1$ and 6.6 ± 0.1, respectively, $p < 0.01$). Furthermore, sodium nitroprusside was unable to fully relax the U46619-induced contractions, so that the highest concentration of sodium nitroprusside tested produced a maximal relaxation of 67 ± 7%. The effects of sodium nitroprusside were also tested in mesenteric arteries precontracted with concentrations of noradrenaline

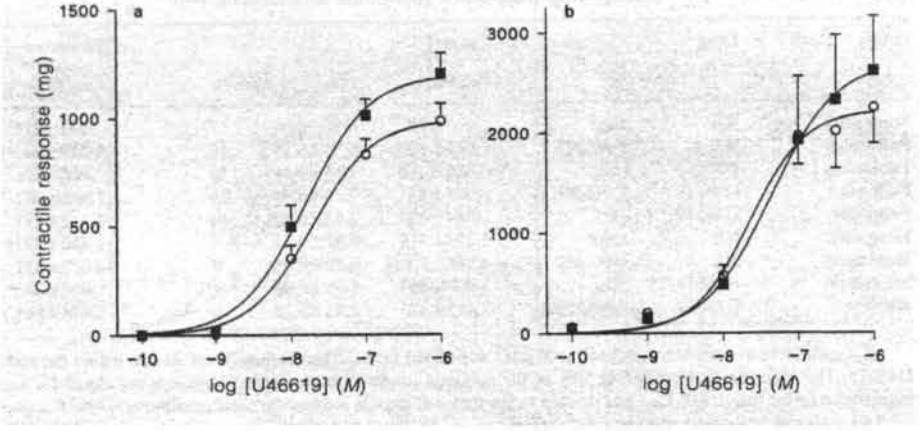

Fig. 4. Effects of *L*-NAME (100 μ*M*) on the concentration-response curves to U46619 in untreated and endotoxin-treated pulmonary artery (**a**) or mesenteric artery (**b**) rings. Concentration-reponse curves to U46619 (100 p*M*–1 μ*M*) were performed in rings previously incubated for 20 h in Krebs solution in the presence of vehicle (control, ○) or endotoxin (1 μg ml^{-1}, ■). *L*-NAME (100 μ*M*) was added 30 min before the addition of noradrenaline. The curves were carried out in the absence of endotoxin. Data are expressed as means ± SEM of 6–19 observations. Ordinate: contractile response (mg). Abscissa: log U46619 concentration (*M*).

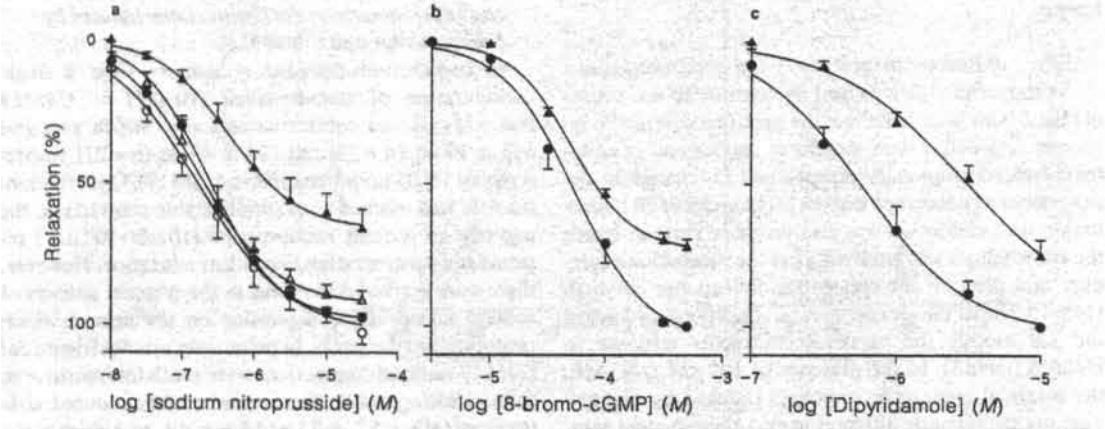

Fig. 5. Relaxant effects of sodium nitroprusside (10 n*M*–30 μ*M*) (**a**), 8-bromo-cyclic GMP (3–500 μ*M*) (**b**) and dipyridamole (100 n*M*–10 μ*M*) (**c**) on the contractions induced by 10 μ*M* noradrenaline (●) or 100 n*M* U46619 (▲) in pulmonary arteries and 1 μ*M* noradrenaline (○) or 1 μ*M* U46619 (△) in mesenteric arteries. Data are expressed as means ± SEM of 5–10 observations. Ordinate: relaxant response (%). Abscissa: log drug concentration (*M*).

(1 μM) and U46619 (1 μM) which induced similar contractile responses (1,525 ± 331 and 1,640 ± 352, respectively; $p > 0.05$). Sodium nitroprusside also produced a concentration-dependent relaxation in mesenteric arteries (fig. 5a) which was not significantly different in noradrenaline- and U46619-contracted muscles ($pD_2 = 6.7 \pm 0.2$ and 6.7 ± 0.1, respectively, $p > 0.05$) and similar to that produced in noradrenaline-precontracted pulmonary arteries. Therefore, the low sensitivity to sodium nitroprusside was specific of U46619-induced contractions in pulmonary arteries.

Furthermore, 8-bromo-cyclic GMP (fig. 5b) and dipyridamole (fig. 5c) also relaxed pulmonary arteries precontracted with noradrenaline (10 μM) or U46619 (100 nM) in a concentration-dependent manner. As reported above for sodium nitroprusside, U46619-induced contractions were also significantly less sensitive to the relaxant effects of 8-bromo-cyclic GMP and dipyridamole than noradrenaline-induced contractions.

Discussion

In the present study we have analyzed the effects of prolonged exposure to endotoxin on the contractile responses induced by noradrenaline and U46619, a stable thromboxane A_2 mimetic, on isolated pulmonary and systemic arteries from neonatal piglets. We have demonstrated that endotoxin reduced the maximum contractile responses to noradrenaline in both pulmonary and mesenteric arteries or to U46619 in mesenteric arteries. The nitric oxide synthase inhibitor *L*-NAME [23, 24], did not modify the vasoconstrictor responses in control arteries but increased the responses in endotoxin-treated arteries resulting in a reversal of the endotoxin-induced hyporesponsiveness. The contractions induced by U46619 in pulmonary arteries, however, appear to be an exception since they were not reduced by endotoxin treatment. Additionally, these contractions were much less sensitive to sodium nitroprusside, 8-bromo-cyclic GMP or dipyridamole when compared to U46619-induced contractions in mesenteric arteries or noradrenaline-induced contractions in pulmonary arteries.

The role of nitric oxide overproduction following iNOS induction in systemic hypotension and decreased responses to vasoconstrictors after endotoxin treatment has been reported by many authors in in vivo, ex vivo or in vitro studies [2]. In a previous study we have already reported that endotoxin or group B streptococcus reduced the contractile responses to noradrenaline in neonatal porcine pulmonary arteries [12]. This hyporesponsiveness was potentiated by *L*-arginine, while *L*-NAME completely reversed it, indicating that overproduction of nitric oxide was responsible for this effect. Dexamethasone, which inhibits the induction of iNOS [25, 26], and cycloheximide, an inhibitor of protein synthesis, when coincubated with endotoxin, completely reversed the reduced response to noradrenaline. Moreover, endotoxin had no effect on the constitutive nitric oxide synthase (cNOS) but induced a marked increase in iNOS activity which was abolished by dexamethasone. All these results indicate that endotoxin-induced pulmonary vascular hyporesponsiveness to noradrenaline is due to enhanced formation of nitric oxide via iNOS induction and requires the novo synthesis of the enzyme in the pulmonary vasculature [12]. In the present study, endotoxin-treated mesenteric arteries also showed decreased contractile responses to noradrenaline or U46619. The reversal by *L*-NAME of these reduced responses in mesenteric arteries strongly suggest that enhanced nitric oxide production was responsible for endotoxin-induced vascular hyporesponsiveness. Although we did not further analyze this possibility, it is likely that the increased nitric oxide production is largely related to the induction of iNOS and may contribute to the latter peripheral vascular failure and the hypotensive response associated with prolonged periods of sepsis caused by gram-negative bacteria.

More interestingly, not all vascular beds respond with depressed responses following endotoxin-mediated induction of iNOS. In the present study, the contractile responses to U46619 in pulmonary arteries were not diminished after prolonged incubation with endotoxin in spite of iNOS induction and decreased pulmonary responsiveness to noradrenaline. Likewise, isolated mesenteric beds from endotoxin-treated rats were not hyporesponsive to various vasoconstrictors, including U46619, despite significant iNOS induction [4]. However, the lack of effect of endotoxin on U46619-induced contractions is not a general finding since in pig mesenteric arteries (present study) or in rabbit [26] or rat [27] perfused hearts the vasoconstrictor responses to U46619 were attenuated by endotoxin. Decreased vasoconstrictor responses to U46619 (but not to angiotensin II) have also been reported in isolated perfused lungs from rats made septic by cecal ligation [28]. Therefore, endotoxin produces opposite effects on the contractions induced by U46619 in neonatal piglet and in adult rat arteries. These contradictory results therefore suggest that the effects of endotoxin on U46619-induced vascular responses may be tissue-, species- and/or age-dependent.

In an attempt to explain the mechanism involved in the lack of hyporesponsiveness to U46619 in the pulmonary arteries we hypothesized that U46619-induced contractions might be insensitive to the relaxant action of nitric oxide, so that iNOS induction and the subsequent increase in nitric oxide release would not affect these contractile responses. In fact, pulmonary arteries precontracted with U46619 were relatively insensitive to the relaxant action of the nitric oxide donor sodium nitroprusside (e.g. 300 n*M* sodium nitroprusside relaxed 61% of noradrenaline- but only 28% of U46619-induced contractions), suggesting an impairment of the signal transduction process leading to vasodilatation. Since nitric oxide is released from sodium nitroprusside independently of nitric oxide synthases [29], the reduced sensitivity to nitric oxide is unlikely to be due to an impairment of nitric oxide synthase activity. Nitric-oxide-induced vasodilatation is mediated through activation of smooth muscle soluble guanylate cyclase which in turn increases intracellular cyclic GMP levels [29]. In order to establish whether these reduced responses to nitric oxide were due to decreased activity of soluble guanylate cyclase, the effects of a stable analog of cyclic GMP, 8-bromo-cyclic GMP, were tested in pulmonary arteries precontracted with noradrenaline or U46619. The U46619-induced contractions were again less sensitive to the relaxant action of 8-bromo-cyclic GMP, which indicated that reduced nitric oxide sensitivity was not due to an interference of U46619 with guanylate cyclase. Furthermore, since these contractions were also less sensitive to the relaxant action of dipyridamole, a specific inhibitor of the cyclic-GMP-specific (type V) phosphodiesterase enzyme [30], the reduced sensitivity to nitric oxide could not be attributed to an enhancement of phosphodiesterase activity after exposure to U46619. Therefore, the low ability of nitric oxide to induce relaxation might be related to impaired activation of cyclic-GMP-dependent kinases or a mechanism located further in the cascade of events leading to vasodilatation. Reduced relaxant responses to sodium nitroprusside have also been reported on U46619-contracted pulmonary arteries from adult rats chronically exposed to hypoxia [31, 32]. Likewise, human umbilical arteries do not relax in response to sodium nitroprusside or phosphodiesterase inhibitors despite the increase of cyclic GMP concentrations [33].

The clinical relevance of the present experiments is unknown. However, the lung is the major target organ in neonatal sepsis [10]. The early pulmonary hypertension in septic shock has been associated with enhanced production of thromboxane A_2, a potent vasoconstrictor [15, 19, 20]. Therefore, it is tempting to speculate that the increased levels of thromboxane A_2 together with the lack of endotoxin-induced hyporesponsiveness to the thromboxane A_2 mimetic U46619 observed in this study may explain the pulmonary hypertension occurring in neonatal septic patients despite iNOS induction in the lung.

In conclusion, endotoxin-treated mesenteric arteries from neonatal piglets show decreased responses to noradrenaline and U46619 due to enhanced nitric oxide release, which may account for the systemic hypotension associated with septic shock. Endotoxin-treated pulmonary arteries exhibited a nitric oxide-mediated hyporesponsiveness to noradrenaline but not to the thromboxane A_2 mimetic U46619. This lack of endotoxin-induced hyporesponsiveness to U46619 may be attributed to a relative insensitivity of these contractions to nitric oxide or cyclic GMP. The absence of pulmonary hyporesponsiveness to U46619 may explain why pulmonary hypertension occurs in septic shock despite iNOS induction in the lung.

Acknowledgments

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Chapter IX. **Effects of group B Streptococcus on the responses to U46619, endothelin-1, and noradrenaline in isolated pulmonary and mesenteric arteries of piglets.**
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Effects of Group B Streptococcus on the Responses to U46619, Endothelin-1, and Noradrenaline in Isolated Pulmonary and Mesenteric Arteries of Piglets[1]

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ABSTRACT

The release of endogenous vasoconstrictors together with changes in the vascular responses are central to the pathophysiology of sepsis. The effects of *in vitro* incubation for 20 h with heat-killed group B Streptococcus (GBS, 3×10^7 colony-forming units mL^{-1}) on the vasoconstrictor responses to noradrenaline (NA, 10^{-8} to 10^{-4} M), the thromboxane A_2 analog 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619; 10^{-10} M to 10^{-6} M) and endothelin-1 (ET-1, 10^{-11} to 3×10^{-9} M) were evaluated on isolated intrapulmonary and mesenteric arteries from 10-17-d-old piglets. The incubation with GBS reduced the maximal contractile response to NA and ET-1 ($p < 0.01$) in both arteries. The nitric oxide (NO) synthase (NOS) inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; 10^{-4} M) completely reversed this hyporesponsiveness. GBS-treated mesenteric arteries also showed a significant reduction of the maximal contractions induced by U46619 ($p < 0.05$) and this effect was inhibited by 10^{-4} M L-NAME. In contrast, the maximal contractile responses to U46619 were similar in control and in GBS-treated pulmonary arteries. Addition of L-NAME did not modify the contractile responses to U46619 in GBS-treated pulmonary arteries. In conclusion, GBS-treated systemic arteries from neonatal piglets showed decreased responses to NA, U46619, and ET-1 due to enhanced NO release. GBS-treated pulmonary arteries also exhibited decreased responses to NA and ET-1 but not to U46619. Induction of NOS in vascular smooth muscle may play a key role in the hypotension and loss of systemic vascular responsiveness that occurs in GBS sepsis. The absence of pulmonary hyporesponsiveness to U46619 may partially explain the coexistence during sepsis of pulmonary hypertension and lung NOS induction. (***Pediatr Res* 40: 827–833, 1996**)

Abbreviations

GBS, Group B Streptococcus
LPS, lipopolysaccharide
ET-1, endothelin-1
TXA_2, thromboxane A_2
NO, nitric oxide
iNOS, inducible nitric oxide synthase
NA, noradrenaline
U46619, 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$
L-NAME, N^{ω}-nitro-L-arginine methyl ester
cfu, colony-forming units

GBS, a Gram-positive bacterium, is one of the most common causal agents of neonatal sepsis (1, 2). In the newborn, GBS produced acute pulmonary hypertension, respiratory failure, arterial hypoxemia, decreased cardiac output, and systemic hypotension, resulting in significant morbidity and mortality (2-4).

Endogenous production and release of several vasoactive agents play a determinant role in the pathophysiology of sepsis (5-16). Thus, sepsis-induced pulmonary hypertension has been divided into an early phase related to the arachidonic acid-derived vasoconstrictor TXA_2 and a late phase associated with development of pulmonary edema (5-7). Additionally, in an attempt to counteract systemic severe hypotension, circulating catecholamines are increased (8, 9). Moreover, elevated levels of the endothelium-derived vasoconstrictor ET-1 have been found in animal models of sepsis (10) as well as in adult patients with septic shock (11). Unfortunately, to our knowl-

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edge, the levels of ET-1 and catecholamines have not been evaluated in GBS sepsis. Together with these vasoconstrictors, endogenous vasodilators are also produced in sepsis (12-16). Currently, there is a great body of evidence that enhanced release of NO via induction of an inducible Ca^{2+}-independent iNOS plays an important role in the loss of systemic (12-14) and pulmonary (15, 16) vascular responsiveness that occurs in Gram-negative sepsis. Gram-positive bacteria have been also recently demonstrated to induce iNOS in several tissues (17, 18). In fact, GBS induced iNOS in macrophages (19, 20) and in pulmonary arteries resulting in a reduced vasoconstrictor response to NA (21).

Therefore, the coexistence of pulmonary hypertension and systemic hypotension may be due to a different sensitivity of the pulmonary and systemic vessels to the vasoactive factors released in sepsis. The aim of the present work was to study the effects of *in vitro* incubation with heat-killed GBS on the vasoconstrictor responses to NA, the stable TXA_2 analog U46619, and ET-1 on isolated pulmonary and mesenteric arteries from piglets.

METHODS

Tissue Preparation and Incubation

Male neonatal piglets (32 animals, 10-17 d of age, 4162 ± 297 g) were used in this study. Piglets were killed by exsanguination, and the lungs and mesenteric vascular beds were rapidly immersed in cold (4°C) Krebs solution of the following composition (mM): NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2.0, KH_2PO_4 1.2, and glucose 11. The pulmonary (third branch, internal diameter 1-2 mm) and mesenteric arteries (internal diameter 1-2 mm) were carefully dissected free of surrounding tissue and cut into rings of 2-3-mm length (21). The arterial rings were incubated in Krebs solution gassed with 95% O_2 and 5% CO_2 at 37°C in the presence of vehicle or heat-inactivated GBS (3×10^7 cfu mL^{-1}) for 20 h. Krebs solution was supplemented with ampicillin (10 μg mL^{-1}) and gentamicin (10 μg mL^{-1}) to avoid bacterial growth during the dissection and incubation procedures. A maximal number of two rings per animal were used in each experimental group. Control and GBS-treated rings from the same animal were always run in parallel. After the incubation, two L-shaped stainless-steel wires were inserted into the arterial lumen, and the rings were introduced in Allhin organ chambers filled with Krebs solution gassed with 95% O_2 and 5% CO_2 and maintained at 37°C. One wire was attached to the chamber and the other to an isometric force-displacement transducer (Grass FT07; Grass Instrument Co., Quincy, MA) connected to a polygraph (Grass model 7). The rings were stretched to a resting tension of 0.5 g (pulmonary rings) or 2 g (mesenteric rings) and allowed to equilibrate for 60-90 min. During this period tissues were restretched and washed every 30 min with warm Krebs solution. In some experiments the endothelium was removed by gently rubbing the intimal surface of the rings with a metal rod. The presence or absence of functional endothelium was verified by testing the relaxant effect of acetylcholine (10^{-6} M) in arteries precontracted with NA 10^{-6} M.

Experimental Protocol

In pulmonary and mesenteric rings previously incubated as described above, concentration-response curves to NA (10^{-8} to 10^{-4} M), U46619 (10^{-10} to 10^{-6} M), or ET-1 (10^{-11} to 3×10^{-9} M), were constructed by increasing the organ chamber concentration by cumulative additions after a steady state response was reached after each increment. In some arteries the response to NA, U46619, or ET-1 were performed in the presence of L-NAME, 10^{-4} M, an inhibitor of NO synthesis), added 30 min before starting the concentration-response curves.

Drugs and Heat-Killed GBS Preparation

The following drugs were used: (−)-noradrenaline bitartrate, acetylcholine chloride, L-NAME, human ET-1, and U46619 (Sigma Chemical Co., London). All drugs were dissolved in distilled deionized water. The concentrations are expressed as a final molar concentration in the tissue chamber.

GBS type III was isolated from the blood of a neonate who developed early-onset sepsis. Bacteria were grown in Todd-Hewitt broth for 18-36 h at 37°C to late log phase and harvested by centrifugation at 5000 rpm for 15 min. Bacteria were resuspended in sterile isotonic saline to a concentration determined by serial viable counts to be 1×10^9 cfu mL^{-1}. Heat-killed bacteria were obtained by heating bacteria to 60°C for 60 min. GBS killing was confirmed by no growth on blood agar. Endotoxin levels in the heat-killed GBS preparation were undetectable as assayed using a standard Limulus assay kit (Sigma Chemical Co.). Aliquots of heat-killed GBS were stored at −80°C until the study day.

Statistical Analysis

Results are expressed as means ± SEM of measurements in n arteries. Individual cumulative concentration-response curves were fitted to a logistic equation. The drug concentration exhibiting 50% of the maximal effect (E_{max}) was calculated from the fitted curve for each ring and expressed as negative log molar (pD_2). Statistically significant differences were calculated by means of an unpaired t test. $p < 0.05$ was considered statistically significant.

RESULTS

Effects of GBS on NA-Induced Contractions

Both pulmonary and mesenteric arterial rings, incubated for 20 h with heat-killed GBS and then transferred to organ baths in the absence of GBS, showed a significant reduction ($p < 0.01$) in the contractile response to NA (10^{-8} to 10^{-4} M) compared with controls (Fig. 1). The reduction of the maximal contraction (E_{max}) was more marked in mesenteric compared with pulmonary arteries (Table 1). However, a decrease of the pD_2 value was observed in pulmonary but not in mesenteric arteries. To determine whether NO production might have influenced the GBS-mediated hyporesponsiveness to NA, the concentration-response curve to NA was performed in the presence of L-NAME (10^{-4} M, a concentration which inhibits

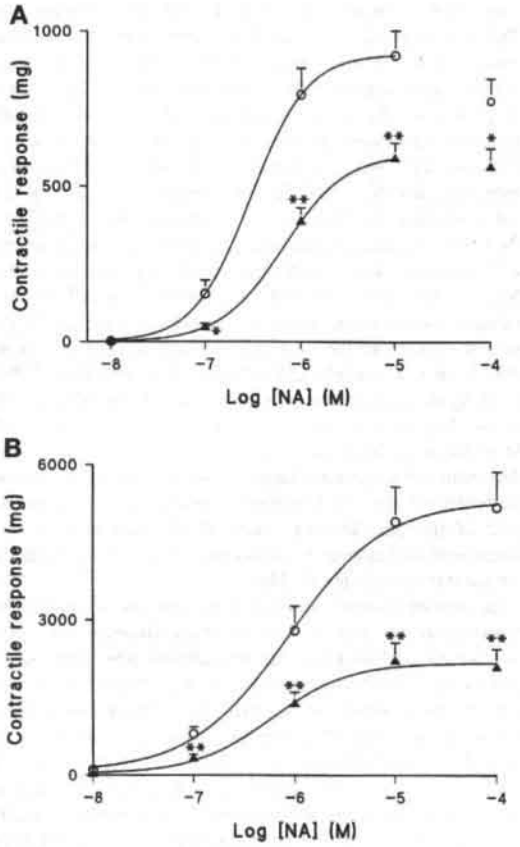

Figure 1. Concentration-response curves to NA (10^{-8} to 10^{-4} M) in pulmonary (*A*) or mesenteric (*B*) artery rings preincubated for 20 h in Krebs solution in the presence of vehicle (○) or GBS (3×10^7 cfu mL^{-1}, ▲). The concentration-response curves to NA were carried out in the absence of GBS. Data are expressed as means ± SEM of 8-21 observations. Ordinate, contractile response (mg); abscissa, log NA concentration (M). $*p < 0.05$ and $**p < 0.01$ compared with control group.

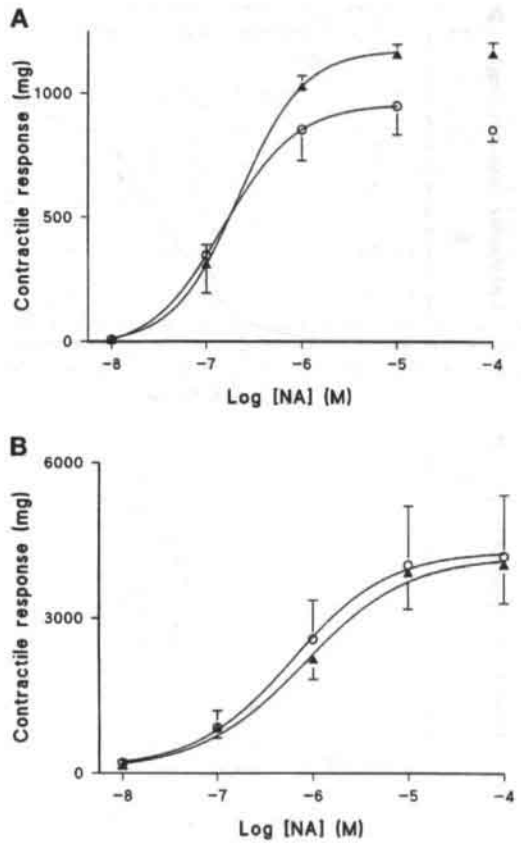

Figure 2. Effects of L-NAME (10^{-4} M) on GBS-induced hyporeactivity to NA in pulmonary (*A*) or mesenteric (*B*) artery rings. Concentration-response curves to NA (10^{-8} to 10^{-4} M) were performed in rings previously incubated for 20 h in Krebs solution in the presence of vehicle (○) or GBS (3×10^7 cfu mL^{-1}, ▲). L-NAME was added 30 min before the addition of NA. The curves were carried out in the absence of GBS. Data are expressed as means ± SEM of seven to nine observations. Ordinate, contractile response (mg); abscissa, log NA concentration (M).

>80% of NO synthesis by the constitutive and inducible isoforms of NOS) (22). Fig. 2 shows that L-NAME induced an upward shift of the concentration-response to NA reversing the hyporesponsiveness to NA induced by GBS in both pulmonary and mesenteric arteries.

Effects of GBS on U46619-Induced Contractions

As shown in Fig. 3*A*, the maximal contractile responses to U46619 were similar in control and in GBS-treated pulmonary arteries and no change in the pD_2 values was observed (Table 1). In the absence of endothelium (Table 1) or in the presence of 10^{-4} M L-NAME (Table 1, Fig. 4*A*), GBS was again unable to modify the contractile responses to U46619.

Fig. 3*B* shows that, in contrast to pulmonary arteries, GBS-treated mesenteric arteries showed a significant reduction ($p < 0.05$) of the maximal contractions induced by U46619 compared with controls without affecting the pD_2 value. Pretreatment with L-NAME induced an upward shift of the concentration-response to U46619 in the GBS-treated but not in untreated mesenteric arteries (Fig. 4*B*). Therefore, L-NAME reversed the hyporesponsiveness to U46619 induced by GBS in mesenteric arteries (Table 1).

Effects of GBS on ET-1-Induced Contractions

As shown in Fig. 5 the incubation for 20 h with GBS produced a marked decrease in the contractile response to ET-1 in both pulmonary and mesenteric arteries. Because the maximal concentration of ET-1 used (3×10^{-9} M) did not reach maximal contractile effect, neither the E_{max} nor the pD_2 values could be calculated in these experiments. The GBS-induced reduction of the maximal contraction to ET-1 was more marked in mesenteric compared with pulmonary arteries (40 ± 5% *versus* 60 ± 4, $p < 0.01$). Fig. 6 shows that GBS-induced

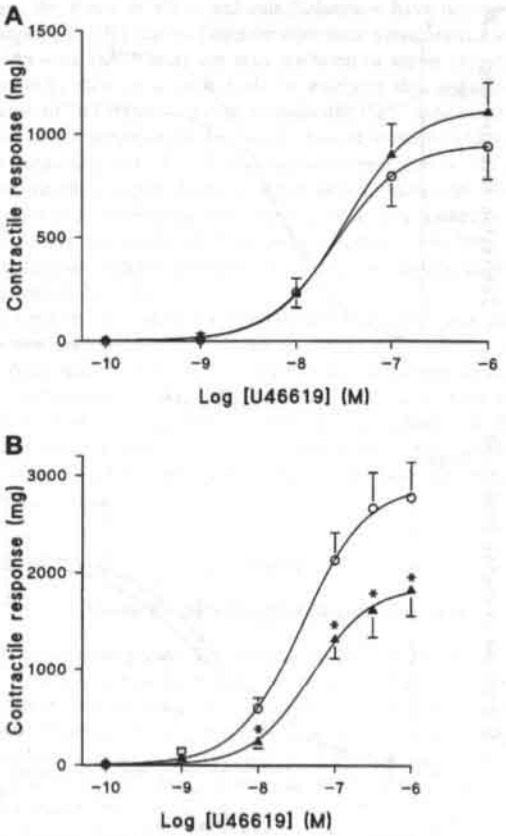

Figure 3. Concentration-response curves to U46619 (10^{-10} to 10^{-6} M) in pulmonary (*A*) or mesenteric (*B*) artery rings preincubated for 20 h in Krebs solution in the presence of vehicle (○) or GBS (3×10^7 cfu mL^{-1}, ▲). The concentration-response curves to U46619 were carried out in the absence of GBS. Data are expressed as means ± SEM of 11-12 observations. Ordinate, contractile response (mg); abscissa, log U46619 concentration (M). *$p < 0.05$ compared with control group.

hyporesponsiveness was completely reversed in the presence of L-NAME.

DISCUSSION

In the present report we have studied the effects of prolonged incubation with heat-killed GBS on the vascular contractile responses induced by NA, the TXA_2 mimetic U46619, and ET-1 on isolated pulmonary and systemic (mesenteric) arteries from piglets. The results demonstrate that GBS reduced the vascular responsiveness to NA, U46619, and ET-1 in mesenteric arteries and this effect was reversed by the presence of the NOS inhibitor L-NAME. In pulmonary arteries, GBS also reduced the response to NA and ET-1, and L-NAME reversed this hyporesponsiveness. In contrast, GBS did not affect the contractions induced by U46619 in pulmonary arteries both in presence or absence of L-NAME.

In a recent study we have reported that prolonged (20 h) incubation with GBS or *Escherichia coli* LPS, reduced the contractile responses to NA in piglet pulmonary arteries (21). This hyporesponsiveness was potentiated by the NO precursor L-arginine and reversed by L-NAME. Dexamethasone, which inhibits the induction of iNOS, and cycloheximide, an inhibitor of protein synthesis, when coincubated with GBS or LPS, completely reversed the reduced response to NA. Moreover, GBS and LPS induced a marked increase in iNOS activity, indicating that the vascular hyporesponsiveness was related to overproduction of NO as a consequence of the induction of iNOS. In the present study, incubation of mesenteric arteries with GBS also decreased the contractile responses induced by NA, U46619, and ET-1. The reversal by L-NAME of these reduced responses suggested that an enhanced NO production may be responsible for GBS-induced vascular hyporesponsiveness. It has been found that LPS leads to the induction of iNOS resulting in an overproduction of NO, which contributes to the severe hypotension seen in Gram-negative sepsis (12-14). More recently, iNOS induction in several tissues has also been demonstrated with some Gram-positive bacteria (17, 18) including GBS (19-21). Moreover, lipoteichoic acid, a component of the peptidoglycan layer of the cell wall in most Gram-positive bacteria, has been described as being responsible for this induction (23, 24).

In spite of the pulmonary hypertension that appears in the sepsis syndrome, induction of iNOS and decreased responses to pressor agents have been reported after *in vitro* incubation of pulmonary arteries from piglets (21) or adult rats (16, 25) with LPS or GBS. Moreover, Curzen *et al.* (26) have described hyporesponsiveness to ET-1 in pulmonary arteries from LPS-treated rats. Unfortunately, they did not evaluate the possible role of NO in this hyporesponsiveness. In the present study we have demonstrated that prolonged *in vitro* incubation with GBS reduces the pulmonary vascular response to NA and ET-1, but not to the TXA_2 mimetic U46619. Interestingly, in mesenteric arteries, the GBS-induced hyporesponsiveness to U46619 was less marked than that to NA or ET-1. The reason for this lack of hyporesponsiveness to U46619 in GBS-treated pulmonary arteries is unknown, but a reduced sensitivity to NO-mediated vasodilatation can be involved. In fact, we have previously reported that pulmonary arteries precontracted with U46619 were relatively insensitive to the relaxant action of the NO donor sodium nitroprusside or 8-bromo-cGMP, the stable analog of cGMP (27). In contrast, inhaled NO was able to reduce the pulmonary hypertension produced by U46619 infusions (28) or GBS-sepsis (29, 30). Interestingly, the response to U46619 after iNOS induction varies among species and vascular beds. Thus, isolated piglet pulmonary arteries incubated with endotoxin (27) or isolated mesenteric beds from endotoxin-treated rats were not hyporesponsive to U46619 despite significant iNOS induction (22), whereas in isolated rat lung (31), isolated piglet mesenteric arteries (27), and isolated perfused rat (32), or rabbit hearts (33), the vasoconstrictor responses to U46619 were attenuated by endotoxin. In addition, increased responses to U46619 have been reported after treatment of isolated perfused guinea pig lungs with tumor necrosis factor-α (34) which is one of the most important cytokines released in septic shock or in experimental models of sepsis induced by Gram-negative and Gram-positive bacteria includ-

Table 1. *Effects of incubation with heat-killed GBS (3×10^7 cfu mL^{-1}) for 20 h on the parameters (E_{max} and pD_2) of the concentration-response curves to NA and U46619 in pulmonary and mesenteric arteries (calculated from Figs. 1–4)*

			Control			GBS		
Artery	Drug	Treatment	E_{max} (mg)	pD_2	n	E_{max} (mg, % of control)	pD_2	n
Pulmonary	NA	None	920 ± 80	6.52 ± 0.07	17	589 ± 50**, 64 ± 5	6.17 ± 0.08**	21
Pulmonary	NA	L-NAME	947 ± 115	6.72 ± 0.11	8	1151 ± 45, 121 ± 6	6.66 ± 0.08	9
Pulmonary	U46619	None	943 ± 159	7.51 ± 0.06	12	1108 ± 142, 117 ± 15‡	7.53 ± 0.09	11
Pulmonary	U46619	L-NAME	984 ± 81	7.61 ± 0.08	18	1081 ± 88, 109 ± 9	7.83 ± 0.10	18
Pulmonary E−	U46619	None	931 ± 106	7.65 ± 0.1	8	1027 ± 139, 110 ± 15	7.57 ± 0.1	9
Mesenteric	NA	None	5175 ± 693	6.07 ± 0.11	8	2072 ± 360**, 40 ± 7†	6.21 ± 0.06	10
Mesenteric	NA	L-NAME	4193 ± 1177	6.22 ± 0.06	8	4010 ± 722, 97 ± 15	6.18 ± 0.7	7
Mesenteric	U46619	None	2777 ± 364	7.33 ± 0.08	11	1825 ± 266*, 66 ± 9†,‡	7.18 ± 0.07	11
Mesenteric	U46619	L-NAME	2248 ± 358	7.43 ± 0.12	6	2591 ± 623, 115 ± 21	7.41 ± 0.08	7

L-NAME (10^{-4}M) was added to the organ bath solution 30 min before the addition of NA or U46619. The concentration exhibiting 50% of the maximal contraction to NA was calculated for each ring and expressed as negative log molar (pD_2). The E_{max} was defined as the maximal tension induced by NA or U46619 in each ring. E− indicates endothelium-denuded arteries. * $p < 0.05$ and ** $p < 0.01$ GBS *vs* control, † $p < 0.05$ pulmonary *vs* mesenteric arteries, ‡ $p < 0.05$ NA *vs* U46619.

ing GBS (20, 35, 36). Thus, a cytokine-mediated increase in pulmonary arterial sensitivity to TXA_2 has been also proposed as a mechanism for the persistence of pulmonary hypertension in sepsis (34). Because TXA_2 is an important mediator in GBS sepsis-related pulmonary hypertension (6, 7), one could speculate that the lack of GBS-induced hyporesponsiveness to U46619 may explain the pulmonary hypertension despite iNOS induction in the lung. However, the relationship between these events is unclear, because TXA_2-mediated pulmonary hypertension appears in the early phase of experimental sepsis (5-7), whereas iNOS induction seems to be a delayed process. In fact, we have observed iNOS induction in piglet pulmonary arteries after incubation for 20 h with GBS but not after 1 or 5 h (21). The uptake of bacteria by pulmonary intravascular macrophages and the subsequent release of inflammatory mediators are central to the pathophysiology of GBS-induced pulmonary hypertension (37). The absence of these cells in our model is another limitation of this study which may explain, at least in part, *in vivo*, *in vitro*, and organ- and species-differences. Moreover, due to its cytotoxic effects, NO may play a role in the edema and vascular injury that accompanies the late phases of sepsis-mediated pulmonary hypertension.

To the best of our knowledge this is the first report of NO-mediated vascular hyporesponsiveness induced by GBS in systemic arteries. Moreover, the percentage of reduction in the contractile response induced by GBS was greater in mesenteric compared with pulmonary arteries for any of the stimuli studied. These results may be relevant due to the association of GBS sepsis and hypotension. In fact, in neonates with early-onset GBS sepsis, the development of hypotension is one of the most sensitive predictors of mortality (4, 38), and in newborns with Gram-positive or Gram-negative sepsis an association between plasma nitrite plus nitrate (metabolites of NO) and shock has been reported (39). Whether iNOS induction is beneficial in sepsis-induced shock or is an undesirable collateral effect remains controversial. Experiments using NO inhibitors have strongly implicated NO as a cytotoxic agent which plays a role in antimicrobial and inflammatory responses (40), whereas mortality related to experimental endotoxemia was abolished in mutant mice lacking the iNOS gene (41).

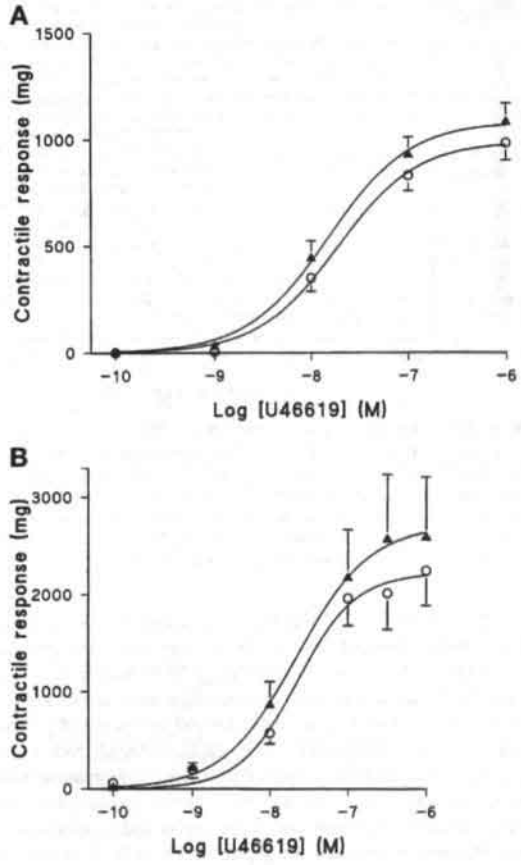

Figure 4. Effects of L-NAME (10^{-4} M) on the concentration-response curves to U46619 in untreated and GBS-treated pulmonary (*A*) or mesenteric (*B*) artery rings. Concentration-response curves to U46619 (10^{-10} to 10^{-6} M) were performed in rings previously incubated for 20 h in Krebs solution in the presence of vehicle (control, ○) or GBS (3×10^7 cfu mL^{-1}, ▲). L-NAME (10^{-4} M) was added 30 min before the addition of U46619. The curves were carried out in the absence of GBS. Data are expressed as means ± SEM of 6-18 observations. Ordinate, contractile response (mg); abscissa, log U46619 concentration (M).

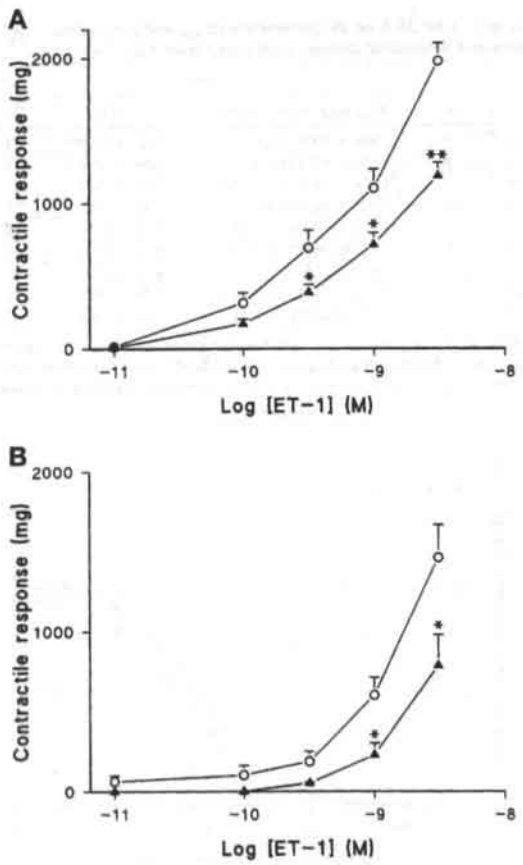

Figure 5. Concentration-response curves to ET-1 (10^{-11} to 3×10^{-9} M) in pulmonary (*A*) or mesenteric (*B*) artery rings preincubated for 20 h in Krebs solution in the presence of vehicle (○) or GBS (3×10^7 cfu mL^{-1}, ▲). The concentration-response curves to U46619 were carried out in the absence of GBS. Data are expressed as means ± SEM of seven to nine observations. Ordinate, contractile response (mg); abscissa, log ET-1 concentration (M). $^*p < 0.05$ and $^{**}p < 0.01$ compared with control group.

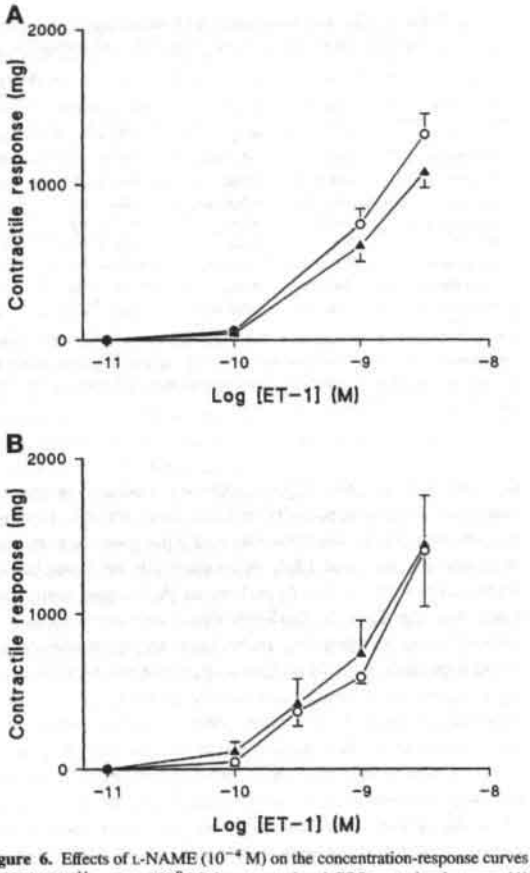

Figure 6. Effects of L-NAME (10^{-4} M) on the concentration-response curves to ET-1 (10^{-11} to 3×10^{-9} M) in untreated and GBS-treated pulmonary (*A*) or mesenteric (*B*) artery rings. Concentration-response curves to ET-1 were performed in rings previously incubated for 20 h in Krebs solution in the presence of vehicle (○) or GBS (3×10^7 cfu mL^{-1}, ▲). L-NAME (10^{-4} M) was added 30 min before the addition of ET-1. The curves were carried out in the absence of GBS. Data are expressed as means ± SEM of 7-10 observations. Ordinate, contractile response (mg); abscissa, log ET-1 concentration (M).

The coexistence of pulmonary hypertension and systemic hypotension in septic shock states important therapeutical problems. Systemic administration of NOS inhibitors to patients with sepsis reversed systemic hypotension but significantly enhanced mean pulmonary arterial pressure and pulmonary vascular resistances (42). Thus, inhaled NO (as a treatment for pulmonary hypertension) in combination with NOS inhibitors (as a treatment for systemic hypotension) has been studied in a porcine model of sepsis and proposed as a new therapeutic regimen for septic shock (43). However, in experimental models of GBS sepsis where systemic hypotension is not observed, probably due to the short duration of the GBS infusion, treatment with NOS inhibitors worsens the hemodynamic situation, increasing both pulmonary and systemic vascular resistances and decreasing cardiac output (44, 45). These data partially contradicts our findings, because L-NAME did not enhance the U46619-induced contractions in GBS-incubated pulmonary arteries. Further studies, including the vascular responses of pulmonary and systemic vessels after prolonged in vivo GBS exposure, would be necessary to elucidate the pathophysiologic and therapeutical implications of sepsis-induced iNOS induction and the possible involvement of the changes in vascular tone which accompanies this process.

In conclusion, prolonged incubation of piglet mesenteric arteries with heat-killed GBS produced a marked hyporesponsiveness to NA, U46619, and ET-1 due to an enhanced NO release, suggesting a role for iNOS induction in the systemic hypotension associated with GBS sepsis. GBS-treated pulmonary arteries exhibited a NO-mediated hyporesponsiveness to NA and ET-1 but not to the TXA_2 mimetic U46619. This absence of pulmonary hyporesponsiveness to U46619 may

contribute to the persistence of pulmonary hypertension in GBS sepsis despite iNOS induction in the lung.

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Chapter X. **Involvement of protein kinase C in reduced relaxant responses to the NO/cyclic GMP pathway in piglet pulmonary arteries contracted by the thromboxane A_2-mimetic U46619.**

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Involvement of protein kinase C in reduced relaxant responses to the NO/cyclic GMP pathway in piglet pulmonary arteries contracted by the thromboxane A_2-mimetic U46619

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1 Impairment of nitric oxide (NO)/cyclic GMP production and/or increased activities of thromboxane A_2 (TXA_2) and endothelin-1 (ET-1) have been associated with pulmonary hypertension. We have analysed the interactions of noradrenaline (NA), the TXA_2-mimetic U46619 and ET-1 with the relaxation induced via cyclic GMP in isolated piglet intrapulmonary arteries.

2 The contractions induced by NA were augmented by endothelium removal or by methylene blue and pre-contracted rings were fully relaxed by acetylcholine, sodium nitroprusside (SNP), atrial natriuretic peptide and 8-bromo-cyclic GMP. In contrast, U46619- and ET-1 induced contractions were endothelium-independent and only partially relaxed by the latter vasodilators. Whereas the reduced responses to SNP in arteries contracted by U46619 were independent of the U46619-induced tone, a higher concentration of ET-1 (tone higher than that induced by NA) was required to reduce the vasodilator responses to SNP. NA, U46619 and ET-1 had no effect on the SNP-induced increases in cyclic GMP.

3 The reduced relaxant responses to SNP in arteries pre-contracted by U46619 were specific for piglet pulmonary arteries since they were not observed in piglet mesenteric or coronary arteries or in rat pulmonary arteries. Furthermore, there were no differences in the relaxant response to the adenylate cyclase activator forskolin in piglet pulmonary arteries pre-contracted by either NA, U46619 or ET-1.

4 SNP-induced relaxation was inhibited by thapsigargin (but not by inhibition of the membrane Na^+/K^+ ATPase nor K^+ channels) indicating a role for Ca^{2+} sequestration by the Ca^{2+} ATPase in the effects of SNP.

5 The phorbol ester 12-myristate, 13-acetate inhibited the relaxant response to SNP. The inhibitory effect of U46619 on SNP-induced relaxation was abolished by the protein kinase C inhibitor (PKC) staurosporine suggesting that PKC may be a part of the signal transduction mechanism.

6 In summary, piglet pulmonary arteries when activated by a TXA_2-mimetic show abnormally reduced relaxant responses to the NO/cyclicGMP pathway. This effect appears to be mediated by activation of PKC.

Keywords: Thromboxane A_2; endothelin-1; nitric oxide; cyclic GMP; pulmonary artery

Introduction

The nitric oxide (guanosine 3':5'-cyclic monophosphate) (NO/cyclic GMP) pathway plays a key role in the maintenance of vasodilator tone in the vascular bed (Moncada *et al.*, 1991; Warner *et al.*, 1994). Under physiological conditions, the endothelium is the main source of NO for vascular smooth muscle relaxation whereas in several pathological states the induction of the inducible NO synthase (iNOS) in smooth muscle cells and macrophages may account for a large production of NO (Moncada *et al.*, 1991). NO acting as an autocrine or paracrine mediator, activates the soluble guanylate cyclase and increases intracellular levels of cyclic GMP in vascular smooth muscle cells (Warner *et al.*, 1994; Barnes & Liu, 1995). Alterations of this pathway, at the level of NO and cyclic GMP synthesis or action in vascular smooth muscle have been associated with a number of vascular diseases. In the pulmonary system, NO is crucial for maintaining low vascular resistances and arterial pressure (Cremona *et al.*, 1991; Barnes & Liu, 1995). Reduced NO or cyclic GMP activities have been related to the maintenance of high pulmonary pressure during foetal life (Abman *et al.*, 1990), experimental persistent pulmonary hypertension of the newborn (McQueston *et al.*, 1995), hypoxia-induced pulmonary vasoconstriction as well as primary and secondary pulmonary hypertension (Dinh-Xuan *et al.*, 1991; Giaid & Saleh, 1995). In addition, in both adults and neonates, inhalation of NO has recently been introduced as a life-saving therapeutic approach with beneficial results in many subjects (Abman & Kinsella, 1995; Roberts *et al.*, 1997; Neonatal Inhaled Nitric Oxide Study Group, 1997).

On the other hand, increased activity of the pulmonary vasoconstrictors thromboxane A_2 (TXA_2) and endothelin-1 (ET-1) has also been implicated in several forms of pulmonary hypertension. TXA_2 has been shown to be responsible for the early phase of sepsis-induced pulmonary hypertension (Weitzberg *et al.*, 1995). It has also been implicated in other experimental models of pulmonary hypertension induced by heparin/protamine (Montalescot *et al.*, 1990), leukotriene D_4 (Noonan & Malik, 1986), microembolism (García-Szabo *et al.*, 1988) and ischaemia-reperfusion (Zamora *et al.*, 1993). Furthermore, elevated levels of thromboxane B_2, the metabolite of TXA_2, have been found in neonatal pulmonary hypertension (Dobyns *et al.*, 1994). Augmented levels of ET-1 have also been shown to be associated with several forms of experimental and clinical pulmonary hypertension, including that induced by sepsis, hypoxia and monocrotaline (Weitzberg *et al.*, 1996), persistent pulmonary hypertension of the newborn (Rosenberg *et al.*, 1993), primary pulmonary hypertension (Giaid *et al.*, 1993) and

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pulmonary hypertension associated with congenital heart disease (Yoshibayashi *et al.*, 1991) or congestive heart failure (Cody *et al.*, 1992).

In physiological situations, pulmonary vascular tone results from the balance of vasoconstrictors and vasodilators. Whether the imbalance which occurs in pulmonary hypertension is due to alteration of a single factor or multiple vasoactive agents remains unclear. Therefore, the aim of the present investigation was to study the interactions of the vasoconstrictors noradrenaline (NA), the TXA_2 mimetic, U46619, and ET-1 with the NO/cyclic GMP pathway as well as the mechanisms involved in NO/cyclic GMP-induced relaxation in piglet isolated intrapulmonary arteries. The specificity of these interactions was analysed by comparing these results with those mediated through: (a) the cyclic AMP pathway in piglet pulmonary arteries, and (b) the NO/cyclic GMP pathway in piglet mesenteric and coronary arteries and in rat pulmonary arteries.

Methods

Tissue preparation

Two week old male piglets (10–17 days, 3–5 kg) were used in this study. Some experiments were also carried out on adult Wistar rats (250–300 g) and on 2–3 month old piglets (15–25 kg). Piglets were killed in the local abattoir by exsanguination and the lungs, hearts and mesenteric vascular beds were rapidly immersed in cold (4°C) Krebs solution (composition in mM: NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2.0, KH_2PO_4 1.2 and glucose 11) and transported to the laboratory. Rats were killed in the laboratory by a sharp blow on the head followed by exsanguination. The intrapulmonary arteries (third branch), mesenteric and left descending coronary arteries from piglets (all with an internal diameter 1–2 mm) and the right and left branches of the main rat pulmonary artery were carefully dissected free of surrounding tissue and cut into rings of 2–3 mm length (Pérez-Vizcaíno *et al.*, 1996; Villamor *et al.*, 1996a,b). Except where stated otherwise, the endothelium was removed by gently rubbing the intimal surface of the rings with a metal rod. The endothelium removal procedure was verified by the inability of acetylcholine (ACh, 10^{-6} M) to relax arteries precontracted with 10^{-6} M NA. Two L-shaped stainless-steel wires were inserted into the arterial lumen and the rings were introduced into Allhin organ chambers filled with Krebs solution (gassed with 95% O_2 and 5% CO_2 at 37°C). One wire was attached to the chamber and the other to an isometric force-displacement transducer coupled to a signal amplifier (Model PRE 206–4, Cibertec, Madrid) and connected to a Hewlett Packard computer via an A/D interface. Contractile tension was recorded by a REGXPC computer program (Cibertec, Madrid). The preparations were stretched to a resting tension of 0.5 g (pulmonary rings), 1 g (coronary rings) or 2 g (mesenteric rings) and allowed to equilibrate for 60–90 min. During this period tissues were re-stretched and washed every 30 min with warm Krebs solution.

Experimental protocols

After equilibration, rings were contracted with either NA, U46619 or ET-1. When the contractile response to each agonist reached a stable tension, cumulative concentration-response curves to methylene blue, ACh, sodium nitroprusside (SNP), atrial natriuretic peptide (ANP), 8-bromo-guanosine-3′-5′-cyclic monophosphate (8-Br-cyclic GMP), dipyridamole or forskolin, were carried out by cumulative addition of drugs after a steady-state response was reached after each increment. In some experiments the relaxant effect of SNP was tested in arteries precontracted with 3×10^{-7} M phorbol 12-myristate, 13-acetate (PMA). The relaxant effect of SNP was also analysed in arteries treated with thapsigargin (2×10^{-6} M), staurosporine (10^{-8} M and 10^{-7} M), PMA (3×10^{-8} M), meclofenamate (10^{-5} M) or K^+-free solution (without KCl and KH_2PO_4 replaced with NaH_2PO_4) for 45 min before a contraction was induced with NA, U46619 or ET-1. Some arteries were contracted with 80 mM KCl (replacing NaCl isotonically), then relaxed with 10^{-6} M nifedipine and, thereafter, NA or U46619 were added before the concentration-response curve to SNP was carried out. In other experiments, after a contraction had been induced with NA or U46619, arteries were treated with 10^{-6} M 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), 3×10^{-7} M dipyridamole or 10^{-7} M charybdotoxin for 20–30 min before concentration-response curves were constructed to SNP.

Cyclic GMP assay

Pulmonary rings were mounted in the organ chambers and tension was recorded as described above. After equilibration, they were exposed to vehicle, 10^{-5} M NA, 10^{-6} M U46619 or 3×10^{-9} M ET-1 for 40 min and finally, were either untreated or treated with 10^{-5} M SNP for 3 min. At the end of the 3 min, rings were rapidly removed from the organ chamber and quickly frozen on dry ice and stored at −80°C. The rings were then homogenized in 600 μl of 10% trichloroacetic acid, centrifuged at 10,000 *g* for 10 min at 4°C and the supernatant extracted 4 times in 3 volumes of water-saturated diethylether. The cyclic GMP concentrations were determined by radioimmunoassay by use of an acetylated Amersham [^{125}I]-cyclic GMP assay kit (Amersham International, Buckinghamshire, UK). The cyclic GMP was expressed as pmol g^{-1} wet tissue.

Drugs

The following drugs were used: (−)-noradrenaline bitartrate, acetylcholine chloride, sodium nitroprusside (SNP), human atrial natriuretic peptide (ANP), 8-bromo-cyclic GMP, 5-hydroxytryptamine (5-HT) creatine phosphate complex, adenosine 5′-trisphosphate magnesium salt (ATP), [Arg^8]-vasopressin, U46619 (9,11-dideoxy-11α, 9α-epoxymethano-prostaglandin $F_{2\alpha}$ methyl acetate solution), thapsigargin, endothelin-1 (ET-1), methylene blue, methoxamine hydrochloride, forskolin, staurosporine, phorbol 12-myristate, 13-acetate and dipyridamole (Sigma Chemical Co., London), charybdotoxin (RBI, Natick, MA), meclofenamate (Warner Lambert Co., U.S.A.), nifedipine (Bayer, Leverkussen, Germany) and ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (Tocris Cookson Ltd, Bristol, U.K.). All drugs were dissolved initially in distilled deionized water (except for dipyridamole, staurosporine, thapsigargin, PMA and forskolin which were dissolved in dimethyl sulphoxide and nifedipine in ethanol) to prepare a 10^{-2} M, 10^{-3} M or 10^{-4} M stock solution and further dilutions were made in PSS. The concentrations expressed are final molar concentrations in the tissue chamber.

Statistical analysis

Results are expressed as means ± s.e.mean of measurements in *n* arteries. Individual cumulative concentration-response curves were fitted to a logistic equation. The drug concentration exhibiting 50% of the maximal effect (E_{max}) was calculated from the fitted concentration-response curves for each ring and expressed as negative log molar concentration (pD_2). Statistically significant differences between groups were calculated by ANOVA followed by Newman Keuls test. $P < 0.05$ was considered statistically significant.

Results

Contractile effects of NA, U46619, ET-1, vasopressin, 5-HT and ATP in piglet pulmonary arteries

In previous experiments in piglet endothelium-denuded pulmonary arteries, 10^{-5} M NA induced a maximally effective

contractile response (99±4% of the E_{max} to NA), 3×10^{-8} M, 10^{-7} M and 10^{-6} M U46619 induced a response of 56±9%, 80±9% and 96±4%, respectively, of the E_{max} to U46619 and 10^{-9} M and 3×10^{-9} M ET-1 induced a response of 65±5% and 86±4%, respectively, of the E_{max} to ET-1. The magnitude of the steady-state contractile responses induced by 10^{-4} M methoxamine, 3×10^{-8} M or 10^{-7} M U46619 or 10^{-9} M ET-1 were not significantly different from those induced by 10^{-5} M NA (Table 1). In endothelium-denuded rings NA (10^{-5} M), methoxamine (10^{-4} M), U46619 (10^{-6} M) and ET-1 (3×10^{-9} M) induced contractile responses with a clearly different time-course (Figure 1a). NA and methoxamine induced a rapid increase in tension which reached a peak in about 1–2 min and thereafter, slowly decreased to reach a lower steady-state tension at about 10 min. This response probably reflects an initial inositol 1,4,5-triphosphate(IP_3)-induced Ca^{2+} release followed by a secondary component due to Ca^{2+} entry. In contrast, U46619 and ET-1 induced a progressive monophasic contractile response which required about 20 and 40 min, respectively, to reach a plateau. Addition of NA 10^{-5} M on top of a steady-state contraction induced by 10^{-6} M U46619 (maximally effective concentration) was still able to increase tone (from 1192±190 mg to 1595±174 mg, $n=7$, $P<0.01$). 5-HT (up to 10^{-5} M), vasopressin (up to 10^{-6} M) and ATP (up to 10^{-3} M) produced no measurable contractile effects in resting pulmonary arteries ($n=4-6$).

In endothelium-intact arteries the steady-state contractile response to NA was significantly smaller than in endothelium-denuded arteries, whereas endothelium removal did not significantly affect the responses to U46619 and ET-1 (Figure 1b).

In endothelium-denuded arteries addition of the guanylate cyclase inhibitor methylene blue (10^{-6} M and 10^{-5} M) on top of a steady-state contraction induced by 10^{-5} M NA or 10^{-6} M U46619 produced a concentration-dependent contraction (Figure 1c). This contractile response was more marked in NA- than in U46619-precontracted vessels, so that 10^{-5} M methylene blue abolished the differences in the amplitude of the contractile responses between NA and U46619.

Vasorelaxant responses of ACh, SNP, ANP and 8-Br-cyclic GMP on NA-, U46619-, ET-1-and methoxamine-induced contractions in piglet pulmonary arteries

As shown in Figure 2a, in endothelium-intact arteries ACh induced a concentration-dependent relaxant response. However, ACh induced complete relaxation in 10^{-5} M NA-precontracted rings, while it relaxed only about 50% of the contractions induced by 10^{-6} M U46619. Similarly, the relaxant responses induced by SNP, ANP and 8-Br-cyclic GMP were significantly less marked in endothelium-denuded arteries precontracted with U46619 than with NA (Figure 2b, c and d).

To evaluate the role of the precontractile tone on SNP-induced relaxation, the effects of SNP (10^{-8} M – 3×10^{-5} M) were also compared in pulmonary arteries precontracted by NA (10^{-5} M), U46619 (3×10^{-8} M, 10^{-7} M and 10^{-6} M), ET-1 (10^{-9} M and 3×10^{-9} M) and the combination of 10^{-6} M U46619 plus 10^{-5} M NA. Parameters for the concentration-response curves are shown in Table 1. In rings precontracted by equieffective concentrations of U46619 and NA (10^{-7} M and 10^{-5} M, respectively), SNP produced complete relaxation of NA-induced contractions, while it only relaxed the contractions induced by U46619 by 66%. A similar result was observed in pulmonary arteries precontracted to lower tension levels by 3×10^{-8} M U46619. The relaxant effects of 8-Br-cyclic GMP were much more marked in arteries precontracted with 10^{-5} M NA than with 10^{-7} M U46619 (not shown). In rings contracted by the combination of 10^{-6} M U46619 plus 10^{-5} M NA, the relaxant response to SNP was not different from that obtained in arteries contracted by U46619 alone. In arteries contracted by 10^{-9} M ET-1 (equieffective to 10^{-5} M NA) the vasorelaxant response to SNP was similar to that observed in arteries contracted by 10^{-5} M NA, whereas with contractions induced by higher concentrations of ET-1 (3×10^{-9} M), SNP only induced a partial relaxation. Meclofenamate (10^{-5} M) did not modify the contractile response to 3×10^{-9} M ET-1 (1275±159 mg ($n=4$)) or the relaxant response to SNP ($pD_2=5.88\pm0.18$ and $E_{max}=68\pm7\%$, $P>0.05$ vs control values obtained with 3×10^{-9} M ET-1 in Table 1).

Pulmonary arteries contracted by the selective α_1-adrenoceptor agonist methoxamine (10^{-4} M) showed a similar relaxant response to SNP as when activated by the mixed α_1 and α_2 adrenoceptor agonist NA (Table 1).

Vasorelaxant responses of forskolin on NA-, U46619- and ET-1-induced contractions in piglet pulmonary arteries

Table 2 shows that the adenylate cyclase activator forskolin (10^{-9} M – 10^{-6} M) produced a similar full relaxation in pulmonary arteries contracted by either 10^{-5} M NA, 10^{-6} M U46619 or 3×10^{-9} M ET-1. Thus, in contrast to the results obtained with the vasodilators acting through the cyclic GMP pathway, the relaxant response to forskolin was independent of the agonist employed to induce tone.

Effects of SNP on NA-, U46619- and ET-1-induced contractions in piglet coronary and mesenteric arteries

In contrast to pulmonary arteries, SNP (10^{-8} M – 3×10^{-5} M) produced a complete relaxation in mesenteric and coronary arteries which was independent of the agonist employed to raise tone (10^{-6} M NA, 10^{-6} M U46619 or 3×10^{-9} M ET-1) and similar in both arteries (Table 3). As previously shown (Ohgushi *et al.*, 1993), NA produced minimal contractile effects in pig coronary arteries and, therefore, the relaxant effects of SNP could not be evaluated.

Table 1 Relaxant effects of SNP in endothelium-denuded pulmonary arteries precontracted by NA, U46619, the combination of U46619 and NA, ET-1 and methoxamine

	n	*Tension* (mg)	pD_2	E_{max}(%)
NA (10^{-5} M)	13	654±41	6.62±0.08	104±2
U46619 (3×10^{-8} M)	6	561±43	5.65±0.07**	73±4**
U46619 (10^{-7} M)	12	720±40	6.20±0.10**	66±7**
U46619 (10^{-6} M)	14	1220±59**	5.92±0.10**	66±4**
U46619 (10^{-6} M) + NA (10^{-5} M)	7	1595±174**	5.92±0.11**	75±5**
ET-1 (10^{-9} M)	6	744±92	6.52±0.08	100±2
ET-1 (3×10^{-9} M)	11	1015±77**	5.68±0.17**	75±6**
Methoxamine (10^{-4} M)	4	575±64	6.49±0.17	107±2

Tension is the pre-contraction value induced by the vasoconstrictor and pD_2 and E_{max} values refer to the effects of SNP. Results are means±s.e.means of n number of experiments. *$P<0.05$, **$P<0.01$, respectively, vs 10^{-5} M NA. Data with 10^{-5} M NA and 10^{-6} M U46619 were calculated from Figure 2b.

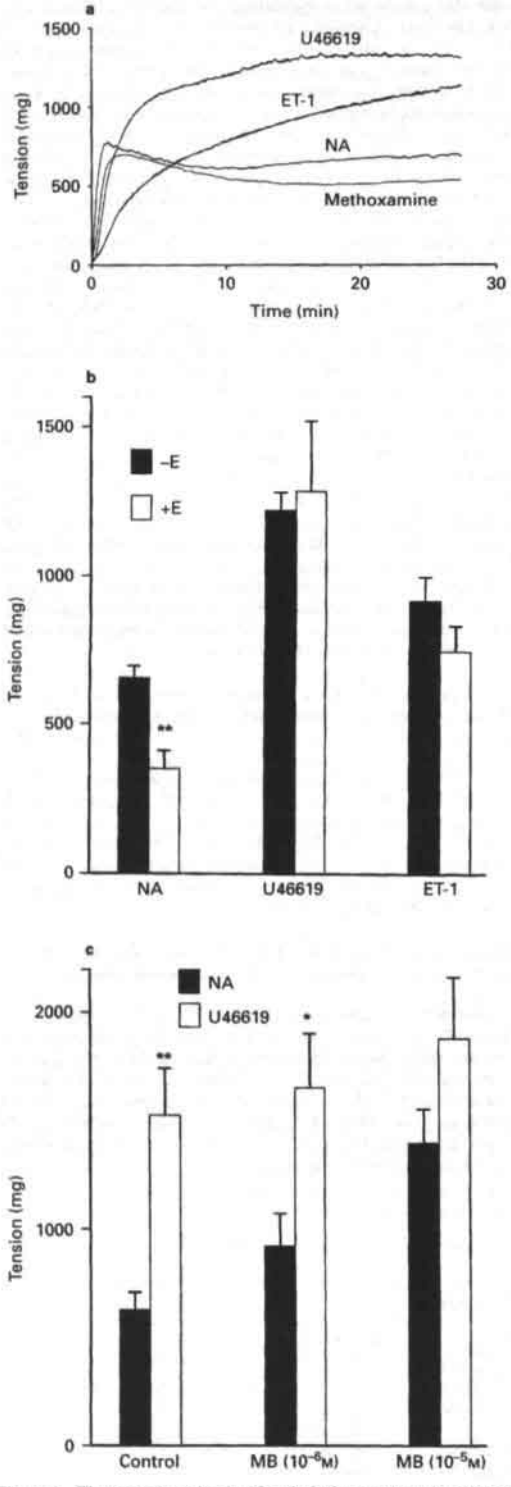

Figure 1 Time-course and role of endothelium and cyclic GMP in the contractile responses induced by NA (10^{-5} M), U46619 (10^{-6} M), ET-1 (3×10^{-9} M) and methoxamine (10^{-4} M) in piglet pulmonary arteries. (a) Time-course of the contractions induced by the four vasoconstrictors in endothelium-denuded arteries. Each trace represents the averaged recordings from 5–6 arteries. (b) Endothelial-dependence of the sustained contractions induced by the NA, U46619 and ET-1. Solid columns indicate endothelium-denuded arteries (−E) and open columns endothelium-intact arteries (+E). Each column represents the mean±s.e.mean of 6–17 experiments. $^{**}P<0.01$ endothelium-denuded vs endothelium-intact arteries. (c) Effects of methylene blue (MB, 10^{-6} M and 10^{-5} M) on the contractions induced by NA and U46619 in endothelium-denuded arteries. Each column represents the mean±s.e.mean of 7–8 arteries. $^{*}P<0.05$ and $^{**}P<0.01$ NA vs U46619.

Effects of SNP on NA- and U46619-induced contractions in adult rat and 2–3 months old piglet pulmonary arteries

Endothelium-denuded rat pulmonary arteries showed no differences in their maximal responses to NA and U46619 (351±82 mg and 275±29 mg, respectively, $P>0.05$, $n=5$). SNP (10^{-10} M–10^{-6} M) was equally effective at relaxing the contractions induced by maximally effective concentrations of NA (10^{-7} M) and U46619 (3×10^{-6} M) (Table 4).

The relaxant effects of SNP were similar in pulmonary arteries from 2–3 month old (Table 4) or 2 week old piglets (Table 1), i.e. again U46619-induced contractions were less sensitive to SNP than those induced by NA.

Effects of NA, U46619 and ET-1 on SNP-induced increase in cyclic GMP in pulmonary arteries

Figure 3 shows the effects of SNP, alone or in combination with NA, U46619 and ET-1 on cyclic GMP levels in endothelium-denuded pulmonary arteries. SNP (10^{-5} M) increased the cyclic GMP content in resting arteries by about three fold after 3 min. The increase in cyclic GMP levels induced by SNP was unchanged in arteries pre-contracted with NA (10^{-5} M), U46619 (10^{-6} M) or ET-1 (3×10^{-9} M).

Effects of dipyridamole, ODQ, KCl, charybdotoxin, K^+-free solution and thapsigargin on the vasorelaxant responses to SNP in piglet pulmonary arteries

Addition of the cyclic GMP-dependent phosphodiesterase inhibitor dipyridamole (3×10^{-7} M) relaxed NA- and U46619-contracted arteries by 33±4% and 9±2%, respectively ($P<0.05$), and significantly shifted the concentration-response curves to SNP to the left (Figure 4a). This leftward shift was similar in arteries contracted by either NA or U46619 (6.3 and 6.6 fold, respectively). However, pretreatment with dipyridamole had no effect on the maximal vasodilator response to SNP. In NA-contracted arteries, addition of 10^{-6} M ODQ, a specific inhibitor of the soluble guanylate cyclase, raised tone by 102±47% over previous tone and inhibited the relaxant response to SNP ($pD_2=5.96\pm0.22$, $E_{max}=81\pm7\%$, $n=6$; $P<0.05$ as compared to ODQ-untreated arteries).

In resting arteries, 80 mM KCl (replacing NaCl isotonically) induced a sustained contraction averaging 876±162 mg ($n=11$) which was relaxed by 74±3% with 10^{-6} M nifedipine. Thereafter, a contractile response was induced by 10^{-5} M NA (final tension 1131±107 mg, $n=6$) or 10^{-6} M U46619 (final tension 1585±350 mg, $n=5$). As can be observed in Figure 4b, under these conditions, SNP induced a relaxant response in both NA-and U46619-contracted vessels which was slightly but significantly more potent ($P<0.05$) than in arterial rings not treated with KCl plus nifedipine (2.8 and 3.0 fold leftward shift in NA and U46619 contracted vessels, respectively). In NA-contracted arteries, addition of the Ca^{2+}-activated K^+ channel blocking agent charybdotoxin (10^{-7} M) induced a weak contractile response (16±3% over previous tone) but did not modify the relaxant response to SNP ($pD_2=6.69\pm0.11$, $E_{max}=103\pm3$, $n=4$, $P>0.05$ as compared to charybdotoxin-untreated arteries).

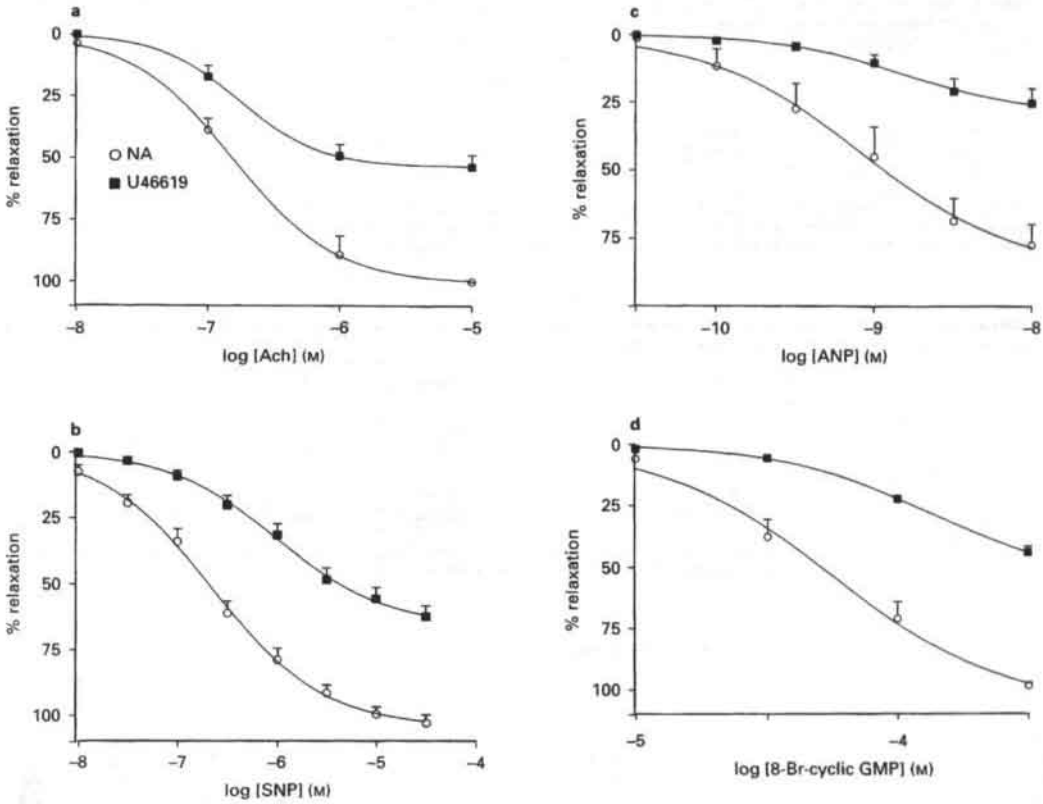

Figure 2 Effects of stimulation of the cyclic GMP pathway. Concentration-dependent relaxant effects of (a) ACh, (b) SNP, (c) ANP and (d) 8-Br-cyclic GMP in piglet pulmonary arteries precontracted with NA and U46619. The responses to ACh were studied in endothelium-intact arteries and to the other agonists in endothelium-denuded arteries. Each point represents the mean of 6–14 arteries; vertical lines show s.e.mean.

The sarcoplasmic reticulum Ca^{2+} ATPase inhibitor thapsigargin (2×10^{-6} M) induced a slowly developing contractile response in resting arteries (322 ± 74 mg, $n=25$). In the presence of thapsigargin, the contractile responses induced by 10^{-5} M NA and 10^{-6} M U46619 averaged 964 ± 102 mg ($n=12$) and 1060 ± 153 mg ($n=13$), respectively. Figure 4c shows that under these conditions, the vasorelaxant response to SNP was strongly inhibited, thus E_{max} was reduced to $60\pm9\%$ and $28\pm4\%$, respectively ($P<0.01$).

Exposure of pulmonary arteries to a K^+-free solution induced a contractile response (112 ± 46 mg, $n=5$) but did not modify the contractile response to 10^{-6} M NA (779 ± 77 mg) or the relaxant effect of SNP ($pD_2=6.80\pm0.04$ and $E_{max}=105\pm4\%$).

Role of protein kinase C in the impaired response to SNP in piglet pulmonary arteries precontracted by U46619

The protein kinase C (PKC) activator PMA (3×10^{-8} M and 3×10^{-7} M) produced a contractile response which reached steady-state within 60–90 min (77 ± 17 mg, $n=5$ and 498 ± 61 mg, $n=10$, respectively). Figure 5a shows that in arteries contracted with 3×10^{-8}M PMA plus 10^{-5} M NA (final tension $=618\pm103$ mg), the relaxant response to SNP was significantly inhibited ($E_{max}=72\pm7\%$, $n=5$) as compared to NA alone ($P>0.05$), whereas in arteries precontracted with 3×10^{-7} M PMA alone, SNP induced only a small vasorelaxant effect ($E_{max}=25\pm4\%$).

Table 2 Relaxant effect of forskolin (10^{-9} M – 10^{-6} M) on the contractions induced by NA, U46619 and ET-1 in piglet endothelium-denuded pulmonary arteries

	n	pD_2	E_{max} (%)
NA (10^{-5} M)	6	7.18 ± 0.10	99 ± 7
U46619 (10^{-6} M)	6	7.15 ± 0.04	96 ± 3
ET-1 (3×10^{-9} M)	7	6.97 ± 0.14	100 ± 4

Results are means ± s.e.means of n number of experiments. No significant differences were found between the three groups.

The effects of pretreatment for 45 min with the PKC inhibitor staurosporine (10^{-8} M or 10^{-7} M) on the responses to SNP in arteries precontracted by 10^{-5} M NA or 10^{-6} M U46619 are shown in Figure 5b and c, respectively. This pretreatment had no effect on the contractile responses to NA (574 ± 98 mg, $n=7$, and 674 ± 84 mg, $n=7$, for 10^{-8} M and 10^{-7} M staurosporine, respectively, $P>0.05$ vs control in Table 1). Furthermore, staurosporine did not modify the relaxant responses to SNP in arteries precontracted with NA. In contrast, 10^{-8} M and 10^{-7} M staurosporine decreased the contractile response to U46619 (895 ± 121 mg, $n=8$, and 852 ± 176 mg, $n=6$ for 10^{-8} M and 10^{-7} M staurosporine, respectively, $P<0.01$ vs controls in Table 1) and augmented, in a concentration-dependent manner, the vasorelaxant response to

Table 3 Relaxant effect of SNP (10^{-8} M–3×10^{-5} M) on the contractions induced by NA, U46619 and ET-1 in endothelium-denuded mesenteric and coronary arteries

	Mesenteric arteries				*Coronary arteries*			
	n	*Tension* (mg)	pD_2	E_{max} (%)	n	*Tension* (mg)	pD_2	E_{max} (%)
NA (10^{-6} M)	5	1480±394	6.55±0.24	107±3	5	–	–	–
U46619 (10^{-6} M)	7	1640±351	6.74±0.13	92±4	7	1190±145	6.57±0.11	96±2
ET-1 (3×10^{-9} M)	9	1957±230	6.42±0.10	99±3	6	1216±190	6.52±0.17	108±6

Tension is the pre-contraction value induced by the vasoconstrictor and pD_2 and E_{max} values refer to the effects of SNP. Results are means±s.e.means of *n* number of experiments. No significant differences were found between the three groups. NA produced very weak contractile effects in coronary arteries and, therefore, the effects of SNP were not studied.

Table 4 Relaxant effects of SNP on endothelium-denuded pulmonary arteries from adult rats or 2–3 month old piglets contracted by maximally effective concentrations of NA and U46619

		n	*Tension* (mg)	pD_2	E_{max} (%)
Adult rat					
	NA (10^{-7} M)	5	351±82	7.9±0.1	101±4
	U46619 (3×10^{-6} M)	5	275±29	7.8±0.1	100±4
2–3 month piglets					
	NA (10^{-5} M)	5	1081±129	6.5±0.3	107±4
	U46619 (10^{-6} M)	7	2034±169**	6.1±0.1*	64±6**

Tension is the pre-contraction value induced by the vasoconstrictor and pD_2 and E_{max} values refer to the effects of SNP. Results are mean±s.e.means of *n* number of experiments. *$P<0.05$, **$P<0.01$, respectively, vs 10^{-5} M NA.

SNP, increasing ($P<0.01$ vs controls in Table 1) both the pD_2 (6.26±0.15 and 6.83±0.19, respectively) and the E_{max} values (95±2% and 101±4%, respectively). Thus, in the presence of 10^{-7} M staurosporine the concentration-response curve to SNP was similar in arteries precontracted with NA and U46619. However, staurosporine (10^{-8} M) did not enhance the vasodilator effect of SNP when it was previously inhibited by 2×10^{-6} M thapsigargin ($pD_2=5.68\pm0.18$ and 5.49±0.10 and $E_{max}=62\pm9\%$ and 59±16% in the absence $n=13$, from Figure 4c, and in the presence of staurosporine, $n=5$, respectively).

Discussion

In the present study we have demonstrated that piglet pulmonary arteries when activated by the thromboxane A_2-mimetic U46619 show abnormally low relaxant responses to either NO or cyclic GMP. This effect was specific to piglet pulmonary arteries, since it was not present in rat pulmonary arteries or in piglet mesenteric or coronary arteries. The activation of protein kinase C (PKC) by the phorbol ester PMA inhibited the relaxant responses to SNP whereas the inhibition of PKC by staurosporine potentiated the relaxant response to SNP in U46619 pre-contracted arteries. The relaxant effect of SNP in piglet pulmonary arteries was inhibited by the sarcoplasmic reticulum Ca^{2+} ATPase inhibitor thapsigargin but not by K^+-free solution, high KCl plus nifedipine or charybdotoxin.

Role of basal cyclic GMP

Resting pulmonary arteries have detectable basal levels of cyclic GMP which are reduced, but not abolished, when the endothelium is removed or by NO synthase inhibitors, indicating that endothelial release of NO partially accounts for the basal cyclic GMP concentration (Ignarro *et al.*, 1987). In accordance with previous studies (Levy *et al.*, 1995), in the piglet pulmonary artery the tonic contractile response to NA was augmented by endothelium removal. We found basal levels of cyclic GMP even in endothelium-denuded piglet pulmonary arteries, as detected by radioimmunoassay, and its inhibition by the guanylate cyclase inhibitor methylene blue augmented the contractile response induced by NA. Furthermore, dipyridamole induced a relaxant response in endothelium-denuded

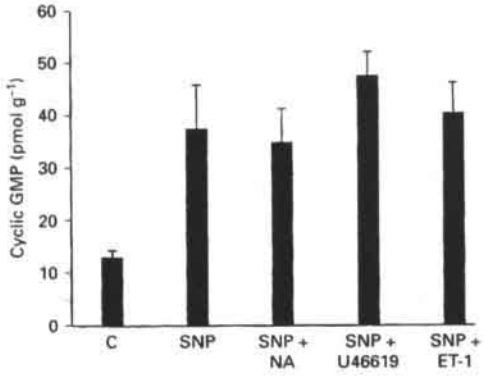

Figure 3 Effects of NA, U46619 and ET-1 on the formation of cyclic GMP stimulated by SNP. Results were obtained in endothelium-denuded arteries under control conditions (C) and in the presence of 10^{-5} M SNP alone or in combination with 10^{-5} M NA, 10^{-6} M U46619 or 3×10^{-9} M ET-1. Each column represents the mean±s.e.mean of 7–8 experiments.

pulmonary arteries. The vasodilator response to dipyridamole in the pulmonary circulation has been attributed to an increase in cyclic GMP by specifically inhibiting its degradation by cyclic GMP-dependent (type V) phosphodiesterase and not to its inhibitory effects of the uptake of adenosine (Ziegler *et al.*, 1995). However, this latter possibility cannot be fully excluded in our experiments. Thus, it can be concluded that basal formation of cyclic GMP modulates the contractile effects of NA and that the endothelium, by releasing NO, is partly responsible of cyclic GMP production. In contrast, the contractile responses to U46619 and ET-1 were unaffected by endothelium removal. The maximal U46619-induced tonic contractions were of higher magnitude than those induced by NA and this difference could be abolished by methylene blue, an inhibitor of soluble guanylate cyclase. From these results it seems that U46619 inhibits the synthesis or action of basal cyclic GMP in pulmonary smooth muscle cells. Alternatively, the differences

between NA and U46619 can be explained on the basis that NA might increase cyclic GMP and induce relaxation. This has been found following the activation of endothelial α_2-adrenoceptors in piglet pulmonary arteries by the non selective α-adrenoceptor agonist phenylephrine or the selective α_2-adrenoceptor agonist clonidine, but not by the selective α_1-adrenoceptor agonist methoxamine (Pepke-Zaba *et al.*, 1993). However, this was not the case in our study, since the experiments were performed in endothelium-denuded arteries and even in these conditions methylene blue induced a contractile

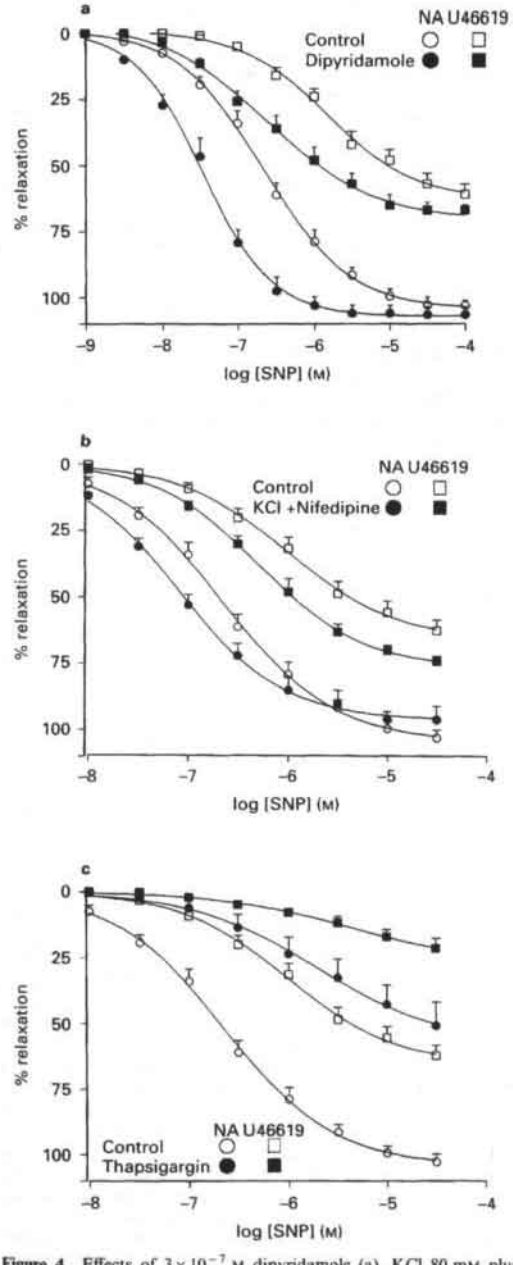

Figure 4 Effects of 3×10^{-7} M dipyridamole (a), KCl 80 mM plus 10^{-6} M nifedipine (b) and 2×10^{-6} M thapsigargin (c) on the responses to SNP in piglet endothelium-denuded pulmonary arteries contracted by 10^{-5} M NA and 10^{-6} M U46619. Each symbol represents the mean of 5–13 arteries; vertical lines show s.e.mean.

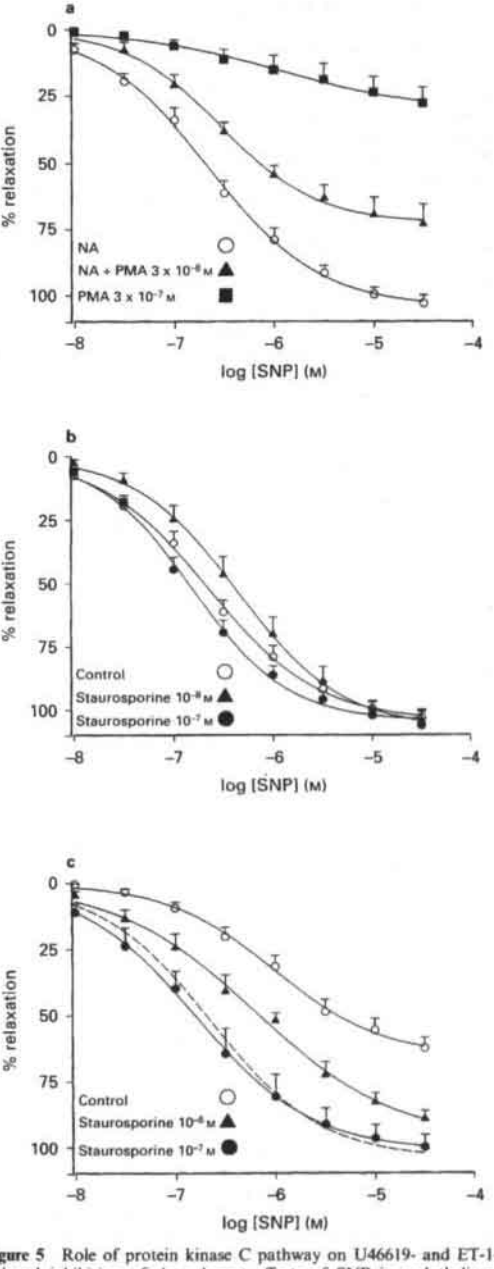

Figure 5 Role of protein kinase C pathway on U46619- and ET-1-induced inhibition of the relaxant effects of SNP in endothelium-denuded piglet pulmonary arteries. (a) The relaxant effects of SNP in arteries contracted by 10^{-5} M NA, 3×10^{-8} M PMA plus 10^{-5} M NA or 3×10^{-7} M PMA alone. (b) and (c) The effects of staurosporine 10^{-8} M and 10^{-7} M (controls are from Figure 2b) on the relaxant effects of SNP in arteries contracted by 10^{-5} M NA (b) and 10^{-6} M U46619 (c). In (c) the dashed line representing the fitted curve of the effects of SNP in NA-contracted arteries under control conditions (taken from (b)) is given for reference. Each symbol represents the mean of 6–10 arteries; vertical lines show s.e.mean.

response in arteries precontracted by NA. Furthermore, when NA was applied on top of a maximally effective U46619-induced contraction, it further contracted the artery.

Effects of stimulation of cyclic GMP

Cyclic GMP can be synthesized by the soluble and the membrane-bound isoforms of guanylate cyclase which are mainly activated by NO and ANP, respectively (Warner *et al.*, 1994). NO can be raised by stimulating its release from endothelial cells (e.g. with ACh) or by the administration of NO donors such as SNP. Most of the effects of cyclic GMP are mediated by stimulation of the cyclic GMP-dependent protein kinase (PKG) (Warner *et al.*, 1994; Lincoln *et al.*, 1994), which can also be activated by membrane permeable and more stable cyclic GMP analogues such as 8-Br-cyclic GMP. In the present study, various activators of the cyclic GMP pathway (i.e., ACh, SNP, ANP and 8-Br-cyclic GMP) fully relaxed NA-contracted arteries, while only a partial relaxation was observed on the contractions induced by U46619. Likewise, expression of inducible nitric oxide synthase (iNOS) by endotoxin in these arteries depressed the contractile responses to NA but did not affect the responses to U46619 (Pérez-Vizcaino *et al.*, 1996). Dipyridamole, an inhibitor of type V phosphodiesterase, produced a similar leftward shift of the concentration-response curve to SNP in NA- and U46619-contracted vessels, but was unable to enhance its maximal response, which suggests that U46619 was not increasing the degradation of cyclic GMP. Moreover, U46619 did not affect SNP-induced increase in cyclic GMP synthesis. Taken together, these results suggest that U46619 inhibits the NO/cyclic GMP pathway for smooth muscle relaxation beyond the level of cyclicGMP synthesis, probably by inhibiting the activity of PKG or the mechanisms by which PKG induces its relaxant effects. An alternative explanation for the reduced relaxant responses to NO/cyclicGMP is that U46619 might induce a contractile response through a different signal transduction pathway from NA. This pathway could be less sensitive to inhibition by cyclic GMP. Assuming this possibility, U46619 would not actively inhibit the NO/cyclicGMP pathway but the contractions induced by U46619 would just be less sensitive to it. Because U46619 induced greater maximal contractile responses than NA, the effects of SNP were also studied (Table 1) in arteries contracted with lower concentrations of U46619 which produced a contractile response similar to that induced by NA. Under these conditions, arteries pre-contracted by U46619 (3×10^{-8} M or 10^{-7} M) still showed a reduced relaxant response to SNP as compared to NA. Therefore, the inhibitory action of U46619 on SNP-induced relaxation is independent of previous tone. Since the relaxant response to SNP was similar in arteries treated with U46619 alone or in combination with NA, differences in the relaxant response to SNP between NA and U46619 must be due to an U46619-induced impairment of the SNP-induced relaxation, rather than to a NA-induced potentiation of SNP-induced relaxation. A possible interference of NA with α_2-adrenoceptors can be ruled out since similar results were obtained with methoxamine (selective α_1-adrenoceptor agonist). Pulmonary arteries precontracted with ET-1 (10^{-9} M) showed similar relaxations to SNP as NA pre-contracted arteries, whereas at 3×10^{-9} M ET-1, which induced a contraction higher than 10^{-5} M NA, the relaxant response to SNP was greatly reduced, therefore, this higher tone might be responsible for the inhibitory action. However, it should be noted that 10^{-5} M NA-induced contractions were maximal or near maximal and they could be fully inhibited by SNP, i.e. an activation of the NO/cyclicGMP pathway can abolish vascular tone in the presence of any amount of NA. In contrast, if ET-1 concentrations were raised over a certain level, the increased vascular tone could not be fully inhibited even with a maximal activation of the NO/cyclic GMP pathway.

Specificity of U46619-and ET-1-induced effects

In order to assess the specificity of the impaired response to SNP observed in 2 week old piglet pulmonary arteries, we also studied the effects of SNP in endothelium-denuded mesenteric and coronary arteries from the same animals, pulmonary arteries from older piglets (2–3 months old) and pulmonary arteries from adult rats. SNP fully relaxed mesenteric and coronary arteries precontracted by NA, U46619 or ET-1 as well as those induced by NA and U46619 in adult rat pulmonary arteries. However, in pulmonary arteries from 2–3 month old piglets the vasorelaxant response induced by SNP was similar to that observed in 2 week old piglets contracted by either NA or U46619 (i.e. again U46619-induced contractions were partially resistant to the relaxant effects of SNP).

The adenylate cyclase activator forskolin (Seamon & Daly, 1986), which increases cyclic AMP levels, produced complete vasorelaxation of the contractions induced by NA, U46619 and ET-1. The mechanism by which cyclic AMP causes smooth muscle relaxation is not fully understood. While activation of the specific cyclic AMP-dependent protein kinase has been shown to be involved, more recent data suggest that cyclic AMP could also stimulate PKG and relax smooth muscle (Lincoln *et al.*, 1990). Due to the differences in the relaxant response between cyclic AMP- and cyclic GMP-dependent vasodilators, our data might suggest that cyclic AMP- and cyclic GMP act via different pathways in these arteries. However, this requires further investigation. All these results showed that the abnormal relaxant response to the NO/cyclic GMP pathway is a singular feature of piglet pulmonary arteries when activated by U46619.

Mechanism of action of the vasorelaxant effect of SNP in piglet pulmonary arteries

SNP causes vascular smooth muscle relaxation by releasing NO which, in turn, activates guanylate cyclase and increases intracellular cyclic GMP levels (Rapoport *et al.*, 1985; Ignarro *et al.*, 1986; Kowaluk *et al.*, 1992). In the present study, SNP raised intracellular cyclic GMP by about three fold and its vasorelaxant effect was potentiated by dipyridamole and inhibited by ODQ, which indicates that SNP-induced relaxation in piglet pulmonary arteries is mediated by stimulation of cyclic GMP synthesis.

Cyclic GMP and PKG may control a large number of cellular activities to regulate vascular smooth muscle tone (Lincoln *et al.*, 1994), including inhibition of the synthesis and/or action of IP_3 (Hirata *et al.*, 1990), activation of the sarcoplasmic reticulum Ca^{2+}-ATPase (Rashatwar *et al.*, 1987; Luo *et al.*, 1993), activation of Ca^{2+}-dependent K^+ channels (Archer *et al.*, 1994), inhibition of Ca^{2+} entry and possibly activation of the Na^+/K^+-ATPase (Rapoport *et al.*, 1985). The role of each of these mechanisms may be different depending on the vascular bed (Ferrer *et al.*, 1995). All these effects may lead to decreased cytosolic Ca^{2+} levels and/or Ca^{2+} sensitivity of the contractile apparatus of vascular smooth muscle leading to relaxation (Lincoln *et al.*, 1994; Warner *et al.*, 1994). Using pharmacological tools we have investigated the role of several of these mechanisms in the piglet pulmonary artery. The role of K^+ channel opening was studied in preparations exposed to high KCl concentrations, thus eliminating the chemical gradient for K^+ efflux so that the opening of K^+ channels would not result in net K^+ flow and hyperpolarization. Nifedipine was included in these experiments to avoid an excessive intracellular Ca^{2+} load induced by KCl depolarization. Under these conditions, the relaxant effect of SNP was not inhibited and instead a weak but significant increase was observed, suggesting that K^+ channel activation is unlikely to mediate the vasodilator effects of SNP. Moreover, the specific inhibitor of large conductance Ca^{2+}-activated K^+ channels charybdotoxin (10^{-7} M) had no effect on SNP-induced relaxation which is consistent with a lack of involvement of Ca^{2+}-activated K^+ channels in SNP-induced relaxation. Ca^{2+} sequestration by the

sarcoplasmic reticulum Ca^{2+}-ATPase leads to decreased cytosolic Ca^{2+} levels and smooth muscle relaxation. Thapsigargin, a specific inhibitor of this Ca^{2+}-ATPase (Thastrup *et al.*, 1990), markedly suppressed SNP-induced relaxation in arteries contracted by either NA or U46619, which indicated that increased Ca^{2+} uptake by the sarcoplasmic reticulum is an important mechanism by which SNP decreases $[Ca^{2+}]_i$ and induces its relaxant effects in piglet pulmonary arteries. Activation of the membrane electrogenic Na^+/K^+ ATPase leads to vascular smooth muscle hyperpolarization and relaxation, whereas its inhibition produces the opposite effects (Rapoport *et al.*, 1985). Incubation in a K^+-free solution, which inhibits the Na^+/K^+-ATPase, had no effect on SNP-induced relaxation, suggesting that an activation of this pump is not involved in its vasorelaxant effect in piglet pulmonary arteries. This is in accordance with previous results from our group (Villamor *et al.*, 1996a), showing that the relaxant effects of ACh in these arteries were not modified by incubation with a Mg^{2+}-free solution which also inhibits the activity of the pump.

Possible mechanisms of reduced NO/cyclic GMP relaxation in U46619 pre-contracted arteries

We further investigated the mechanisms involved in the inhibition of the relaxant effects of SNP by U46619. Because both U46619 and ET-1 inhibit several K^+ channels (Miyoshi *et al.*, 1992; Scornik & Toro, 1992) this effect might be mediating the inhibition of SNP-induced relaxation. However, as described above, K^+ channels do not appear to be involved in SNP-induced relaxation in pulmonary arteries. Furthermore, the inhibitory action of U46619 and ET-1 on K^+ channels has been shown in porcine coronary artery myocytes (Miyoshi *et al.*, 1992; Scornik & Toro, 1992) which showed normal relaxation to SNP in our experiments.

G-protein linked membrane receptors for several vasoconstrictors including NA, thromboxane A_2 (TXA_2) and ET-1 (α_1-adrenoceptors, TP and ET_A receptors, respectively) are coupled with phospholipase C (Strader *et al.*, 1995). Activated phospholipase C catalyzes hydrolysis of phosphoinositol 1,4,5-diphosphate (PIP_2) into IP_3, which releases Ca^{2+} from intracellular stores, and diacylglycerol, which activates PKC. In our experiments, activation of PKC by the phorbol ester PMA, alone or in combination with NA, inhibited the relaxant response to SNP, suggesting that one potential mechanism of inhibition of cyclic GMP-induced vasodilatation by U46619 might be related to the activation of PKC. This has also been shown in the rat aorta where SNP was more effective in relaxing methoxamine- than phorbol ester-induced contractions (Morrison & Pollock, 1990). Staurosporine, a potent inhibitor of protein kinase C (Hidaka & Kobayashi, 1992), did not modify the relaxant response to SNP in arteries precontracted by NA, but abolished the inhibition induced by U46619. Thus, these results suggest that U46619 may activate PKC and thus, inhibit the relaxant effects of cyclic GMP. Staurosporine also depressed the contractile response to U46619, indicating that inhibition of PKC might increase the sensitivity not only to stimulated cyclic GMP but also to basal cyclic GMP. ET-1 has been shown to stimulate the release of TXA_2 in guinea-pig lung (De Nucci *et al.*, 1988), so that ET-1 might inhibit SNP-induced relaxation by releasing TXA_2. However, this was not the case in the present study, because the cyclo-oxygenase inhibitor meclofenamate did not change either the contractile effect of ET-1 or the relaxant effect of SNP.

Implications of TXA_2, ET-1 and impaired NO/cyclic GMP pathway in the pulmonary vascular bed

The finding that U46619 inhibits the NO/cyclic GMP pathway in the pulmonary vasculature may help to explain why: (a) The induction of iNOS by endotoxin or Group B *Streptococcus* and the subsequent large increase in NO concentrations did not modify the contractile responses to U46619 in piglet isolated pulmonary arteries despite reducing the contractions to NA (Villamor *et al.*, 1996b; Pérez-Vizcaíno *et al.*, 1996). (b) The administration of inhaled NO to piglets did not modify (10 p.p.m) (Weitzberg *et al.*, 1993) or only slightly reduced (50 p.p.m) (Klemm *et al.*, 1995) the TXA_2-mediated early phase but inhibited the TXA_2-independent late phase of endotoxin-induced pulmonary hypertension (Weitzberg *et al.*, 1993; Klemm *et al.*, 1995). (c) In piglets with endotoxin-induced pulmonary hypertension, the administration of NO synthase inhibitors did not further increase pulmonary vascular resistance (Klemm *et al.*, 1995; Weitzberg *et al.*, 1995). (d) Inhaled NO (50 p.p.m) had no effect on the pulmonary hypertension induced by continuous i.v. administration of U46619 in dogs (Welte *et al.*, 1995). In contrast, inhaled NO (5–80 p.p.m) inhibited U46619-induced vasoconstriction in sheep (Frostell *et al.*, 1991). In our study, species-dependent differences were also observed, since U46619 inhibited the vasodilator effect of the NO donor SNP in piglet but not in rat pulmonary arteries.

Furthermore, our results showed that ET-1 only impaired the NO/cyclic GMP induced relaxation when inducing a relatively high contraction. In addition, very high concentrations of ET-1 (2×10^{-8} M and 2×10^{-7} M) inhibited SNP-induced relaxation in human pulmonary arteries and veins but, in contrast to the present study, it decreased cyclic GMP synthesis (Pussard *et al.*, 1995). To our knowledge it is not known if inhaled nitric oxide can reduce pulmonary hypertension induced by infusion of ET-1. However, the late phase of pulmonary hypertension induced by endotoxin in piglets, which has been related to increased production of ET-1 (Weitzberg *et al.*, 1996), can be inhibited by inhaled NO (Weitzberg *et al.*, 1993; Klemm *et al.*, 1995). We have also recently demonstrated that iNOS induction by Group B *Streptococci* reduces the contractile responses to ET-1 (Villamor *et al.*, 1996b).

Pulmonary hypertension is accompanied by an increase in pulmonary vascular resistances due to an imbalance between vasoactive mediators. Increased ET-1 and TXA_2 levels and decreased activity of NO/cyclic GMP are the most recognized mechanisms in the vasoconstrictor and vasodilator responses, respectively (see Introduction). It is becoming clear that no single mechanism can be responsible for the different clinical forms of pulmonary hypertension. Impairment of the NO/cyclic GMP pathway at the level of NO synthesis, activation of guanylate cyclase and/or the activity of cyclic GMP may depend on several factors, including the species, age and the factor causing the pulmonary insult. Our results provide evidence for a novel mechanism of NO/cyclic GMP impairment mediated by PKC through TXA_2 receptor activation at the level of cyclic GMP activity. Inhaled NO has been shown to be effective in vasodilating the ventilated lung areas and improving gas exchange in newborn and adult patients with pulmonary hypertension (Kinsella *et al.*, 1993; Falke, 1993; Abman & Kinsella, 1995). However, about half of the patients do not respond to inhaled NO (Roberts *et al.*, 1997; Neonatal Inhaled Nitric Oxide Study Group, 1997). A possible theoretical explanation for this lack of response is an alteration of the smooth muscle responsiveness to NO (Cremona *et al.*, 1991; Abman & Kinsella, 1995). Because anatomical development of neonatal pig lung is similar to that of human lung and neonatal piglets have been widely used as an experimental model of pulmonary hypertension of the newborn (e.g. Gibson *et al.*, 1987), it is tempting to speculate that an impairment of the NO/cyclic GMP pathway similar to that herein described may play a role in human nonresponders to inhaled NO. Understanding the precise mechanisms regulating the NO/cyclic GMP signalling under physiological and pathological conditions will certainly help in the therapeutic management of patients with pulmonary hypertension.

In conclusion, piglet pulmonary arteries pre-contracted by the TXA_2-mimetic U46619 showed reduced relaxant responses to the NO/cyclic GMP-pathway. This effect was not associated with changes in cyclic GMP content and was not observed in

piglet mesenteric or coronary arteries or in rat pulmonary arteries. SNP-induced relaxation in piglet pulmonary arteries was inhibited by the sarcoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin (but not by inhibition of either the membrane Na^+/K^+-ATPase or K^+ channels) indicating a role for Ca^{2+} uptake by the sarcoplasmic reticulum in these relaxant effects. The effects of U46619 on the relaxation of SNP could be abolished by inhibition of PKC, suggesting that PKC may be a part of signal transduction in the U46619-induced inhibitory effect.

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Chapter XI. **Pulmonary versus systemic effects of vasodilator drugs: an in vitro study in isolated intrapulmonary and mesenteric arteries of neonatal piglets.**

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Pulmonary versus systemic effects of vasodilator drugs: an in vitro study in isolated intrapulmonary and mesenteric arteries of neonatal piglets

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Abstract

The ability of several vasodilators to inhibit the responses to noradrenaline and U46619 (a thromboxane A_2 analog) in isolated pulmonary and mesenteric arteries of neonatal piglets was compared. In pulmonary arteries, acetylcholine produced endothelium-dependent relaxations (pIC_{50} = about 6.8) while, in mesenteric arteries, a relaxant ($\leq 10^{-7}$ M) or a contractile response ($\geq 10^{-6}$ M) was observed. Sodium nitroprusside produced relaxant effects in pulmonary and mesenteric arteries contracted by noradrenaline (pIC_{50} = 6.6 and 6.0, respectively) and U46619 (pIC_{50} = 5.4 and 6.7, respectively). ATP induced an endothelium-independent relaxation in pulmonary arteries (pIC_{50} = about 4) but in mesenteric arteries it produced weak relaxant effects. In resting mesenteric arteries, ATP induced a concentration-dependent contraction which was not observed in pulmonary arteries. Prostaglandin E_1 induced a concentration-dependent relaxation in pulmonary arteries (pIC_{50} = about 6). In mesenteric arteries, prostaglandin E_1 at $< 10^{-6}$ M produced a contractile effect whereas, at higher concentrations, a relaxant response was observed. The α-adrenoceptor antagonist tolazoline had no effect on arteries contracted by U46619 but relaxed arteries contracted by noradrenaline being slightly more potent in mesenteric than in pulmonary arteries (pIC_{50} = 5.1 and 4.8, respectively). Nifedipine ($> 10^{-7}$ M) relaxed both arteries, mesenteric being more sensitive than pulmonary arteries and noradrenaline more sensitive than U46619-induced contractions. In conclusion, differences in the relaxant effects for all vasodilators were found depending on the artery, the vasoconstrictor used or both. However, ATP was the only drug which, regardless of the concentration or vasoconstrictor used, produced greater relaxant effects in pulmonary than in mesenteric arteries.

Keywords: Pulmonary artery; Prostaglandin E_1; ATP; Acetylcholine; Tolazoline; Nifedipine; Sodium nitroprusside

1. Introduction

The normal adult pulmonary circulation is a low pressure, low resistance circuit (Barnes and Liu, 1995). In contrast, in the fetus, gas exchange occurs in the placenta and not in the lung and, therefore, right ventricular cardiac output is directed through the ductus arteriosus to the systemic circulation. Consequently, the fetal pulmonary vascular resistance is high and the pulmonary blood flow is low (Fineman et al., 1995). The transition from the fetal to the adult pulmonary circulation in the perinatal period is a delicate process which takes place at birth and in the following weeks (Fineman et al., 1995). Failure of the pulmonary circulation to undergo this transition results in persistent pulmonary hypertension of the newborn. Persistent pulmonary hypertension of the newborn is characterized by an increased pulmonary vascular resistance resulting in right to left shunting of blood across a patent foramen ovale and/or ductus arteriosus, severe hypoxemia and acidosis (Roberts and Shaul, 1993). Various intravenous vasodilators including the α-adrenoceptor blocker tolazoline (Stevenson et al., 1979; Ward, 1984; Gouyon and Francoise, 1992), prostaglandins I_2 and E_1 (Gouyon and Francoise, 1992), the Ca^{2+} channel blocker nifedipine (Simmoneau et al., 1981), $MgSO_4$ (Abu-Osba et al., 1992), acetylcholine (Tripp et al., 1980), the nitric oxide donor sodium nitroprusside (Benitz et al., 1985) and ATP (Fineman et al., 1990; Konduri and Woodard, 1991) have been used to reduce the increased pulmonary vascular resistance (Drummond and Lock, 1984; Kulik and Lock, 1984; Gouyon and Francoise, 1992; Roberts and Shaul, 1993). Since the magnitude of the shunt depends on the difference between pulmonary artery and aortic pressure, systemic vasodilation would increase the flow across the ductus

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reducing the blood supply to the lung and worsening the pulmonary gas exchange. Therefore, the ideal drug for the treatment of persistent pulmonary hypertension of the newborn should be a vasodilator with selectivity for pulmonary over systemic vessels (Roberts and Shaul, 1993; Drummond and Lock, 1984). Recently, the direct delivery of drugs to the lung by inhalation or endotracheal administration has been tested successfully in human newborns with nitric oxide (NO, Roberts et al., 1992; Kinsella et al., 1992) and tolazoline (Welch et al., 1995) and in animal experimental models with cGMP analogs (Lawson et al., 1995) and prostaglandin I_2 (Zobel et al., 1995) which reduce pulmonary artery pressure with minimum systemic effects. This strategy has the additional advantage that the best ventilated areas of the lung are those receiving higher concentrations of drugs and, therefore, those areas will receive higher flow allowing a higher gas exchange. However, this approach is not extensively available yet and may have other undesirable effects, particularly in chronic treatment.

The neonatal circulatory system responds quite differently to drugs than does the mature circulation but the effects of vasodilators in pulmonary neonatal vessels have been poorly investigated (Fineman et al., 1995). Therefore, the aim of the present work was to compare the ability of several vasodilators to inhibit the responses to noradrenaline and the thromboxane A_2 mimetic U46619 in the isolated pulmonary and mesenteric arteries of neonatal piglets.

2. Materials and methods

2.1. Tissue preparation

Male piglets (10–17 days of age, 4679 ± 267 g) obtained from the local abattoir were killed by exsanguination and the lungs and mesenteric beds were rapidly immersed in cold (4°C) Krebs solution (composition in mM: NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2.0, KH_2PO_4 1.2 and glucose 11) and transported immediately to the laboratory. Pulmonary and mesenteric arteries (i.d. 1–2 mm) were carefully dissected free of surrounding tissue and cut into rings of 2–3 mm of length (Pérez-Vizcaíno et al., 1994; Villamor et al., 1995). Two L-shaped stainless-steel wires were inserted into the arterial lumen and the rings were introduced in Allhin organ chambers filled with Krebs solution (gassed with 95% O_2 and 5% CO_2 at 37°C). One wire was attached to the chamber and the other to an isometric force-displacement transducer coupled to a signal amplifier (model PRE 206-4; Cibertec, Madrid, Spain) and connected to a Hewlett Packard computer via an A/D interface. Contractile tension was recorded by a REGXPC computer program (Cibertec). The rings were initially stretched to a resting tension of 0.5 *g* (pulmonary rings) or 2 *g* (mesenteric rings) and allowed to equilibrate for 60–90 min. During this period, tissues were re-stretched and washed every 30 min with warm Krebs solution. In some experiments, the endothelium was removed by gently rubbing the intimal surface of the rings with a metal rod. The presence of functional endothelium was tested by addition of acetylcholine (10^{-7} M) in arteries pre-contracted with 3×10^{-7} M noradrenaline. The ability of acetylcholine to induce relaxation of unrubbed rings was taken as an indicator of the presence of functional endothelium.

2.2. Experimental protocol

After equilibration, the rings were contracted with either 10^{-5} M noradrenaline or 10^{-6} M U46619. When the contractile response to each agonist reached a stable tension, cumulative concentration-response curves to acetylcholine, ATP, prostaglandin E_1, sodium nitroprusside, nifedipine and tolazoline were carried out by cumulative addition of drugs after a steady-state relaxant response was reached after each increment. In some arteries, the effects of vasodilators were evaluated in the presence of the nitric oxide synthase inhibitor N^G-nitro-L-arginine-methyl ester (L-NAME, 10^{-4} M) or in endothelium-denuded arteries. In another set of rings, the vasoconstrictor effects of ATP were tested in endothelium intact arteries under resting conditions.

2.3. Drugs

The following drugs were used: (−)-noradrenaline bitartrate, acetylcholine chloride, L-NAME, sodium nitroprusside, U46619, prostaglandin E_1, ATP-$MgCl_2$, tolazoline hydrochloride (Sigma, London, UK) and nifedipine (Bayer, Leverkusen, Germany). Drugs were dissolved in deionized distilled water (except nifedipine in absolute ethanol) and further dilutions were carried out in Krebs solution. Noradrenaline (10^{-2} M) was dissolved in 10^{-4} M ascorbic acid to prevent oxidation. The concentrations are expressed as final molar concentration in the tissue chamber.

2.4. Statistical analysis

Results are expressed as mean ± S.E.M. of measurements in *n* arteries. The vasoconstrictor and vasodilator responses were expressed in mg and as a percentage of the pre-contraction value, respectively. Individual cumulative concentration-response curves were fitted to a logistic equation. For the concentration-response of vasoconstrictors, the maximal effect (E_{max}) and the drug concentration exhibiting 50% of the E_{max} (pD_2, expressed as negative log molar) were calculated. For vasodilators, the negative log drug concentrations producing a 50% relaxation of the control contraction (pIC_{50} values) were calculated. Statistically significant differences were calculated by means of

an unpaired Student's t-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of noradrenaline and U46619

The parameters of the concentration-response curves to noradrenaline (10^{-8}-10^{-4} M) and U46619 (10^{-10}-10^{-6} M) in pulmonary and mesenteric arteries are shown in Table 1. The maximal response (E_{max}) to both noradrenaline and U46619 was significantly greater in mesenteric than in pulmonary arteries ($P < 0.01$). In mesenteric arteries, noradrenaline induced greater maximal contractions than U46619 but the opposite occurred in pulmonary arteries. The pD_2 values for U46619-induced contractions were similar in both arteries ($P > 0.05$) whereas the pD_2 value to noradrenaline was greater in mesenteric than in pulmonary arteries ($P < 0.05$). The effects of vasodilators reported below were analyzed on the contractions induced by near-maximally effective concentrations of noradrenaline (10^{-5} M) and U46619 (10^{-6} M).

3.2. Vasodilator effects of acetylcholine

Addition of acetylcholine (10^{-8}-10^{-5} M) resulted in a concentration-dependent relaxation in pulmonary arteries pre-contracted with noradrenaline and U46619 (Fig. 1, Table 2). The relaxant effects of acetylcholine were more pronounced in arteries pre-contracted with noradrenaline as compared to U46619 ($P < 0.01$), so that acetylcholine fully relaxed arteries contracted by noradrenaline even when in arteries contracted by U46619 the maximal relaxation achieved was only 53 ± 5%. This relaxant effect was inhibited when the arteries were pre-treated with 10^{-4} M L-NAME or in endothelium-denuded arteries (not shown).

In mesenteric arteries, pre-contracted with either noradrenaline or U46619 the effects of acetylcholine were biphasic (Fig. 1). At 10^{-8} and 10^{-7} M, it produced a relaxant response (of similar magnitude than in pulmonary arteries pre-contracted with noradrenaline). However, when the concentration of acetylcholine was increased ($\geq 10^{-6}$ M), it produced a concentration-dependent contraction in mesenteric arteries that, at 10^{-5} M, averaged 23 ± 13 and 64 ± 32% over control tension in noradrenaline- and U46619-contracted vessels, respectively.

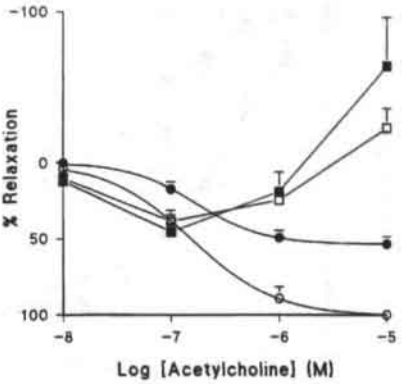

Fig. 1. Relaxant effects of cumulative addition of acetylcholine on pulmonary (circles) and mesenteric arteries (squares) pre-contracted with 10^{-5} M noradrenaline (open symbols) or 10^{-6} M U46619 (solid symbols) of neonatal piglets. Results are expressed as mean ± S.E.M. of 5–10 experiments. Abscissa, % relaxation; ordinate, log acetylcholine concentration (M).

3.3. Vasodilator effects of sodium nitroprusside

The relaxant effect of sodium nitroprusside (10^{-8}-10^{-4} M) was tested in endothelium-denuded arteries. Sodium nitroprusside induced a concentration-dependent relaxation in pulmonary and mesenteric arteries (Fig. 2). However, there were differences in the relaxant potency depending on the artery and the vasoconstrictor employed. Pulmonary arteries contracted by noradrenaline and mesenteric arteries contracted by U46619 were the most sensitive to sodium nitroprusside whereas pulmonary arteries contracted by U46619 were the least (Table 2). Furthermore, sodium nitroprusside was unable to fully relax U46619-induced contractions in pulmonary arteries, so that the highest concentration tested (10^{-4} M) produced a maximal relaxation of 64 ± 5%.

3.4. Vasodilator effects of ATP and effects on resting tension

ATP (10^{-6}-10^{-3} M) induced a concentration-dependent relaxation in pulmonary arteries contracted by either noradrenaline or U46619 ($P > 0.05$ noradrenaline vs. U46619; Fig. 3). However, in mesenteric arteries contracted by noradrenaline, ATP produced only a weak relax-

Table 1
Parameters (pD_2 and E_{max}) of the concentration-response curves to noradrenaline and U46619 in pulmonary and mesenteric arteries

	Pulmonary arteries			Mesenteric arteries		
	pD_2	E_{max} (mg)	n	pD_2	E_{max} (mg)	n
Noradrenaline	6.52 ± 0.07	923 ± 80	17	6.07 ± 0.11 [a]	5273 ± 718 [b]	8
U46619	7.31 ± 0.15 [d]	1279 ± 145 [c]	11	7.11 ± 0.13 [d]	2083 ± 285 [a,d]	7

[a] and [b] $P < 0.05$ and $P < 0.01$ mesenteric vs. pulmonary arteries. [c] and [d] $P < 0.05$ and $P < 0.01$ noradrenaline vs. U46619, respectively.

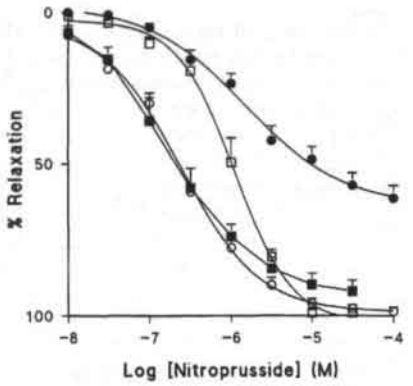

Fig. 2. Relaxant effects of cumulative addition of sodium nitroprusside on pulmonary (circles) and mesenteric arteries (squares) pre-contracted with 10^{-5} M noradrenaline (open symbols) or 10^{-6} M U46619 (solid symbols) of neonatal piglets. Results are expressed as mean ± S.E.M. of 7–9 experiments. Abscissa, % relaxation; ordinate, log nitroprusside concentration (M).

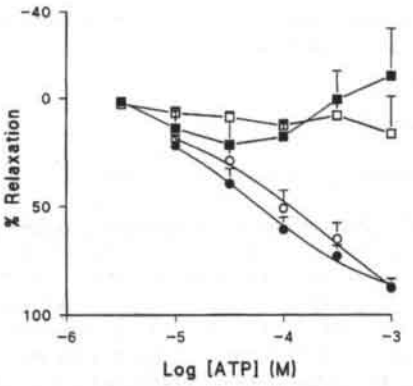

Fig. 3. Relaxant effects of cumulative addition of ATP on pulmonary (circles) and mesenteric arteries (squares) pre-contracted with 10^{-5} M noradrenaline (open symbols) or 10^{-6} M U46619 (solid symbols) of neonatal piglets. Results are expressed as mean ± S.E.M. of 5–7 experiments. Abscissa, % relaxation; ordinate, log ATP concentration (M).

ant effect whereas, in those contracted with U46619, a biphasic effect was observed. At concentrations of $\leq 10^{-4}$ M, ATP produced a relaxant effect while, at higher concentrations, a progressive increase in contractile force was observed, so that at the highest concentration tested reached tension values similar to control values. The effects of ATP were also tested in endothelium-denuded pulmonary arteries and in rings pre-treated for 20 min with L-NAME and then contracted by U46619. In both cases, ATP produced a relaxant effect similar to that observed in control endothelium intact pulmonary arteries (not shown).

Increasing concentrations of ATP (10^{-8}-10^{-3} M) produced minimal or no effect in resting pulmonary arteries ($n = 6$; Fig. 4). In contrast, in mesenteric arteries, ATP ($\geq 10^{-6}$ M) produced a concentration-dependent contraction ($n = 5$; Fig. 4). These contractions were fast but transient, reaching a peak in about 1–2 min and were followed by a slower decay to a lower tone.

3.5. Vasodilator effects of prostaglandin E_1

Addition of prostaglandin E_1 (10^{-8}-10^{-5} M) to pulmonary arteries resulted in a concentration-dependent relaxation (Fig. 5). The maximal relaxant effect was greater when the pulmonary arteries were contracted by noradrenaline than by U46619 ($P < 0.05$; Table 2). In mesenteric arteries, the response to prostaglandin E_1 was biphasic. At low concentrations, prostaglandin E_1 produced a contractile effect which was maximum at 10^{-7}-3×10^{-7} M while, at higher concentrations, prostaglandin E_1 produced a relaxant effect, so that, at 10^{-5} M, it produced almost full relaxation when the arteries were contracted by noradrenaline but only 41 ± 11% when contracted by U46619.

Table 2
Parameters of the concentration-response curves to acetylcholine, sodium nitroprusside, ATP, prostaglandin E_1 and tolazoline calculated from Figs. 1–3 and 5–7

	Pulmonary arteries						Mesenteric arteries					
	Noradrenaline			U46619			Noradrenaline			U46619		
	pIC_{50}	Max (%)	n	pIC_{50}	Max (%)	n	pIC_{50}	Max (%)	n	pIC_{50}	Max (%)	n
Acetylcholine	6.8 ± 0.2	101 ± 2	5	5.9 ± 0.4 [d]	53 ± 5 [d]	5	B.E.	B.E.	7	B.E.	B.E.	8
Nitroprusside	6.6 ± 0.1	100 ± 1	9	5.4 ± 0.3 [d]	61 ± 4 [d]	9	6.0 ± 0.1 [b]	106 ± 4	7	6.7 ± 0.2 [b,d]	92 ± 4	7
ATP	3.9 ± 0.2	87 ± 4	6	4.1 ± 0.1	87 ± 4	7	N.E.	N.E.	7	N.E.	N.E.	7
Prostaglandin E_1	6.1 ± 0.1	96 ± 3	9	5.7 ± 0.2	67 ± 6 [d]	10	5.8 ± 0.1	87 ± 7	8	> 5	41 ± 11 [b,d]	7
Tolazoline	4.8 ± 0.1	91 ± 3	7	N.E.	N.E.	7	5.1 ± 0.1 [a]	88 ± 3 [a]	7	N.E.	N.E.	5
Nifedipine	5.0 ± 0.1	51 ± 8	5	> 5	15 ± 3 [d]	7	5.7 ± 0.1 [b]	75 ± 5 [a]	7	> 5	28 ± 8 [d]	6

pIC_{50} is the negative logarithm of concentration which relaxed 50% and Max is the maximal relaxant effect achieved with the highest concentration of vasodilator tested.

B.E., biphasic effect; relaxation and contraction depending on drug concentration (see text and figures for details). N.E., minimum or no effect.

[a] and [b] $P < 0.05$ and $P < 0.01$ mesenteric vs. pulmonary arteries. [d] $P < 0.01$ noradrenaline vs. U46619, respectively.

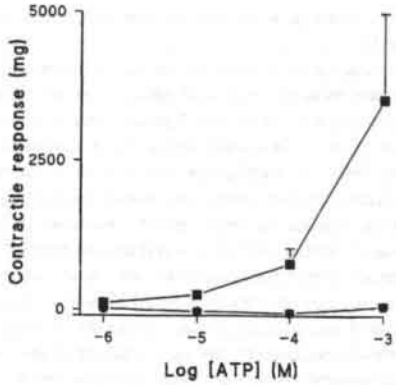

Fig. 4. Contractile effects of cumulative addition of ATP on pulmonary (circles) and mesenteric arteries (squares) on resting tension. Mesenteric arteries responded to ATP with a fast contraction reaching a peak in about 1–2 min and were followed by a decay in tone to a lower sustained level (peak contractile values are shown). Results are expressed as mean ± S.E.M. of 6 experiments. Abscissa, % relaxation; ordinate, log ATP concentration (M).

3.6. Vasodilator effects of tolazoline

The α-adrenoceptor antagonist tolazoline (10^{-6}-5×10^{-4} M) fully relaxed pulmonary or mesenteric arteries contracted by noradrenaline (Fig. 6), this effect being slightly more potent in mesenteric arteries ($P < 0.05$; Table 2). However, tolazoline had no effect on arteries contracted by U46619.

3.7. Vasodilator effects of nifedipine

Fig. 7 shows that, at concentrations of $\leq 10^{-7}$ M, nifedipine produced no relaxant effect on pulmonary or mesenteric arteries. At higher concentrations, it produced a concentration-dependent relaxation in both arteries, even when the maximal relaxant effect could not be reached at the maximal concentration tested (10^{-5} M). Higher concentrations could not be used because the vehicle (ethanol) had significant effects at these concentrations. The relaxant responses to nifedipine were more pronounced when the arteries were contracted by noradrenaline than by U46619 ($P < 0.05$) and in mesenteric than in pulmonary arteries ($P < 0.05$).

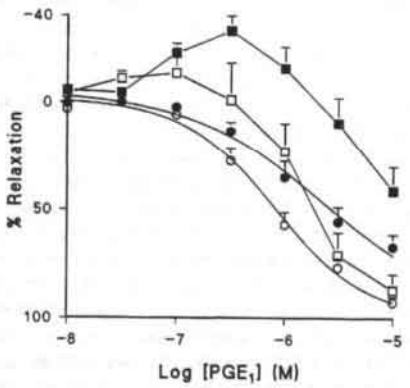

Fig. 5. Relaxant effects of cumulative addition of PGE_1 on pulmonary (circles) and mesenteric arteries (squares) pre-contracted with 10^{-5} M noradrenaline (open symbols) or 10^{-6} M U46619 (solid symbols) of neonatal piglets. Results are expressed as means ± S.E.M. of 7–10 experiments. Abscissa, % relaxation; ordinate, log PGE_1 concentration (M).

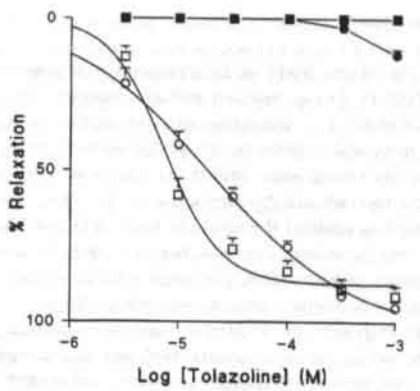

Fig. 6. Relaxant effects of cumulative addition of tolazoline on pulmonary (circles) and mesenteric arteries (squares) pre-contracted with 10^{-5} M noradrenaline (open symbols) or 10^{-6} M U46619 (solid symbols) of neonatal piglets. Results are expressed as mean ± S.E.M. of 6–7 experiments. Abscissa, % relaxation; ordinate, log tolazoline concentration (M).

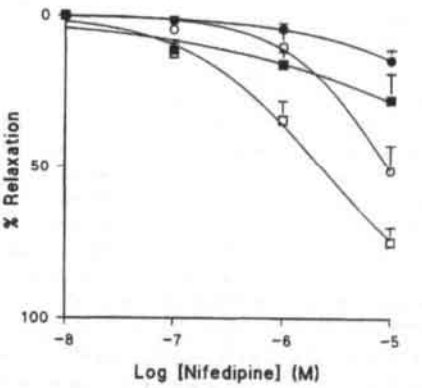

Fig. 7. Relaxant effects of cumulative addition of nifedipine on pulmonary (circles) and mesenteric arteries (squares) pre-contracted with 10^{-5} M noradrenaline (open symbols) or 10^{-6} M U46619 (solid symbols) of neonatal piglets. Results are expressed as mean ± S.E.M. of 5–7 experiments. Abscissa, % relaxation; ordinate, log nifedipine concentration (M).

4. Discussion

In the present study we have compared the effects of six vasodilators (acetylcholine, sodium nitroprusside, ATP, prostaglandin E_1, tolazoline and nifedipine) in isolated pulmonary and mesenteric arteries of neonatal piglets. For any of the vasodilators studied, we have found differences in their relaxant effects depending on the artery, the agonist used to contract the artery or both. ATP was the only drug which, at any concentration and regardless of the contractile agonist used, produced relaxant effects more marked in pulmonary than in mesenteric arteries.

The responses of isolated arteries to vasodilator drugs depend on the species, vascular bed, age, sex, pre-existing tone, endothelial preservation, vasoconstrictor used to increase tone and arterial diameter among other factors. Therefore, we must rise the following methodological considerations. We have studied the effects of vasodilators under conditions of a high vascular tone, i.e. after inducing maximal or near-maximal contractions, which presumably reflect what happens in persistent pulmonary hypertension of the newborn. Noradrenaline and the thromboxane A_2 mimetic U46619 were chosen as contractile agonists since noradrenaline is considered to be one of the most important factors regulating systemic and pulmonary vascular tone (Bülbring and Tomita, 1987; Barnes and Liu, 1995) and thromboxane A_2 has been associated with several forms of persistent pulmonary hypertension of the newborn (Dobyns et al., 1994). The same concentrations of vasoconstrictors were used in pulmonary and mesenteric arteries and, therefore, the vasodilator effects were not evaluated under equieffective concentrations of noradrenaline and U46619 for a given artery. Mesenteric arteries of a similar diameter than pulmonary arteries were chosen as representatives of systemic arteries. However, the effects of vasodilators on the whole systemic vascular resistance is the sum of the effects in all vascular beds and, thus, extrapolation of mesenteric arteries to an universal systemic artery has to be done with caution.

Acetylcholine has been reported to produce endothelium-dependent vasodilatation and both endothelium-dependent and -independent contraction (Furchgott and Zawadki, 1980; Altiere et al., 1986). In the present study, acetylcholine induced a concentration-dependent relaxation in pulmonary arteries and this effect was inhibited by the nitric oxide synthesis inhibitor L-NAME indicating an acetylcholine-induced nitric oxide release from the endothelium (Furchgott and Zawadki, 1980). However, when the arteries were pre-contracted by U46619, acetylcholine was unable to induce full relaxation. In contrast, in mesenteric arteries, acetylcholine produced relaxation at concentrations of $\leq 10^{-7}$ M, but contraction at higher concentrations. Therefore, at high concentrations, acetylcholine showed greater relaxant effects on pulmonary than on mesenteric arteries, although its relaxant effect was significantly less marked when the arteries were pre-contracted by U46619.

The vasodilator effects of sodium nitroprusside have been attributed to the release of nitric oxide which, in turn, stimulates soluble guanylate cyclase and increases the intracellular levels of cGMP (Ignarro and Kadowitz, 1985; Feelisch, 1991). The effects of this drug were analyzed in endothelium denuded arteries to avoid interferences with endothelial release of nitric oxide. Addition of sodium nitroprusside produced full vasorelaxant effects in mesenteric arteries contracted by either U46619 or noradrenaline and in pulmonary arteries contracted by noradrenaline but, as occurred with acetylcholine, it induced only partial relaxation in pulmonary arteries contracted by U46619. Thus, as recently reported (Pérez-Vizcaíno et al., 1996), activation of thromboxane A_2 receptors by U46619 in pulmonary arteries seems to reduce the sensitivity to nitric oxide.

ATP had no effect on resting tension but relaxed pulmonary arteries pre-contracted by noradrenaline or U46619. In contrast, addition of ATP ($> 10^{-6}$ M) to mesenteric arteries at resting tone induced a concentration-dependent contraction. The vasoactive effects of ATP have been attributed to the activation of membrane P_2-purinoceptors. Activation of P_{2x}-purinoceptors located on smooth muscle mediate contraction in both rat and human pulmonary arteries whereas P_{2y}-purinoceptors mediating relaxation are located on the endothelium in rat and on the smooth muscle in human pulmonary arteries (Liu et al., 1989a,b). The present results show that ATP-induced relaxation in neonatal piglet pulmonary arteries are endothelium- and nitric oxide-independent. The purinoceptor subtype mediating this effect is unknown but based on the similarities of the response with human pulmonary arteries it might be tempting to speculate that it is mediated by P_{2y}-purinoceptors located on smooth muscle. In contrast to human pulmonary arteries (Liu et al., 1989a), ATP produced minimal contractile effect in piglet pulmonary arteries at resting tone. At present, we do not know if differences are species- or age-dependent. In pre-contracted mesenteric arteries, ATP produced both relaxant and contractile effects. A weak relaxant response was observed at low concentrations ($\leq 10^{-4}$ M) whereas at higher concentrations, ATP produced a weak contractile effect, so that the average tension level was not significantly different to the initial tension value. Therefore, the vasodilator effect of ATP was selective for pulmonary over mesenteric arteries regardless of the agonist used to contract the arteries.

Prostaglandin E_1 is a non-selective agonist of prostanoid EP_1, EP_2 and EP_3 receptors (Coleman et al., 1994). In general, EP_1 and EP_3 receptor subtypes mediate contraction of smooth muscle, and EP_2 receptors mediate smooth muscle relaxation. Therefore, both prostaglandin E_1-induced vasodilation and vasoconstriction have been reported (Kadowitz et al., 1976; Bergström et al., 1968; Qian

et al., 1994). In the present study, prostaglandin E_1 relaxed pulmonary arteries but this effect was significantly more pronounced when the arteries were pre-contracted by noradrenaline. In contrast, in mesenteric arteries, low concentrations of prostaglandin E_1 induced a small contractile response whereas, at higher concentrations, a relaxation was observed. Therefore, prostaglandin E_1 showed a weak selectivity for pulmonary over mesenteric arteries.

Tolazoline is considered a non-selective α-adrenoceptor blocker (Ruffolo et al., 1991) that exhibits other non-adrenoceptor-mediated vasodilators effects (Drummond and Lock, 1984). In the present study, tolazoline almost fully relaxed pulmonary and mesenteric arteries pre-contracted with noradrenaline, which consistent with its α-adrenoceptor blocking properties and this effect was slightly but significantly more potent in mesenteric than in pulmonary arteries. Tolazoline, however, had no effect in pulmonary or mesenteric arteries contracted by U46619, which suggested that a direct pulmonary vasodilator effect unrelated to α-adrenoceptor blockade was absent.

The potency of the L-type Ca^{2+} channel blocker nifedipine to inhibit the contractions induced by noradrenaline or other agonists is highly variable, depending on the role of Ca^{2+} entry through L-type Ca^{2+} channels in the contractile response (Cauvin et al., 1983; Godfraind et al., 1986). In the present study, only at very high concentrations of nifedipine ($\geq 10^{-6}$ M) induced relaxant responses in noradrenaline- or U46619-precontracted arteries. However, at these concentrations, it is very unlikely that the vasodilator effects of nifedipine can be related to Ca^{2+} entry blockade. The relaxant response to nifedipine was more marked in both pulmonary or mesenteric arteries pre-contracted by noradrenaline as compared to U46619 contracted vessels. Nevertheless, mesenteric were more sensitive than pulmonary arteries to nifedipine.

Since lowering pulmonary artery pressure, while maintaining systemic vascular resistance and good cardiac output, is crucial for newborns with persistent pulmonary hypertension of the newborn, a search for selective pulmonary vasodilators has been constant in the last two decades (Roberts and Shaul, 1993). Most clinical studies in neonates suffering persistent pulmonary hypertension of the newborn, however, have been carried out in small number of patients, were not randomized, and no direct measurements of pulmonary artery pressure were made, so that arterial pO_2 or clinical improvement was used to indirectly evaluate pulmonary vasodilation. Therefore, conclusions regarding drug pulmonary selectivity cannot be drawn and most data come from animal models of persistent pulmonary hypertension of the newborn. Tolazoline, the most widely used drug in the treatment of persistent pulmonary hypertension of the newborn, produced systemic hypotension in $> 50\%$ of patients (Stevenson et al., 1979; Ward, 1984; Starling et al., 1981; Gouyon and Francoise, 1992) and, therefore, it can be considered as a poor pulmonary selective drug. Similar systemic deleterious effects have been reported in animal models of pulmonary hypertension after infusion of acetylcholine (Tripp et al., 1980), nifedipine (Dickstein et al., 1984) or prostaglandin E_1 (Tripp et al., 1980; Starling et al., 1981). The limited use of sodium nitroprusside in persistent pulmonary hypertension of the newborn has rendered variable results (Benitz et al., 1985). The present in vitro results with tolazoline and nifedipine demonstrated that systemic arteries dilate at least as much as pulmonary arteries, supporting the poor selectivity observed in clinical studies. The results obtained with acetylcholine, prostaglandin E_1 or sodium nitroprusside are difficult to interpret in terms of pulmonary vs. systemic selectivity due to the biphasic (contractile and relaxant) responses or to agonist-dependent differences. ATP has demonstrated selective pulmonary vasodilating effects in animal models of pulmonary hypertension (Konduri and Woodard, 1991; Fineman et al., 1990) and our in vitro results also provide evidence of its selective pulmonary vasodilator effect. Indeed, ATP was able to induce contractile responses in mesenteric but not in pulmonary arteries under resting conditions. Very recently, the administration of low doses of ATP in pulmonary hypertensive newborns and infants produced a decrease in pulmonary vascular resistance without effects on systemic blood pressure, suggesting that ATP may be a selective pulmonary vasodilator, although at higher doses it produced mild systemic effects (Brook et al., 1995).

In conclusion, the vasodilators studied exhibited differences in the relaxant effects depending upon the artery and/or the agonist used to contract the vessel. However, ATP was the only drug which, at all concentrations and regardless of the contracting agent used, relaxed the pulmonary artery but not the mesenteric artery.

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Chapter XII. In vitro effects of magnesium sulfate in isolated intrapulmonary and mesenteric arteries of piglets.

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In Vitro Effects of Magnesium Sulfate in Isolated Intrapulmonary and Mesenteric Arteries of Piglets

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ABSTRACT

Magnesium sulfate ($MgSO_4$) has been proposed to be an efficient treatment in persistent pulmonary hypertension of the newborn. We compared the ability of $MgSO_4$ to inhibit the responses to several vasoconstrictors in isolated intrapulmonary and mesenteric arteries from 10–17-d-old piglets. $MgSO_4$ (3–100 mM) produced a slight vasodilator effect in pulmonary arteries precontracted with the thromboxane A_2 mimetic U46619 (10^{-6} M), noradrenaline (10^{-5} M), and KCl (80 mM) (15.1 ± 3.7%; 20 ± 3.33%; 10.4 ± 0.9% at 100 mM $MgSO_4$, respectively). In contrast, in mesenteric arteries $MgSO_4$ produced a marked vasodilation (80.4 ± 4.0%, 93.1 ± 3.46%, and 87.5 ± 1.93% at 100 mM $MgSO_4$, respectively, $p < 0.01$ *versus* pulmonary arteries). The vasodilator effect of $MgSO_4$ was endothelium-independent and reversed by increasing the extracellular Ca^{2+} concentration. After incubation for 1 h of pulmonary arteries with three different $MgSO_4$ concentrations (0, 1.2, and 4.8 mM) there were no differences in the contractile responses to U46619 nor in the vasodilator effects of acetylcholine or sodium nitroprusside. Rapid removal of Mg^{2+} from bath medium produced a transient vasodilation which was more marked in pulmonary than in mesenteric arteries and was greatly reduced by the removal of endothelium or by the nitric oxide synthase inhibitor L-NAME (10^{-4} M). We conclude that $MgSO_4$ is a poor vasodilator of pulmonary arteries *in vitro* and at physiologic concentrations appears to inhibit nitric oxide release from the pulmonary endothelium. Thus, the possible beneficial clinical effects of $MgSO_4$ in persistent pulmonary hypertension of the newborn do not seem to be related to a direct effect on pulmonary vascular smooth muscle. (***Pediatr Res* 39: 1107–1112, 1996**)

Abbreviations

PPHN, persistent pulmonary hypertension of the newborn
$[X]_i$, X intracellular concentration
$[X]_o$, X extracellular concentration
TXA_2, thromboxane A_2
NO, nitric oxide
ACh, acetylcholine
NA, noradrenaline
SNP, sodium nitroprusside
VSM, vascular smooth muscle
U46619, 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$ (TXA_2 analog)
L-NAME, N^{ω}-nitro-L-arginine methyl ester

PPHN is characterized by a increased pulmonary vascular resistance resulting in right to left shunting of blood across a patent foramen ovale and/or ductus arteriosus, severe hypoxemia, and acidosis, which may produce further pulmonary vasoconstriction (1). PPHN appears as a common complication of several pulmonary and nonpulmonary diseases, including meconium aspiration syndrome, congenital diaphragmatic hernia, sepsis, and asphyxia (1, 2). Because the magnitude of the shunt depends on the ratio of systemic and pulmonary vascular resistance, a selective pulmonary vasodilator would dramatically improve the treatment and outcome of PPHN. Recent studies have demonstrated that inhaled nitric oxide (NO) is a potent and selective vasodilator which causes marked improvement in many newborn infants with PPHN (3–5). However, current pharmacologic treatment of PPHN often resorts to intravenous vasodilators that are nonselective for pulmonary circulation and may sometimes exacerbate cardiovascular and intrapulmonary shunting of venous blood and cause systemic hypotension (1, 2, 6).

Extracellular Mg^{2+} concentrations exert an important role in the modulation of VSM tone and reactivity (7, 8). Extracellular Ca^{2+} and Mg^{2+} elicit mutually antagonistic or reciprocal actions on VSM, so that Mg^{2+}, nature's physiologic Ca^{2+} channel blocker (9), antagonizes Ca^{2+} entry into the VSM cell,

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thereby promoting vasodilation (8, 10). Increased Mg^{2+} relaxed VSM, lowered blood pressure, and attenuated VSM cell contractile responses to calcium and other vasoactive agents, whereas decreased Mg^{2+} caused the opposite effects (7, 8, 11). Furthermore, Mg^{2+} also influences NO release (11–13) and arachidonic acid metabolism (11, 14), so that prostacyclin production increased when Mg^{2+} was elevated (15). Animal studies have shown that $MgSO_4$ can prevent and reduce hypoxic pulmonary hypertension (16, 17), and two clinical reports have shown its benefit in the management of PPHN (18, 19). Thus, it was suggested that $MgSO_4$ may exert a beneficial effect in the management of PPHN when other treatments fail, are contraindicated, or not available (18, 19).

Therefore, the aim of the present work was to study the vasodilator effects of $MgSO_4$ in isolated pulmonary as compared with systemic (mesenteric) arteries of piglets. The role of Mg^{2+} on the vascular reactivity and endothelial regulation of NO in these arteries was also analyzed.

METHODS

Tissue preparation. Male piglets (10–17 d of age, 4277 ± 343 g) were killed by exsanguination, and the lungs and mesenteric beds were rapidly immersed in cold (4°C) Krebs solution (composition in mM: NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2.0, KH_2PO_4 1.2, and glucose 11) and transported immediately to the laboratory. The pulmonary arteries (third branch) or mesenteric arteries (internal diameter 1–2 mm) were carefully dissected free of surrounding tissue and cut into rings 2–3 mm in length. Two L-shaped stainless steel wires were inserted into the arterial lumen, and the rings were introduced in Allhin organ chambers filled with Krebs solution (gassed with 95% O_2 and 5% CO_2 at 37°C). One wire was attached to the chamber and the other to an isometric force-displacement transducer (Grass FT07) and connected to a polygraph (Grass, model 7) as previously described (20, 21). The rings were initially stretched to a resting tension of 0.5 g (pulmonary rings) or 2 g (mesenteric rings) and allowed to equilibrate for 60–90 min. During this period tissues were restretched and washed every 30 min with warm Krebs solution. In some experiments the endothelium was removed by gently rubbing the intimal surface of the rings with a metal rod. The presence of functional endothelium was verified by addition of ACh (10^{-6} M) in arteries precontracted with NA. The ability of ACh to induce relaxation of unrubbed rings was taken as an indicator of the presence of functional endothelium.

Experimental protocols. After equilibration, the rings were contracted with either 10^{-5} M NA, 10^{-6} M U46619, or 80 mM KCl. When the contractile response to each agonist reached a stable tension, cumulative concentration-response curves to $MgSO_4$ (0.3–100 mM) were carried out by cumulative increments of $MgSO_4$ concentration after a steady state relaxant response was reached at each increment. After the maximal relaxant response to 100 mM $MgSO_4$ had developed in pulmonary and mesenteric arteries precontracted with 80 mM KCl, $CaCl_2$ (2–12 mM) was added to the bath medium in a cumulative fashion.

In another group of experiments, pulmonary and mesenteric artery rings were incubated for 1 h in Krebs solution containing 0, 1.2, or 4.8 mM $MgSO_4$. After the incubation, the vasoconstrictor effects of U46619 were tested by cumulative increases in the concentration of U46619 (10^{-10} to 10^{-6} M). The vasodilator effects of ACh (10^{-8} to 10^{-4} M) or SNP (10^{-8} to 10^{-4} M) were also tested in arteries precontracted with 10^{-6} M U46619 previously incubated in 0, 1.2, or 4.8 mM $MgSO_4$.

In a third group of experiments we studied the effects of acute extracellular Mg^{2+} removal using a protocol similar to that previously described (11). After equilibration in Krebs solution ($MgSO_4$ concentration 1.2 mM) pulmonary and mesenteric arteries were maximally precontracted with U46619 (10^{-6} M). When the contractile response to U46619 reached a stable tension, the bath medium was replaced by the same solution containing U46619 but without $MgSO_4$. The same protocol was repeated in endothelium-denuded arteries and in the presence of the NO synthase inhibitor L-NAME (10^{-4} M).

Drugs. The following drugs were used: (−)-NA bitartrate, ACh chloride, L-NAME, SNP, U46619, and $MgSO_4 7H_2O$ (Sigma Chemical Co., London). Drugs were dissolved in deionized distilled water, and further dilutions were carried out in Krebs solution. NA (10^{-2} M) was dissolved in 0.2% ascorbic acid to prevent oxidation and then diluted in Krebs solution. The concentrations are expressed as final molar concentration in the tissue chamber.

Statistical analysis. Results are expressed as means ± SEM of measurements in n arteries. The vasoconstrictor and vasodilator responses are expressed in milligrams and as a percentage of the precontraction value, respectively. Individual cumulative concentration-response curves were fitted to a logistic equation. Statistically significant differences were calculated by means of an unpaired t test. $p < 0.05$ was considered statistically significant.

RESULTS

Vasodilator responses to $MgSO_4$ and reversal by Ca^{++}. The concentration response curves to $MgSO_4$ (3–100 mM) in pulmonary and mesenteric arteries precontracted with U46619 (10^{-6} M), NA (10^{-5} M) or KCl (80 mM) are shown in Figure 1, *a*, *b*, and *c*, respectively. In pulmonary arteries, U46619, NA, and KCl induced a contraction averaging 1085 ± 148 mg (n = 6), 504 ± 81 mg (n = 7), and 1408 ± 83 mg (n = 6), respectively. When $MgSO_4$ was stepwise increased from 1.2 mM (normal concentration in Krebs) to 100 mM, it produced a slight vasodilator effect in pulmonary arteries precontracted with U46619, NA, and KCl which averaged 15.1 ± 3.7%, 20.0 ± 3.3%, and 10.4 ± 0.9% in the presence of 100 mM $MgSO_4$, respectively. The relaxant response induced by $MgSO_4$ on U46619-induced contraction was slightly but significantly increased when endothelium was removed (Fig. 1*a*). In contrast, in mesenteric arteries precontracted with U46619 (2655 ± 525, n = 8), NA (2315 ± 289, n = 5), and KCl (3158 ± 845 mg, n = 6), $MgSO_4$ produced a much more marked relaxant effect which averaged 80.4 ± 4.0%, 93.1 ± 3.4%, and 87.5 ± 1.9% at 100 mM $MgSO_4$, respectively ($p < 0.01$ *versus* pulmonary arteries in the three groups). Furthermore, Figure 1*a* shows that

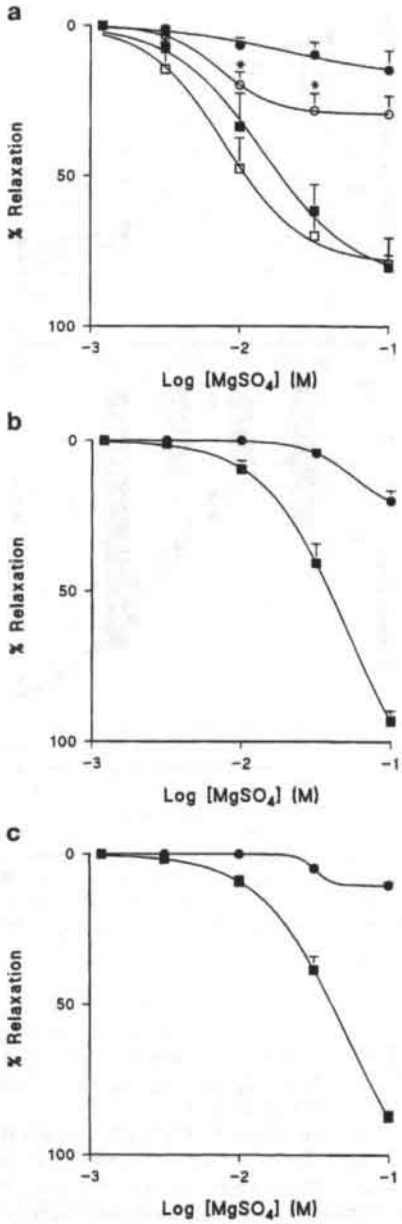

Figure 1. Concentration-response curves to $MgSO_4$ (3–100 mM) in piglet endothelium intact (●, ■) or endothelium denuded (○, □) pulmonary (●, ○) and mesenteric arteries (■, □) precontracted with the TXA_2 mimetic U46619 (10^{-6} M) (*a*), NA (10^{-5} M) (*b*), or KCl (80 mM) (*c*). Ordinate: relaxation (percentage of precontraction tone). Abscissa: log $MgSO_4$ concentration (M). Each symbol represents the mean ± SEM of five to eight experiments.

the relaxant effects of $MgSO_4$ on U46619-induced contractions were not significantly different in mesenteric arteries in which the endothelium was mechanically removed. No differences were observed when the concentration-response curves to $MgSO_4$ were carried out in pulmonary arteries previously incubated in Mg^{2+}-free Krebs solution (data not shown).

As illustrated in Figure 2, after the maximal relaxant response to 100 mM $MgSO_4$ had developed in pulmonary and mesenteric arteries precontracted with 80 mM KCl, addition of $CaCl_2$ (2–12 mM) to the bath medium produced a progressive reversal of the $MgSO_4$-induced vasodilator effect. Moreover, in pulmonary arteries the precontractile response was obtained when the Ca^{2+} was increased over 2.5 mM, whereas in mesenteric arteries exposure to 12 mM $CaCl_2$ recovered 81.6% of $MgSO_4$-induced vasodilation.

Effects of $MgSO_4$ on the contractile response to U46619 and in the vasodilator response to ACh or SNP. The incubation for 1 h in Krebs solution containing different $MgSO_4$ concentrations (0, 1.2 and 4.8 mM) did not influence the contraction-response curves to U46619 (10^{-10} to 10^{-6} M) both in pulmonary (Fig. 3*a*) and mesenteric arteries (Fig. 3*b*).

To determine the effects of Mg^{2+} on agonist-induced endothelium-dependent relaxation, the effects of cumulative addition of ACh on U46619-precontracted pulmonary arteries were studied in the presence of three concentrations of extracellular Mg^{2+}. In pulmonary arteries contracted with U46619 (10^{-6} M), ACh produced a concentration-dependent relaxation (Fig. 4*a*). Increasing the $MgSO_4$ concentration (0, 1.2, and 4.8 mM) in the bathing media tended to reduce (but not significantly) the vasorelaxant effect of ACh. As shown in Figure 4*b*, the vasorelaxant effect of SNP was not significantly different when the arteries were incubated in 1.2 or 4.8 mM $MgSO_4$.

Response of pulmonary and mesenteric arteries to rapid removal of extracellular Mg^{++}. Rapid removal of $MgSO_4$ from the bath medium in pulmonary and mesenteric arteries maximally precontracted with U46619 (10^{-6} M) produced a rapid and transient endothelium-dependent relaxation followed by a progressive spontaneous recovery of tension to previous values (Fig. 5*a*). This relaxant response was more marked in pulmonary than in mesenteric arteries ($p < 0.01$). In pulmo-

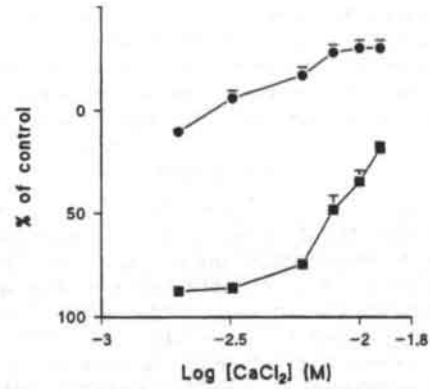

Figure 2. Reversion of the vasodilatory effect induced by 100 mM $MgSO_4$ by cumulative increases of $CaCl_2$ concentration in pulmonary (●) and mesenteric arteries (■). Both arteries were precontracted with 80 mM KCl, then relaxed with 100 mM $MgSO_4$, and the $CaCl_2$ was stepwise increased from 2 to 12 mM. Ordinate: percentage of precontraction tone. Abscissa: log $CaCl_2$ concentration (M). Each symbol represents the mean ± SEM of six experiments.

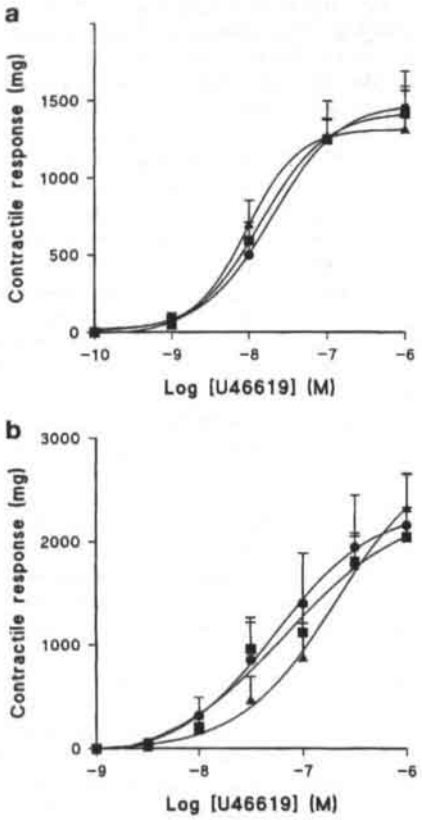

Figure 3. Concentration-response curves to the TXA_2 mimetic U46619 in piglet isolated intrapulmonary (*a*) and mesenteric arteries (*b*) incubated in Krebs solution containing 0 (●), 1.2 (■), and 4.8 mM (▲) $MgSO_4$. Ordinate: contractile response (mg). Abscissa: log U46619 concentration (M). Each symbol represents the mean ± SEM of 7–10 experiments.

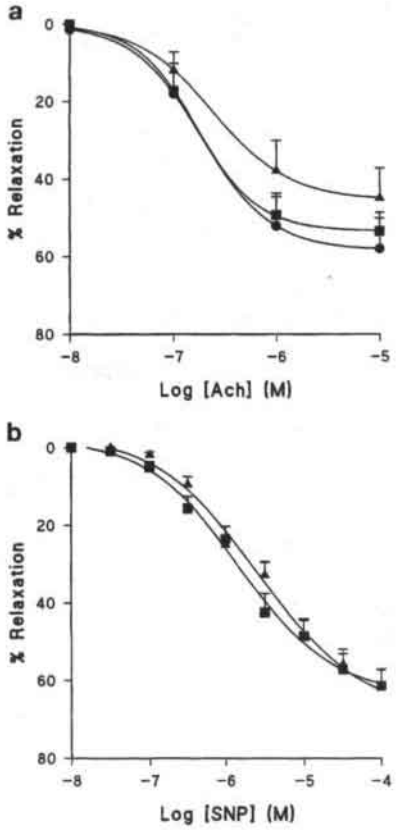

Figure 4. Concentration-response curves to ACh (*a*) and SNP (*b*) in isolated intrapulmonary arteries incubated in Krebs solution containing 0 (●), 1.2 (■), and 4.8 mM (▲) $MgSO_4$ and precontracted with 10^{-6} M U46619. Abscissa: log ACh or SNP concentration (M). Each symbol represents the mean ± SEM of six to eight experiments.

nary arteries this relaxant effect was significantly reduced to almost a similar extent in endothelium-denuded rings or in rings pretreated with the NO synthase inhibitor L-NAME (10^{-4} M) (Fig. 5*b*). Pretreatment of mesenteric arteries with L-NAME abolished the relaxation produced by Mg^{2+} removal.

DISCUSSION

The present results demonstrated that, at least in an *in vitro* porcine model, $MgSO_4$ exhibited a poor pulmonary vasodilator activity. In intrapulmonary arteries (third branch) precontracted with NA, KCl, or U46619, $MgSO_4$ produced a slight vasodilator effect as compared with the marked vasodilation produced in systemic (mesenteric) arteries of a similar diameter. Furthermore, at the normal concentration (1.2 mM), $MgSO_4$ inhibited the vasodilator effect of NO, as proved by the fact that in mesenteric and pulmonary arteries precontracted by U46619 the rapid removal of $MgSO_4$ from the bath medium produced a transient endothelium-dependent vasodilation, which was greatly reduced after removal of the endothelium or by L-NAME. In addition, $MgSO_4$ did not seem to play a marked role in the pulmonary VSM reactivity, because the vasoconstrictor effects of U46619 or the vasodilator effects of the endothelium-dependent vasodilator ACh or the NO donor SNP were not affected by changing the extracellular Mg^{2+} concentration from 0 to 4.8 mM.

Mg^{2+} plays an important role in VSM tone and reactivity. Therefore, Mg^{2+} has been used for decades as an antihypertensive agent (22). However, the use of $MgSO_4$ in PPHN treatment remains controversial. It has been recently reported that $MgSO_4$ produced a significant fall in mean pulmonary pressure both in an adult (16) and newborn sheep model of pulmonary hypertension induced by hypoxia (17) without adversely affecting systemic arterial pressure). After these observations, Abu-Osba *et al.* (18) and Tolsa *et al.* (19) treated 9 and 11 newborns with PPHN, respectively, with $MgSO_4$. Even when they did not measure pulmonary arterial pressure, they observed an increase in baseline arterial oxygen tension and hemoglobin oxygen saturation, which in the absence of changes on systemic arterial pressure was attributed to a decrease in pulmonary vascular resistance and thus right to left

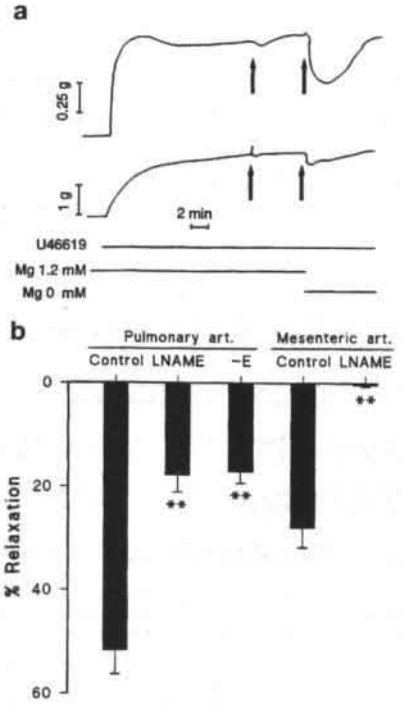

Figure 5. (*a*) Typical tracings of the effects of extracellular Mg^{2+} removal in piglet isolated intrapulmonary (*upper panel*) and mesenteric arteries (*lower panel*) precontracted with 10^{-6} M U46619. Pulmonary and mesenteric rings were contracted by 10^{-6} M U46619, after they reached a steady state contraction the bathing medium was changed by the same solution (Krebs solution containing U46619 and 1.2 mM $MgSO_4$) as indicated by the *first arrow*. Note that minimal washing artifact was observed. When the medium was changed to a $MgSO_4$-free solution (as indicated by the *second arrow*), a transient relaxation was observed. (*b*) Graph shows the influence of endothelium removal or the presence of the NO synthase inhibitor L-NAME (10^{-4} M) on the vasodilatory effect of extracellular Mg^{2+} removal. Relaxation values are expressed as a percentage of precontraction tone and are mean ± SEM of five to six experiments. $^{**}p < 0.01$ *vs* control arteries.

shunt. In contrast, in newborn piglets subjected to hypoxia, the reduction in pulmonary arterial pressure and pulmonary vascular resistance produced by the infusion of $MgSO_4$ was associated with a proportional fall in systemic and pulmonary vascular resistances, indicating that in this animal model $MgSO_4$ was not a specific pulmonary vasodilator (2). A decrease in systemic arterial pressure and systemic vascular resistance has also been observed in newborn sheep with sepsis-mediated pulmonary hypertension treated with $MgSO_4$ (17). In accord with these latter results, we found that $MgSO_4$ exhibited a higher vasodilator activity in systemic than in pulmonary arteries. In fact, the pulmonary vasodilator effect was very weak. However, extrapolation of these *in vitro* results to the clinical situation of PPHN should be done with caution. The present experiments were performed under conditions (pH 7.4 and 95% O_2) quite different from those observed in whole animal experimental models or in patients with PPHN where both acidosis and hypoxia are present. Furthermore, our experiments were performed in 10–17-d-old piglets, whereas PPHN is observed during the first days after birth (1). Even when important developmental differences in pulmonary vascular reactivity have been reported during the first days of life (23), 2–3-wk-old piglet or sheep have been widely used as experimental models of PPHN (*e.g.* see Refs 17 and 26).

Although the mechanism of Mg^{2+} vascular action is not fully understood, Mg^{2+} deficiency-induced potentiation of VSM contraction has been attributed generally to a reciprocal increase in Ca^{2+} influx into VSM cells (7, 8). Elevated $[Mg^{2+}]_o$ relaxes VSM, primarily by decreasing $[Ca^{2+}]_i$ through Ca^{2+}-channel blockade (24), so that it has been considered as nature's physiologic Ca^{2+}-channel blocker (9). The finding that $MgSO_4$-induced vasodilation was reversed by increasing $[Ca^{2+}]_o$ both in pulmonary and mesenteric arteries suggests that $MgSO_4$ may have exerted Ca^{2+}-channel blocking activity in our experiments. However, Mg^{2+} is a weak Ca^{2+} antagonist, several orders of magnitude less potent than typical Ca^{2+} channel blocking agents (8, 25) and less potent than other multivalent cations (La^{3+}, Cd^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}) to inhibit Ca^{2+} influx stimulated by agonists (10). Moreover, the efficacy of Ca^{2+} channel blockers in experimental models and in patients with pulmonary hypertension has been equivocal because they also produced a marked systemic hypotension (6, 26). In fact, in our *in vitro* model, nifedipine was also less potent to induce vasodilation in pulmonary than in mesenteric arteries (E. Villamor and F. Pérez-Vizcaíno, unpublished results).

The vascular effects of $MgSO_4$ have also been related to the production and release of several arachidonic acid metabolites. Eicosanoids are present in high concentrations in infants with PPHN and decreased after resolution of their disease (27). Mg^{2+} increased the release of prostacyclin by endothelial cells, and cyclooxygenase inhibitors abolished the vascular effects of $MgSO_4$ infusion (15). Furthermore, it has been proposed that high $[Mg^{2+}]$ in body fluids could bind to arachidonic acid, altering the synthesis or release of vasoconstrictor eicosanoids, mainly TXA_2 (14). In the present experiments, the incubation of pulmonary and mesenteric arteries with a $[MgSO_4]$ four times greater than normal, close to the concentration which produced pulmonary vasodilation in experimental models (16, 17), did not modify the vascular response to U46619. Therefore, changes of vasodilator or vasoconstrictor eicosanoids production does not appear to be present in our experiments. However, we must consider that nonvascular sources of eicosanoids were absent in our experiments, and that eicosanoids in PPHN may not come from the endothelium (28).

Mg^{2+} has been implicated in the regulation of the L-arginine-NO pathway. Inhaled NO has been shown to be a specific pulmonary vasodilator in animal models of pulmonary hypertension (3, 28) and in infants with PPHN (4, 5). Mg^{2+} withdrawal-induced vasodilation in canine coronary arteries was inhibited by both hemoglobin and dichlorobenzamil but not by nifedipine, suggesting that the major site of Mg^{2+} inhibition of the NO probably involves endothelial Ca^{2+} influx via the Na^+/Ca^{2+} exchange system (12, 13). Furthermore, rapid removal of extracellular Mg^{2+} also produced a transient

endothelium- and Ca^{2+}-dependent vasodilation and cyclic GMP accumulation in ovine pulmonary arteries (11). We have observed a transient endothelium-dependent vasodilation upon rapid $MgSO_4$ removal in pulmonary and mesenteric arteries that was inhibited by L-NAME, indicating that Mg^{2+} removal produced a transient NO release from the endothelium. Because Ca^{2+} is obligatory for both VSM contraction and endothelial NO formation, Mg^{2+} could oppose the actions of Ca^{2+} at both sites (11). In spite of the endothelial NO synthase activation produced by acute removal of extracellular Mg^{2+}, NO release and activity in VSM seem to be independent of the presence of $MgSO_4$. In fact, we found similar pulmonary vasodilator effects of ACh and SNP and similar vasoconstrictor effects of U46619 in the presence of three different $MgSO_4$ concentrations. Thus, the antagonism of NO release by physiologic concentrations of $MgSO_4$ seems to act only acutely. In contrast, Mg^{2+} appears to be required for endothelium-dependent relaxation in canine coronary arteries (13, 30), but not in ovine pulmonary (11) or in cat cerebral or mesenteric arteries (31). The reasons for these differences between species and different blood vessels are presently unknown.

In conclusion, the present results demonstrated that in an *in vitro* porcine model $MgSO_4$ exhibits a poor pulmonary selectivity. Furthermore, acute $MgSO_4$ removal enhanced the release and/or action of the endogenous vasodilator NO. Thus, we speculate that the beneficial clinical effects of $MgSO_4$ in PPHN may not be related to a direct vasodilator effect on pulmonary vascular smooth muscle.

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Chapter XIII. **Relaxant effects of carbon monoxide compared with nitric oxide in pulmonary and systemic vessels of newborn piglets** (Pediatr Res. 2000; 48:546-553).

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Relaxant Effects of Carbon Monoxide Compared with Nitric Oxide in Pulmonary and Systemic Vessels of Newborn Piglets

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ABSTRACT

Nitric oxide (NO) has been implicated in a number of diverse physiologic processes, including regulation of vascular tone. Carbon monoxide (CO) is another endogenously generated diatomic gas that may play an important physiologic role in vascular smooth muscle homeostasis. The purpose of this study was to compare the responses to exogenous NO and CO in isolated vessels (pulmonary arteries, pulmonary veins, and mesenteric arteries) from 12- to 24-h-old and 2-wk-old piglets. Vessels precontracted with the thromboxane A_2 mimetic U46619 (10^{-7} M) relaxed in response to CO (2×10^{-6} to 2×10^{-4} M) and NO (2×10^{-9} to 2×10^{-7} M); these effects were not affected by endothelium removal but were completely abolished by the soluble guanylate cyclase inhibitor ODQ (10^{-5} M). In pulmonary arteries, the maximal relaxation to NO increased with postnatal age from 33 ± 4% of the precontraction value to 56 ± 5%, in 12- to 24-h-old and 2-week-old piglets, respectively ($p < 0.01$), but the response to CO decreased from 25 ± 3% to 12 ± 1%, respectively ($p < 0.01$). The maximal response to CO was greater in pulmonary veins than in pulmonary or mesenteric arteries for both age groups ($p < 0.01$). Vasorelaxation induced by endogenous NO (stimulated by acetylcholine) was also greater in pulmonary veins when compared with pulmonary arteries and increased with postnatal age in both vessels. In contrast, no age-related differences were observed in the vasorelaxation induced by the cGMP analog 8-bromo cGMP in pulmonary arteries. When the response to NO was analyzed under three different extracellular O_2 concentrations (Po_2 4.51 ± 0.03, 19.32 ± 0.17, and 86 ± 0.62, kPa), no significant differences were found. However, in the presence of superoxide dismutase (100 U/mL), the response to CO remained unchanged, and the response to NO improved in pulmonary arteries from 2-week-old but not from newborn piglets. In conclusion, both NO and CO relaxed neonatal vessels through soluble guanylate cyclase activation. However, when compared with NO, CO exhibited a poor vasorelaxant activity. Pulmonary vasorelaxation induced by NO increased with postnatal age, whereas that induced by CO decreased. Changes in extracellular oxygen concentration did not alter the pulmonary vascular response to NO. However, the presence of superoxide dismutase improved the response to NO, indicating that oxidant activity limits the vasorelaxant response to NO but not to CO. **(*Pediatr Res* 48: 546–553, 2000)**

Abbreviations

NO, nitric oxide
CO, carbon monoxide
sGC, soluble guanylate cyclase
HO, heme oxygenase
SOD, superoxide dismutase
U46619, 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$ (thromboxane A_2 analog)
L-NAME, N^{ω}-nitro-L-arginine methyl ester
ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (sGC inhibitor)

NO is known to be involved in the regulation of multiple physiologic processes, including the regulation of pulmonary vascular tone (1). On the other hand, another diatomic gas, CO, traditionally considered as a toxic pollutant, poisons by binding to the iron-containing heme group found in Hb and other enzymes (2). Recently, evidence is accumulating that CO can be also a physiologic endogenous regulator (2, 3). CO appears to mimic many of the actions of NO, including smooth muscle relaxation and inhibition of platelet aggregation (4), which are mainly mediated through the activation of sGC (5).

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CO is produced endogenously by two sources, *i.e.* enzymatic peroxidation of microsomal lipids and heme destruction catalyzed by HO (2, 3). HOs are rate-limiting enzymes that catalyze the conversion of heme into CO, iron, and biliverdin (3). Two distinct forms of HO have been characterized, including an inducible HO-1 and a constitutively expressed HO-2 (3). HO-2 has been localized in several tissues, including endothelial cells and adventitial nerves of blood vessels (6). In contrast, HO-1 is scarcely expressed under basal conditions, but it is induced widespread after several types of stressful stimuli, including hypoxia, endotoxins, and ischemia-reperfusion (7, 8). Furthermore, a protective role of HO-1 in several inflammatory conditions has been suggested (3, 9).

The ability of CO to induce vasorelaxation has long been known (10). CO-induced vasodilation has been described in many vascular beds from several species (11–13). However, it is not a universal finding (12), and the sensitivity of the different vessels to CO is variable (11–13). A vasoregulatory role for endogenous CO produced by constitutive HO-2 has been postulated in the maintenance of sinusoidal tone in the perfused rat liver (14) and the vascular tone of porcine distal pulmonary arteries (6). Additionally, endogenously released CO as a consequence of HO-1 induction participated in the regulation of vascular contractility in rat aorta (15) and fetal lamb ductus arteriosus (16). An interaction between CO and NO may also significantly contribute to the fine-regulation of vascular tone (3, 17).

At birth, important structural and functional changes are produced in the pulmonary circulation to replace the placenta for gas exchange (18). This transformation is not limited to the first moments of extrauterine life, but it extends during the subsequent weeks or even months (18, 19). The mechanisms regulating birth-related changes in pulmonary circulation are incompletely understood, and numerous vasoactive factors are involved (1, 20). As mentioned above, a possible role for CO has been considered in the control of ductus arteriosus tone (16) but not in pulmonary or other vessels during the perinatal period. However, in the newborn period, the substrate for CO production, heme, is readily available, and increased HO activity, and consequently, an increased CO production, has been described in newborns compared with adults (21). In fact, the pulmonary excretion rate of CO and end-tidal breath CO have been proposed as methods to estimate bilirubin production (21). The possible physiologic role of this increased production of CO remains unknown.

Unfortunately, to the best of our knowledge, neither exogenous CO-induced vasodilation nor the role of endogenous CO in vascular tone has been studied in vessels from newborn animals. We hypothesized that if CO plays a role in the control of neonatal vascular tone, exogenous CO should present the ability to relax neonatal vessels. In the present study, we have, therefore, examined the ability and the mechanisms of CO to induce vasorelaxation in pulmonary arteries, pulmonary veins, and mesenteric arteries from 12- to 24-h-old and 2-week-old piglets. In addition, we compared CO- to NO-induced vasorelaxation and studied the response under different oxygen concentrations. The effects of superoxide anions produced within the tissue in modulating the response to CO and NO were also evaluated.

METHODS

Tissue preparation. Male neonatal piglets aged 12–24 h ($n = 9$) and 2 wk ($n = 20$), obtained from a local farm, were killed by exsanguination after being anesthetized with sodium pentobarbitone (100 mg/kg). These procedures were approved by the Complutense University Animal Care and Use Committee. The lungs and mesenteric beds were rapidly immersed in cold (4°C) Krebs solution (composition in mM: NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2.0, KH_2PO_4 1.2, and glucose 11). Pulmonary arteries and veins (third branch, internal diameter, 0.5–2 mm) and mesenteric arteries (internal diameter, 1–2 mm) were carefully dissected free of surrounding tissue and cut into rings of 2–3 mm of length under a dissection microscope (22–24). Except as otherwise stated, the endothelium of the vessels was removed by gently rubbing the intimal surface of the rings with a metal rod, and the lack of functional endothelium was further confirmed by the failure of acetylcholine to relax vessels previously contracted by noradrenaline (10^{-5} M). Two L-shaped stainless-steel wires were inserted into the arterial lumen, and the rings were introduced into Allhin organ chambers filled with Krebs solution at 37°C, gassed with 95% O_2/5% CO_2. One wire was attached to the chamber and the other to an isometric force-displacement transducer coupled to a signal amplifier (model PRE 206–4, Cibertec, Madrid) and connected to a Hewlett Packard computer *via* an analog to digital interface. Contractile tension was recorded by an REGXPC computer program (Cibertec, Madrid), as previously described (22–24). The rings were initially stretched to a resting tension of 0.3 g (pulmonary arteries of 12- to 24-h-old animals), 0.5 g (pulmonary arteries of 2-wk-old animals, pulmonary veins of both groups), 1 g (mesenteric arteries of 12- to 24-h-old animals), or 2 g (mesenteric arteries of 2-wk-old animals) and allowed to equilibrate for 60–90 min. During this period, tissues were restretched and washed every 30 min with warm Krebs solution.

Experimental protocols. After equilibration, the rings were precontracted with the thromboxane A_2 mimetic U46619 (10^{-7} M). In previous experiments, we demonstrated that this concentration produces approximately 80% of the maximal U46619-induced contraction in piglet pulmonary vessels (22, 23). When the contractile response reached a stable tension, concentration-response curves to CO and NO were conducted by addition of increasing volumes of Krebs solution saturated with CO or NO. To prepare these solutions, two vials containing 20 mL of Krebs solution were initially bubbled with N_2 for 10 min and then continuously bubbled with NO (450 ppm) or CO (purity > 99%). Continuous bubbling of the 20-mL Krebs solution vial was started at least 5 min before the addition of the first dose of CO or NO and maintained during the rest of the experiment. The concentrations of NO and CO in the saturated solution were estimated from the solubility of CO and NO in water at 25°C and 1 atm of pressure (9.687×10^{-4} M and 1.931×10^{-3} M, respectively). Actual NO concentrations of Krebs solution saturated with 450 ppm of NO were measured

using a selective NO electrode (ISO-NO), which was calibrated using the titration method (*i.e.* NO_2Na in the presence of H_2SO_4 and KI) according to the manufacturer (WPI Inc., Sarasota, FL, U.S.A.). The measured values in four independent determinations ($8.9 \pm 0.5 \times 10^{-7}$ M) were in good agreement with those calculated from the solubility of NO (8.6×10^{-7} M) considering the dilution factor (450 ppm). The use of NO at 450 ppm instead of pure NO has the advantage that there is no need to further dilute the saturated solution and that the half-life of NO in solution is higher at lower concentrations (25). We assumed that the loss of added CO or NO from the Krebs solution at the time of measuring relaxation was negligible. Because this assumption was not strictly correct, actual concentrations of CO or NO in the organ chamber might be somewhat lower than estimated (11).

To evaluate the role of endothelium in the vascular response to CO and NO, some experiments were performed in endothelium-intact pulmonary arteries. Additionally, the role of sGC stimulation in CO- and NO-induced vasorelaxation was analyzed using the specific inhibitor of this enzyme, ODQ (10^{-5} M; (26). Finally, to evaluate whether superoxide anions produced within the tissue modulate the response to CO and NO, some experiments were performed in the presence of the superoxide scavenger SOD (100 U/mL). Both ODQ and SOD were added after U46619-induced contractions reached steady-state and 15 min before the concentration-response curve to CO or NO.

In another group of experiments, the vascular effects of NO in pulmonary arteries were tested under different O_2 conditions. For that purpose, the organ chambers were bubbled with 21% O_2/5% CO_2/74% N_2, 95% N_2/5% CO_2, or 95% O_2/5% CO_2. The Po_2 values were measured by a blood gas analyzer (BGA electrolyte, Instrumentation Laboratory Inc., Lexington, MA, U.S.A.). Bubbling with the new gas mixture was started 15 min before the addition of U46619 and maintained for the rest of the experiment.

Additionally, concentration-response curves to acetylcholine (10^{-8} to 10^{-5} M) were performed in endothelium-intact vessels in the absence or in the presence of the NO synthase inhibitor L-NAME (10^{-4} M). In these experiments, the vessels were exposed to L-NAME for 20 min before the concentration-response curves were started. Finally, concentration-response curves to the cell membrane-permeable analog of cGMP, 8-bromo cGMP (10^{-5} to 5×10^{-4} M), were also performed.

Drugs. The following drugs were used: acetylcholine, L-NAME, U46619, SOD (from bovine erythrocytes), 8-bromo-cGMP (Sigma Chemical Co., Alcobendas, Spain), ODQ (Tocris Cookson Ltd, Bristol, U.K.), NO (450 ppm, Air liquid, Madrid, Spain), and CO (premier grade > 99% purity, Carburos Metálicos, Barcelona, Spain). All the solid drugs were dissolved initially in distilled deionized water (except ODQ, which was dissolved in DMSO) to prepare a 10^{-2}, 10^{-3}, or 10^{-4} M stock solution, and further dilutions were made in Krebs. Preparation of CO- and NO-saturated solutions has been explained above.

Statistical analysis. Results are expressed as means ± SEM of measurements in *n* arteries. In each protocol, the vessels were obtained from at least four different animals, and a maximum of two vessels per animal was studied. The contractile responses were expressed as absolute values (milligrams), and the relaxant responses as a percentage of the precontractile tone. Statistically significant differences were calculated by means of a one-way ANOVA followed by a Newman-Keuls test. The level of $p < 0.05$ was considered statistically significant.

RESULTS

U46619 (10^{-7} M) induced sustained contractile responses in all vessels studied (Table 1). The responses were significantly greater in pulmonary veins and mesenteric arteries than in pulmonary arteries and in vessels from 2-wk-old than from 12- to 24-h-old animals.

Both CO (2×10^{-6} to 2×10^{-4} M) and NO (2×10^{-9} to 2×10^{-7} M) caused concentration-dependent relaxation of U46619-prestimulated pulmonary arteries, pulmonary veins, and mesenteric arteries from 12- to 24-h-old and 2-wk-old piglets (Figs. 1 and 2). Addition of similar volumes of Krebs solution saturated with N_2 in place of NO or CO had no measurable effect on vessel tone. The vasorelaxant potency of NO was markedly greater than that of CO in all the vessels and in both age groups tested. In fact, the detectable threshold concentrations for the vasorelaxant effects of CO and NO were approximately 2×10^{-5} M and 5×10^{-9} M, respectively. In addition, Figures 1 and 2 show that the relaxing effects of both CO and NO were completely inhibited by ODQ (10^{-5} M), a specific inhibitor of sGC. Pulmonary veins from either 12- to 24-h-old or 2-wk-old animals were the most-sensitive vessels ($p < 0.05$) to the relaxant effect of CO (relaxation at the maximum CO concentration tested was 37.4 ± 2.4% and 33.5 ± 4.1%, respectively) compared with pulmonary (24.5 ± 3.1% and 12 ± 1.1%, respectively) or mesenteric arteries (7.9 ± 1.1% and 10.1 ± 2.4%, respectively). In the 12- to 24-h-old piglets, NO-induced relaxation was significantly ($p < 0.05$) smaller in pulmonary arteries (relaxation at the maximum NO concentration tested was 33.2 ± 3.9%) than in pulmonary veins (71.8 ± 3.6%) or mesenteric arteries (73.1 ± 3.0%), whereas in the 2-wk-old piglets, NO-induced relaxation was very similar in pulmonary arteries and veins (Fig. 2). Therefore, CO-induced vasorelaxation clearly decreased with postnatal age in pulmonary arteries (Fig. 1*A*), whereas no change was observed in pulmonary veins or mesenteric arteries (Fig. 1, *B* and *C*). In contrast, NO-induced relaxation augmented with postnatal age in pulmonary arteries (Fig. 2*A*), but was only weakly modified in pulmonary veins (Fig. 2*B*). Because both CO- and NO-induced relaxation were mediated by an activa-

Table 1. *Contractile responses induced by U46619 (10^{-7} M) in piglet vessels*

Vessel	*n*	12- to 24-h-old Tension (mg)	*n*	2-wk-old Tension (mg)
Pulmonary artery	60	471 ± 26	51	1163 ± 67†
Pulmonary vein	25	1108 ± 110*	20	2913 ± 257†
Mesenteric artery	15	1059 ± 91*	15	2583 ± 298†

* $p < 0.05$ *vs* pulmonary arteries, † $p < 0.05$ *vs* 12- to 24-h-old.

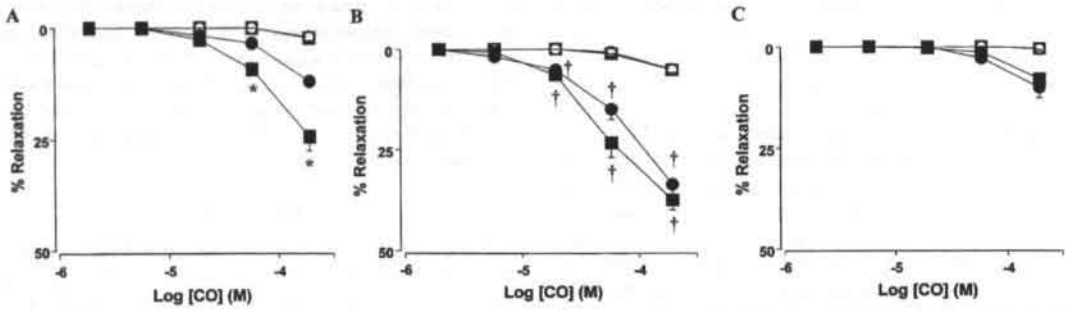

Figure 1. Concentration-dependent relaxant effects of CO in endothelium-denuded pulmonary arteries (*A*), pulmonary veins (*B*), and mesenteric arteries (*C*) of 12- to 24-h-old (■, □) and 2-wk-old piglets (●, ○). Changes in tension induced by CO are expressed as percentage of the contraction induced by U46619 (10^{-7} M). The experiments were performed in the absence (solid symbols) or presence (open symbols) of the sGC inhibitor ODQ (10^{-5} M). Each point represents the mean ± SEM of *n* arteries. Pulmonary arteries: $n = 10$ (control, both groups of age), $n = 6$ (+ODQ, both groups of age). Pulmonary veins: $n = 10$ (control, both groups of age), $n = 6$ (+ODQ, both groups of age). Mesenteric arteries: $n = 8$ (control, both groups of age), $n = 6$ (+ODQ, both groups of age). *$p < 0.05$ 12- to 24-h-old *vs* 2-wk-old. †$p < 0.05$ pulmonary artery *vs* pulmonary vein.

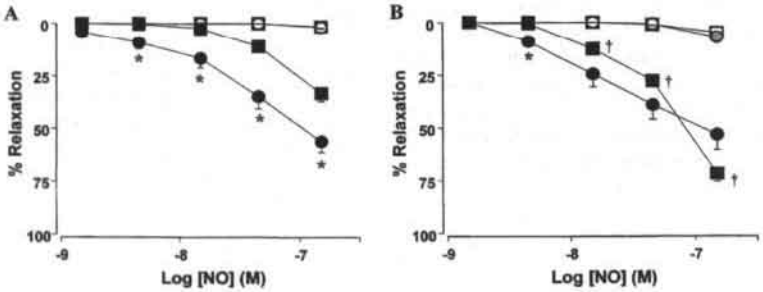

Figure 2. Concentration-dependent relaxant effects of NO in endothelium-denuded pulmonary arteries (*A*) and pulmonary veins (*B*) of 12- to 24-h-old (■, □) and 2-wk-old piglets (●, ○). Changes in tension induced by CO are expressed as a percentage of the contraction induced by U46619 (10^{-7} M). The experiments were performed in the absence (solid symbols) or the presence (open symbols) of the sGC inhibitor ODQ (10^{-5} M). Each point represents the mean ± SEM of *n* arteries. Pulmonary arteries: $n = 15$ (control, 12- to 24-h-old), $n = 13$ (control, 2-wk-old), $n = 6$ (+ODQ, both groups of age). Pulmonary veins: $n = 10$ (control, both groups of age), $n = 6$ (+ODQ, both groups of age). *$p < 0.05$ 12- to 24-h-old *vs* 2-wk-old. †$p < 0.05$ pulmonary artery *vs* pulmonary vein.

tion of sGC (as indicated by the inhibitory effects of ODQ), we analyzed the relaxant effect of the cGMP analog 8-bromo-cGMP. However, no significant differences were observed in the relaxant effects of 8-bromo-cGMP in endothelium-denuded pulmonary arteries from 12- to 24-h-old and 2-wk-old piglets (Fig. 3).

The role of endothelium in the vasodilator effects of CO and NO in pulmonary arteries from 2-wk-old piglets is shown in Figure 4. In endothelium-intact pulmonary artery rings, both CO and NO induced a relaxation that was similar to that induced in endothelium-free rings.

The endothelium-dependent relaxation induced by acetylcholine (10^{-8} to 10^{-5} M) increased with postnatal age ($p < 0.05$) and was significantly greater in endothelium-intact pulmonary veins than in pulmonary arteries for both age groups (Fig. 5). The addition of the NO synthase inhibitor L-NAME (10^{-4} M) increased U46619-induced contractions by 18 ± 2% (pulmonary arteries of 12- to 24-h-old piglets), 32 ± 4% (pulmonary arteries of 2-wk-old piglets, $p < 0.05$ *versus* 12- to 24-h-old piglets), 3.4 ± 0.7% (pulmonary veins of 12- to 24-h-old piglets) and 7 ± 1% (pulmonary veins of 2-wk-old piglets, $p < 0.05$ *versus* 12- to 24-h-old piglets). The increase of the U46619-induced contraction produced by L-NAME

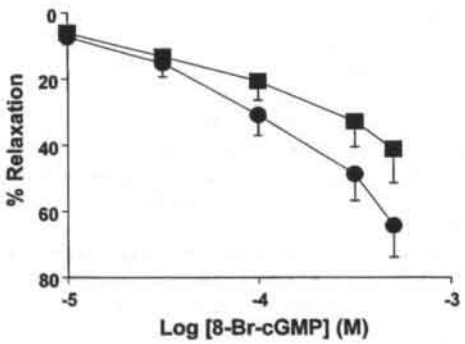

Figure 3. Concentration-dependent relaxant effects of the cGMP analog 8-bromo cGMP (8-Br-cGMP) in endothelium-denuded pulmonary arteries of 12- to 24-h-old (■, $n = 6$) and 2-wk-old piglets (●, $n = 6$). Changes in tension induced by 8-bromo cGMP are expressed as a percentage of the contraction induced by U46619 (10^{-7} M). Each point represents the mean ± SEM of *n* arteries.

reached a stable value after 20 min of exposure. Acetylcholine-induced relaxation was strongly inhibited by L-NAME in both pulmonary arteries and veins (Fig. 5), which indicates that the

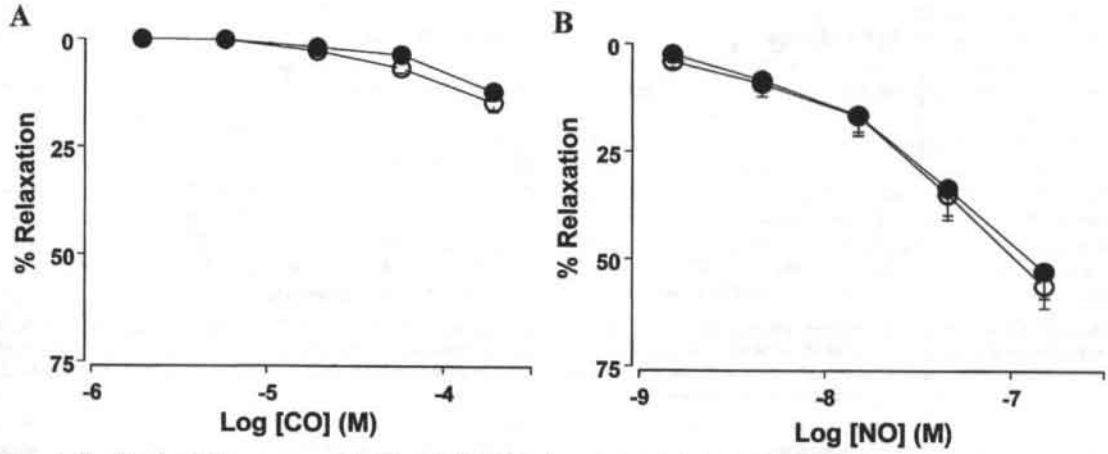

Figure 4. The effect of endothelium presence on (*A*) CO- and (*B*) NO-induced vasorelaxation in 2-wk-old piglet pulmonary arteries. Arteries with endothelium (○, $n = 8$ for both CO and NO experiments) and without endothelium (●, $n = 13$ for both CO and NO experiments) were precontracted with U46619 (10^{-7} M). Changes in tension induced by CO or NO are expressed as percentage of the contraction induced by U46619 (10^{-7} M). Each point represents the mean ± SEM of n vessels.

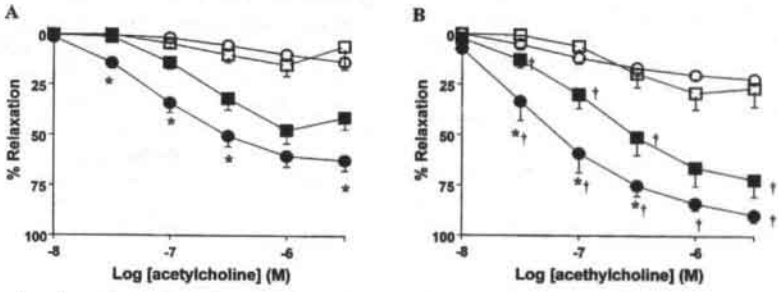

Figure 5. Concentration-dependent relaxant effects of acetylcholine in endothelium-intact pulmonary arteries (*A*) and pulmonary veins (*B*) of 12- to 24-h-old (■, □) and 2-wk-old piglets (●, ○). Changes in tension induced by acetylcholine are expressed as percentage of the contraction induced by U46619 (10^{-7} M). The experiments were performed in the absence (solid symbols) or the presence (open symbols) of the NO synthase inhibitor L-NAME (10^{-4} M). Each point represents the mean ± SEM of n arteries. Pulmonary arteries: $n = 11$ (control, 12- to 24-h-old), $n = 10$ (control, 2-wk-old), $n = 6$ (+L-NAME, both groups of age). Pulmonary veins: $n = 10$ (control, 12- to 24-h-old), $n = 11$ (control, 2-wk-old), $n = 6$ (+L-NAME, both groups of age). *$p < 0.05$ newborn *vs* 2-wk. †$p < 0.05$ pulmonary artery *vs* pulmonary vein.

endothelium-dependent relaxation is mediated mainly by the release of NO from the endothelial cells.

In endothelium-intact pulmonary arteries from 2-wk-old piglets, changing the bubbling gas mixture from 95% O_2 (Po_2, 86 ± 0.62 kPa) to 0% O_2 (Po_2, 4.51 ± 0.03 kPa) or 21% O_2 (Po_2, 19.32 ± 0.17 kPa) had no significant effect on basal tone. Furthermore, these different Po_2 values in the organ chamber did not affect NO-induced vasorelaxation (Fig. 6).

Addition of the superoxide scavenger SOD (100 U/mL) had no significant effect on U46619-induced contractions in endothelium-denuded pulmonary arteries. Furthermore, the vasorelaxant response to CO in SOD-treated arteries from 2-wk-old piglets was similar to that in untreated controls (not shown). However, the response to NO was significantly increased in pulmonary arteries from 2-wk-old (Fig. 7*B*) but not 12- to 24-h-old piglets (Fig. 7*A*).

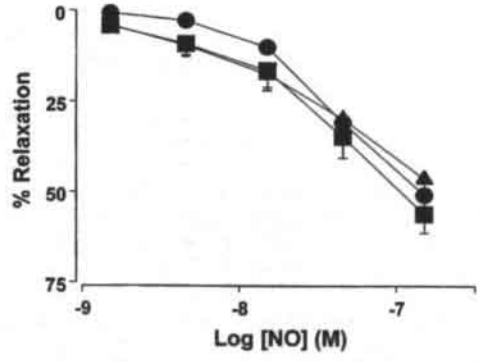

Figure 6. Effects of oxygen concentration on NO-induced vasorelaxation in 2-wk-old piglet pulmonary arteries. Organ chambers were bubbled with 0% (●, $n = 7$), 21% (▲, $n = 9$) or 95% O_2 (■, $n = 13$). Changes in tension induced by NO are expressed as percentage of the contraction induced by U46619 (10^{-7} M). Each point represents the mean ± SEM of n arteries.

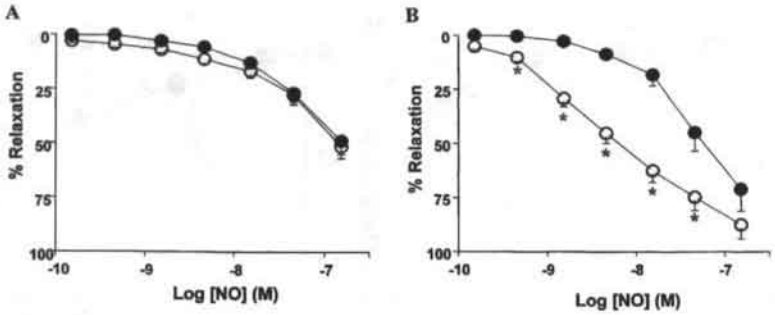

Figure 7. Effects of SOD on NO-induced vasorelaxation in 12- to 24-h-old (*A*) and 2-wk-old (*B*) piglet pulmonary arteries. The experiments were performed in the absence (●) or the presence (○) of SOD (100 U/mL). Changes in tension induced by NO are expressed as percentage of the contraction induced by U46619 (10^{-7} M) Each point represents the mean ± SEM of *n* arteries. $N = 15$ (control, 12- to 24-h-old), $n = 13$ (control, 2-wk-old), $n = 6$ (+SOD, 12- to 24-h-old), $n = 11$ (+SOD, 2-wk-old). $*p < 0.05$ SOD-treated arteries *vs* controls.

DISCUSSION

The present study demonstrated that CO relaxed vessels of 12- to 24-h-old and 2-wk-old piglets. Moreover, CO-induced vasorelaxation was more marked in pulmonary arteries in the first day of extrauterine life than that of 2-wk-old piglets and in pulmonary veins than in pulmonary or mesenteric arteries. Moreover, the vasorelaxant effect of CO was endothelium-independent but abolished by specific inhibition of sGC. However, when compared with NO, CO was a weak vasorelaxant, the relative potency of CO to NO being approximately 1:1000. In contrast to CO, NO- and acetylcholine-induced vasorelaxation increased with postnatal age in piglet pulmonary arteries. Changes in extracellular oxygen concentration did not affect NO-induced vasorelaxation. Finally, in 2-wk-old pulmonary arteries, SOD improved the response to NO but not to CO, whereas in 12- to 24-h-old pulmonary arteries, SOD was without effect on NO- induced vasorelaxation.

Several mechanisms have been proposed to explain the vasorelaxation induced by CO and NO (17) including not only activation of sGC (11) but also stimulation of K^+ channels (17, 27) or inhibition of the cytochrome P450 monooxygenase (28, 29). Hussain *et al.* (30) have shown that ODQ completely abolished relaxation of rabbit aortic rings induced by CO, whereas only a partial attenuation of NO-induced relaxation was achieved. In contrast, we found that ODQ completely abolished CO- and NO-induced relaxation, indicating that sGC was responsible for the effects of both vasorelaxants in piglet neonatal vessels. Similar findings have been reported for NO-induced vasorelaxation in pulmonary arteries of newborn lambs (31). Moreover, we have previously demonstrated that the NO donor sodium nitroprusside increased cGMP levels and relaxed pulmonary arteries from 2-wk-old piglets but neither K^+ channels nor the membrane Na^+/K^+ ATPase were involved in these effects (24). However, in the isolated lamb ductus arteriosus, CO-induced vasorelaxation was not accompanied by a significant accumulation of cGMP (29).

CO produced a weak vasorelaxant effect, particularly when compared with NO. Similar results have been previously reported in several vascular beds (11). Inhaled CO in concentrations up to 1000 ppm in adult rats and fetal lambs had no effect (32, 33), whereas markedly lower concentrations of inhaled NO (< 5 ppm) are required to produce significant pulmonary vasorelaxation (33, 34). Differences in the activation of sGC have been proposed to explain the distinct vasorelaxant potency of CO and NO (35), *i.e.* enzymatic activity of sGC is increased approximately 100- to 200-fold by NO, but only by 4- to 5-fold by CO (35). The activation of sGC by NO appears to be a complex process. First, NO binds to the heme group of the enzyme forming a hexacoordinate complex, which then converts to a pentacoordinate nitrosyl-heme (35–37). CO also forms a complex with the heme moiety of sGC, but unlike NO, only the six-coordinate complex is formed, which results in a weak activation of the enzyme (35–37). On the other hand, nanomolar concentrations of NO competitively prevent the binding of CO to the heme group of the sGC, thus reducing its vascular effect (37). The vasoconstrictor effect of L-NAME in our preparations indicates that NO is released from the endothelium under basal conditions. However, the presence of endothelium did not modify the relaxant effects of CO, indicating that basal release of NO from the endothelium does not modulate the relaxant effect of CO in piglet pulmonary arteries.

Although blood vessels produce CO (38), whether this CO reaches a sufficient concentration to relax vascular smooth muscle remains unclear. The nonselective HO-2 inhibitor tin protoporphyrin IX has been shown to inhibit the endothelium-dependent relaxation induced by acetylcholine after inhibition of NO synthesis, suggesting an involvement of endothelium-derived CO (6). We found that the endothelium-dependent relaxation produced by acetylcholine was strongly reduced by the NO synthase inhibitor L-NAME, indicating that it is mainly mediated by the release of NO. This result, together with the low vasodilator potency of CO, suggests that a possible role for CO as an endothelium-dependent vasodilator in neonatal pulmonary vessels would be small if any. However, this is an *in vitro* study, and, thus, extrapolation of the present results to *in vivo* vascular responses to CO and NO should be done with caution. Moreover, it is unclear whether endogenously generated CO has a vascular effect similar to that of exogenously applied CO. In fact, the physiologic concentrations of CO and NO in the immediate vicinity of vascular smooth muscle cells

in vivo are unknown (17). Nevertheless, studies in brain tissues (39) indicate that the concentrations of CO produced *in vivo* (1–200 μM) are lower than those producing vascular relaxation in the present experiments. In contrast, these concentrations of CO produced significant relaxation in other vascular rings (11, 17).

Superoxide anions produced in several metabolic reactions inactivate NO, forming peroxynitrite (25). In fact, the superoxide scavenger SOD significantly increased the *in vitro* relaxant activity of NO (40). Moreover, the combination of high doses of inhaled NO (100 ppm) and 90% O_2 caused oxidative damage in mechanically ventilated newborn piglets, which was mitigated by the use of recombinant human SOD (41). Therefore, the use of SOD might be suggested as a way of reducing the toxicity and augmenting the response to inhaled NO, which may lead to novel clinical strategies to improve the treatment of neonatal pulmonary hypertension. Even when CO shares with NO some properties (*i.e.* activation of sGC and reaction with Hb), CO is not a free radical and is not expected to react with superoxide. Accordingly, the antioxidant enzyme SOD had no effect on CO-induced vasorelaxation but improved NO-induced vasorelaxation (but only in the 2-wk-old animals).

Fetal pulmonary arteries are exposed to a relatively hypoxic environment, and then at birth they are exposed to 21% O_2. It is well known that acetylcholine-induced endogenous production of NO is markedly influenced by the oxygen tension in the organ chamber (42, 43). Theoretically, extreme hyperoxia can destroy NO by an increased formation of superoxide anions. Additionally, an increased oxidative stress can produce oxidation of the heme group of sGC, resulting in loss of enzyme activity (25, 37). However, no differences in exogenous NO-induced vasorelaxation were observed when the organ chambers were bubbled with 0%, 21%, or 95% O_2. This suggests that high O_2 concentrations did not produce the effects mentioned above (*i.e.* increase in superoxide anions or oxidation of the heme group of sGC) to an extent sufficient to reduce NO-induced vasorelaxation in our experiments.

Pulmonary veins are the major site of action of a number of vasoactive factors in different animal species and at different ages (44, 45). In newborn piglets, we observed that the response not only to exogenous or endogenous NO but also to exogenous CO was greater in pulmonary veins than in pulmonary arteries. This difference was maintained in the 2-wk-old piglets for the response to CO but not for NO. It has been demonstrated that basal concentrations, as well as the increase of cGMP in response to NO, were greater in pulmonary veins than in pulmonary arteries of newborn lambs (45). This could be accounted for, at least partly, by a higher activity of cGMP-specific phosphodiesterases, which produced faster hydrolysis and inactivation of cGMP in pulmonary arteries when compared with pulmonary veins (46).

In the present work, CO-induced relaxation decreased in pulmonary arteries with postnatal age, whereas the cell membrane-permeable analog of cGMP 8-bromo-cGMP produced a similar degree of relaxation in pulmonary arteries from both groups of age. Because 8-bromo-cGMP is very resistant to being hydrolyzed by phosphodiesterases, the age-dependent changes in the effects of CO might be also attributed to an increase in phosphodiesterase activity during the first days of extrauterine life as described in ovine and mouse lung (47). In contrast, the vasorelaxant response to endogenous NO increased with age in piglet (48) and present results), sheep (49), and rabbit pulmonary arteries (43). We also found an age-dependent increase in the vasodilator response to exogenous NO in piglet pulmonary arteries, suggesting that the age-dependent increase to endogenous NO is not only related to increased synthesis but also to an increased action or decreased metabolism of NO. Morecroft and MacLean (43) found that SOD potentiated acetylcholine-induced relaxation in pulmonary arteries from newborn but not from adult rabbits and suggested that the age-dependent increase in the response to endogenous NO was because of an increased accumulation of superoxide in the newborn animals. In contrast, and unexpectedly, SOD did not affect NO-induced relaxation in pulmonary arteries from 12- to 24-h-old piglets but potentiated the relaxant response to NO in mesenteric arteries from these animals (not shown) and in pulmonary arteries from 2-wk-old piglets. Even when the reasons for the lack of effect of SOD in 12- to 24-h-old pulmonary arteries are unclear, several theoretical explanations can be raised: *1*) reduced ability of exogenous SOD to penetrate cell membranes, *2*) reduced endogenous superoxide production or increased endogenous antioxidant activity, and *3*) differential effects of the peroxynitrites. Further studies involving the use of low-molecular-weight membrane-permeant compounds that exhibit SOD-like activity (50) and SOD inhibitors are necessary to elucidate this point. Additionally, the use of SOD from a nonporcine source could have limited its effect in our experiments. However, bovine and porcine SOD have a high degree of homology in their sequences and similar kinetic variables (51) as to reasonably assume identical *in vitro* activity.

In conclusion, CO produced vasorelaxation in neonatal vessels by activation of sGC, but its vasorelaxant potency is markedly reduced compared with NO. Both CO- and NO-induced relaxation was greater in pulmonary veins than in pulmonary arteries. However, whereas NO-induced relaxation in pulmonary arteries was augmented with postnatal age, that induced by CO decreased. Changes in extracellular O_2 concentration did not alter the pulmonary vascular response to NO. In contrast, the presence of SOD improved the response to NO in 2-wk-old piglets, indicating that oxidant activity limits the vasorelaxant response to NO but not to CO. The functional significance of endogenous CO has not been established to date, but it might play, together with NO, a modulatory role in the regulation of vascular contractile responses. However, if the response to exogenous CO reflects the capacity of this gas to relax *in vivo* neonatal pulmonary vessels, its direct participation in the control of pulmonary vascular tone seems unlikely.

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PART III

Chapter XIV. General discussion, summary, and future perspectives.

During the perinatal period, the pulmonary circulation undergoes important structural and functional changes to allow the sudden transition from gas exchange by the placenta to gas exchange by the lungs (1-5). These changes lead to a fall in pulmonary vascular resistance (PVR), with pulmonary blood flow increasing roughly ten-fold and pulmonary arterial pressure falling to one-half systemic values within hours. Failure of the pulmonary circulation to undergo this transition results in persistent pulmonary hypertension of the newborn (PPHN). PPHN is a clinical syndrome of various neonatal cardiopulmonary disorders, which are characterized by sustained elevation of pulmonary vascular resistance after birth, leading to right-to-left shunting of blood across the ductus arteriosus or foramen ovale and severe hypoxemia (6,7).

This thesis is a compilation of work that addresses several unknown aspects of the pathophysiology and treatment of PPHN. Because PPHN is not an homogeneous entity, but a clinical syndrome occurring in a heterogeneous group of diseases with a wide diversity of etiologies (7), its study is severely limited by the experimental approach that is selected (8). In the present thesis, we used an experimental model of PPHN induced by chronic compression of the ductus arteriosus (chapter V), and studies of vascular contractility in isolated intrapulmonary arteries and veins, compared to systemic vessels (chapters VI to XIII).

Experimental models used to study PPHN can be characterized as based on acute perinatal insults, or on chronic intrauterine insults (8). The acute models of PPHN have the advantage of mimicking relevant clinical situations, such as hypoxic stress during labor and delivery (9,10), sepsis (11, 12), or aspiration of meconium (13, 14). On the other hand, only the chronic models are characterized by suprasystemic pressures in the pulmonary vasculature and the associated right-to-left shunting of blood (8, 15). In addition, pulmonary hypertension in the acute models is based on the alteration of the equilibrium between vasoactive factors, but only the chronic models allow the study of a structurally remodeled

pulmonary vascular bed (8, 16). For that reason, chronic models of PPHN have been particularly suited to study alterations in gene expression (8). Chronic intrauterine hypoxia (17, 18), ductal constriction or ligation (19, 20), and chronic NO synthase inhibition (21) are three examples of adverse intrauterine environment that are widely used to experimentally reproduce PPHN. However, only the model of ductal compression or ligation consistently mimics not only the hemodynamic, but also the morphological pulmonary vascular findings of infants with PPHN (8, 16).

Contractility studies in isolated vascular rings have become an invaluable tool for the study of pharmacology, physiology and pathophysiology and very relevant pieces of knowledge of these disciplines have been obtained using this model (22, 23). However, it is, undoubtedly, an approach that is subjected to important limitations. Blood vessels are very sensitive to local changes in intraluminal pressure and flow (24,25), and both stimuli, which play an important role to determine vascular tone, are absent in isolated vascular rings. In addition, important segmental differences in vascular contractility are present in the pulmonary circulation (26, 27). Therefore, information obtained in large or small conduit pulmonary arteries should be cautiously extrapolated to resistance vessels. Another important point in this experimental approach is the level of oxygenation that is achieved. The use of 95% oxygen to bubble organ chambers is a standard method in pharmacological studies. In fact, many previous works have studied fetal and neonatal pulmonary vessels using that oxygen concentration (26, 28-30). Recently, caution has been claimed to this fact due to the substantial lower oxygen environment in which pulmonary arteries are exposed under physiological conditions (31). An extremely high oxygen tension (86 ± 0.62 kPa) is produced when the organ chamber is bubbled with 95% oxygen. Parallelism between this oxygen tension *in vitro* and *in vivo* is, however, not completely suitable due to the absence in the organ chamber of hemoglobin (i.e. the fundamental factor for oxygen transportation *in vivo*). Therefore, *in vitro* oxygen content is only dependent on the oxygen in solution that is expressed by the partial pressure of oxygen (32). Interestingly, it has been demonstrated that high oxygen tensions markedly influenced endothelium-dependent relaxation in foetal but not in neonatal rabbit pulmonary arteries (31). In fact, in the present thesis we describe (chapter

XIII) that bubbling the organ chamber with 95%, 21% or 0% oxygen had no influence on the pulmonary vascular response to NO.

In spite of the limitations of the experimental approaches, we believe that this thesis collects some original contributions to the understanding of PPHN pathophysiology and treatment. In **chapter V** we demonstrated that chronic intrauterine pulmonary hypertension caused by ductus compression decreased eNOS mRNA, protein content, and activity in the late gestation ovine fetus. These findings support previous studies that demonstrated preferential impairment of endothelium-dependent pulmonary vasodilation in this experimental model of PPHN (33,34). Because NO modulates both vascular tone –as a vasodilator- and vascular smooth muscle growth -as an antiproliferative factor- diminished eNOS expression may contribute to both the functional and structural abnormalities that are characteristic of the pulmonary vasculature in PPHN.

Chapter VI is focused on the pulmonary vascular response to hypoxia. Oxygen tension is a determinant regulator of pulmonary vascular tone through the presence of hypoxic pulmonary vasoconstriction (HPV), a rather unique response specific for the pulmonary vascular bed by which circulating blood is diverted to better ventilated alveoli, optimizing the ventilation/perfusion matching (35). During the fetal life, HPV is responsible, at least partially, for maintaining high pulmonary vascular resistance. Reduction of this HPV, by elevation of arterial pO_2, has been suggested as a mechanism to produce the pulmonary vasodilation that occurs at birth (5, 36-38). In addition, chronic intrauterine hypoxia, acute hypoxia at birth, and sustained hypoxia in the neonatal period are mechanisms involved in the development of PPHN (8,39).

In isolated piglet (Landrace-Largewhite strain) pulmonary arteries we observed that hypoxia produced a transient contractile response due to inhibition of basal EDNO production. Other endothelial factors, such as eicosanoids, were not involved in this contractile response. However, we observed a similar response to hypoxia in pulmonary arteries, pulmonary veins, coronary arteries, and mesenteric arteries whereas HPV is unique

to the pulmonary vascular bed. Therefore, we suggest that correlation between the response to hypoxia in isolated pulmonary arteries and HPV should be avoided.

Sepsis is one of the most important factors involved in the etiology of PPHN (40). In **chapters VII, VIII,** and **IX,** we studied sepsis-induced changes in pulmonary and systemic vascular contractility. We analyzed the effects of incubation of piglet pulmonary and mesenteric arteries with heat-inactivated group B *Streptococcus* (GBS) and *Escherichia coli* lipopolysaccharide (LPS) on the vascular responses to several vasoconstrictor agonists. GBS, a gram-positive bacterium, *and E. coli,* a gram-negative bacterium, are among the most common causal agents of neonatal sepsis (41). We observed that prolonged incubation with the bacterial products reduced the contractile responses to noradrenaline in neonatal porcine pulmonary arteries. This hyporesponsiveness was potentiated by the substrate for NO synthesis L-arginine, while the NOS inhibitor L-NAME completely reversed it, indicating that overproduction of NO was responsible for this effect. Dexamethasone, which inhibits the induction of iNOS, and cycloheximide, an inhibitor of protein synthesis, when coincubated with GBS or LPS, completely reversed the reduced response to noradrenaline. Moreover, GBS and LPS induced a marked increase in iNOS activity in lung tissue, which was abolished by dexamethasone. All these results indicated that GBS and LPS-induced pulmonary vascular hyporesponsiveness to noradrenaline was due to an enhanced formation of NO via iNOS induction that required *de novo* synthesis of the enzyme in the pulmonary vasculature. We tested also the effects on systemic vascular contractility of incubation with GBS or LPS. We observed that GBS and LPS reduced the vascular responsiveness to noradrenaline in piglet mesenteric arteries and this effect was reversed by the presence of the NOS inhibitor L-NAME. These data suggest a role for iNOS induction in the systemic vascular dysfunction associated with sepsis, produced not only by gram-negative, but also by gram positive bacteria.

Pulmonary hypertension is the single most reliable circulatory disturbance noted in every laboratory model of neonatal sepsis (40). However, we described lung induction of iNOS and decreased responses to pressor agents in pulmonary arteries after incubation with two common neonatal pathogens. In an attempt to explain this apparent contradiction, we

studied the pulmonary vascular response to the thromboxane A_2 analog U46619, after incubation with LPS or GBS. Thromboxane A_2 has been shown to be the vasoconstrictor responsible for sepsis-induced pulmonary hypertension, at least during the early phases of the process (11, 40). We observed that LPS- or GBS-incubated pulmonary arteries did not show reduced responsiveness to U46619. In contrast, similar incubation, in mesenteric arteries, produced a marked reduction in U46619-induced contraction that was reversed by NOS inhibition. This absence of pulmonary hyporesponsiveness to U46619 may contribute to the persistence of pulmonary hypertension in sepsis, despite iNOS induction in the lung.

In an attempt to explain the differences in the vasoconstriction induced by thromboxane A_2 (i.e. by U46619) and noradrenaline when iNOS was induced in pulmonary arteries, **chapter X** is focussed on the study of the interactions of these agents with the NO/cyclic GMP pathway, as well as the mechanisms involved in NO/cyclic GMP-induced relaxation. We observed that various activators of the cyclic GMP pathway (i.e., ACh, SNP, ANP and 8-Br-cyclic GMP) fully relaxed noradrenaline-contracted arteries, while only a partial relaxation was observed on the contractions induced by U46616. This effect was specific for piglet pulmonary arteries, since it was not present in rat pulmonary arteries or in piglet mesenteric or coronary arteries. Moreover, U466619 was not affecting the rise in cyclic GMP induced by SNP, nor increasing the degradation of cyclic GMP by phosphodiesterases. Our results suggest that U46619 inhibits the NO/cyclic GMP pathway for smooth muscle relaxation beyond the level of cyclic GMP synthesis.

We have studied several mechanisms proposed to explain cyclic GMP-induced vascular relaxation. Our results indicate that neither activation of Ca^{2+}-activated K^+ channels, nor activation of the membrane Na^+/K^+-ATPase were responsible for this relaxation in piglet pulmonary arteries. However, inhibition of the sarcoplasmic reticulum Ca^{2+}-ATPase by thapsigargin, markedly suppressed the relaxation induced by the NO donor SNP in arteries contracted by either noradrenaline or U46619. This finding suggests that increased Ca^{2+} uptake by the sarcoplasmic reticulum is an important mechanism by which cyclic GMP decreases cytosolic Ca^{2+}, and induces its relaxant effects in piglet pulmonary arteries.

In addition, we further investigated the mechanisms involved in the inhibition of the relaxant effects of SNP by U46619. We observed that the activation of protein kinase C (PKC) by the phorbol ester PMA inhibited the relaxant responses to SNP. Conversely, inhibition of PKC by staurosporine potentiated the relaxant response to SNP in U46619 pre-contracted arteries. These results provide evidence for a novel mechanism of NO/cyclic GMP impairment mediated by PKC through TXA_2 receptor activation at the level of the cyclic GMP activity. Further experiments, involving the use of more specific inhibitors of PKC, are needed to confirm the presence of this mechanism.

Since lowering pulmonary artery pressure, while maintaining systemic vascular resistance and good cardiac output, is crucial for newborns with PPHN, the ideal drug for their treatment should be a vasodilator with selectivity for pulmonary over systemic vessels (41,42). Search for this 'selective' pulmonary vasodilator has been constant in the last two decades. In **chapter XI** we have compared the relaxant effects of six putative selective pulmonary vasodilators (acetylcholine, sodium nitroprusside, ATP, PGE_1, tolazoline, and nifedipine) in isolated piglet pulmonary and mesenteric arteries. We have found differences in their relaxant effects depending on the artery, the agonist used to contract the vessel (i.e. noradrenaline or U46619) or both. ATP was the only drug, which, at any concentration and regardless of the contractile agonist used, produced relaxant effects more marked in pulmonary than in mesenteric arteries. Moreover, results with tolazoline and nifedipine demonstrated that systemic arteries dilate at least as much as pulmonary arteries, supporting the poor selectivity observed in clinical studies.

In **chapter XII** we analyzed the *in vitro* relaxant effects of magnesium sulfate ($MgSO_4$), another drug proposed as an alternative and safe treatment for PPHN (44,45). We observed that in intrapulmonary arteries precontracted with noradrenaline, KCl or U46619, $MgSO_4$ produced a weak vasorelaxant effect as compared with the marked vasorelaxation induced in systemic (mesenteric) arteries of a similar diameter. Furthermore, at physiological concentrations, $MgSO_4$ inhibited the vasorelaxant effect of NO, as proved by the fact that in mesenteric and pulmonary arteries precontracted by U46619 the rapid removal of $MgSO_4$

from the bath medium produced a transient endothelium-dependent relaxation, which was greatly reduced after removal of the endothelium or by the NOS inhibitor L-NAME. In addition, $MgSO_4$ did not seem to play a marked role in the pulmonary vascular smooth muscle reactivity, since the vasoconstrictor effects of U46619 or the relaxant effects of the endothelium-dependent vasodilator ACh or the NO donor SNP were not affected by changing the extracellular Mg^{2+} concentration. In view of our results, we conclude that $MgSO_4$ exhibits a poor pulmonary selectivity in vitro, and we speculate that the beneficial clinical effects of $MgSO_4$ in PPHN may not be related to a direct vasodilator effect on pulmonary vascular smooth muscle.

The remarkable basic scientific discovery that the simple gas molecule NO was endogenously released by endothelial cells, producing paracrine vasodilatory effects in the adjacent vascular smooth muscle, enormously boosted the search for a specific treatment for PPHN (46). Administered by inhalation, characterized by a short half-life and the absence of measurable systemic effects, the use of inhaled NO as an adjunct to conventional PPHN therapy is presently, widespread (47). In **chapter XIII** we compared the vasorelaxant effects of NO with another diatomic gas, carbon monoxide (CO), which appears to mimic many of the actions of NO including smooth muscle relaxation (48). The possible role of endogenously produced CO in the control of pulmonary vascular tone during the perinatal period remains unknown. We analyzed the mechanisms involved in NO- and CO-induced relaxation, as well as the age-dependent changes, and the influence of oxygen on NO and CO vascular actions. In addition, we evaluated the effects of superoxide anions produced within the tissue in modulating the response to NO and CO. We observed that both NO and CO relaxed neonatal vessels through soluble guanylate cyclase activation. However, when compared with NO, CO exhibited a 1000-fold lower vasorelaxant activity. The maximal response to NO and CO was greater in pulmonary veins than in pulmonary arteries. In addition, pulmonary vasorelaxation induced by NO increased with postnatal age, whereas that induced by CO decreased, suggesting a different mechanism of soluble guanylate cyclase activation. Changes in extracellular oxygen concentration did not alter the pulmonary vascular response to NO. However, the presence of superoxide dismutase improved the

response to NO indicating that oxidant activity limits the vasodilator response to NO but not to CO. We concluded that if the response to exogenous CO reflects the capacity of this gas to relax *in vivo* neonatal pulmonary vessels, it seems unlikely its direct participation in the control of pulmonary vascular tone.

FUTURE PERSPECTIVES

Our understanding of the molecular and cellular changes in the pulmonary circulation during fetal life, the normal adaptation to extrauterine life, and early postnatal development is still deficient to identify the early crucial factors, which instigate the cascade of abnormal structural and functional changes that manifest themselves clinically as PPHN (39). Blood vessels in the lung undergo profound structural remodeling as pulmonary hypertension develops. Changes include cellular hypertrophy, hyperplasia, and increased deposition of structural matrix proteins such as collagen and elastin in the vessel wall (49). The identification of a number of defined growth factors, observations in genetically manipulated mice, and the recognition of the importance of cell-cell interactions have greatly expanded our understanding of the regulation of vascularization under physiological and pathological conditions. The paracrine actions of a variety of polypeptide growth factors, including platelet-derived growth factor, vascular endothelial growth factor, transforming growth factor-beta, and the angiopoietins, appear to be orchestrated in a complex sequence of steps that lead to the development of the vascular system (50-53). Some of these factors stimulate increases in a variety of immediate-early genes (54). Thus, communication between the forming vasculature and the tissue parenchyma, as well as interactions among cells of the vascular wall, influence vascular development and growth (49). Elucidation of the signaling pathways which are activated under adverse perinatal environment may help to develop new strategies to treat PPHN, to avoid complications of present therapies, and ultimately to develop new therapies aimed at preventing the early cellular and molecular changes prompted by pulmonary hypertensive stimuli.

Understanding the precise mechanisms regulating the NO/cyclic GMP signaling under physiological and pathological conditions, and the interactions between cyclic GMP-induced relaxation and the action of vasoconstrictors, will certainly help in the therapeutic management of patients with PPHN. Vasoconstriction results from increased Ca^{2+}-calmodulin dependent myosin light chain kinase activation, but also from Ca^{2+}-independent mechanisms (often referred as Ca^{2+}-sensitization). The latter may involve inhibition of myosin light chain phosphatase, actin-linked regulatory mechanisms, or availability of calmodulin (55). Conversely, vasodilation may result from a reduction in intracellular Ca^{2+} concentration or from Ca^{2+}-independent effects. Therefore, it is expected that the sensitivity to a given vasodilator depends on the mechanism by which agonists induce their contractile response, e.g. a Ca^{2+}-dependent contractile response should be preferentially relaxed by vasodilators lowering Ca^{2+}. Ca^{2+}-independent mechanisms of vasodilation have been reported following the activation of the cyclic nucleotide dependent protein kinases (55, 56). In addition, vasoconstrictors involved in the pathophysiology of PPHN, such as thromboxane A_2 and endothelin-1, induce part of their activity, in pulmonary arteries, through an increase in Ca^{2+} sensitivity (57, 58). Further studies are necessary to understand the interactions, at the level of Ca^{2+}-dependent and –independent transduction mechanisms, between the vasoconstrictors and vasorelaxants pathways involved in the pathophysiology of PPHN.

In addition, vascular maturation involves important shifts in the mechanisms mediating vascular smooth muscle pharmacomechanical coupling. Thus, in ovine cerebral arteries, normal development involves a reduction in the Ca^{2+} sensitizing effects of agonists, with parallel increases in the agonist-induced intracellular Ca^{2+} release (59, 60). Until the present moment, studies of pulmonary arteries focused on developmental changes in Ca^{2+} sensitivity have not been performed. Such studies might explain why vascular reactivity varies with age. Moreover, from a pathophysiologic perspective a hypothetical failure to shift from the increased Ca^{2+} sensitivity typical of immature arteries, might lead to vascular hyperreactivity in the neonatal or adult life.

Randomized controlled trials have demonstrated that inhaled NO is an effective treatment for PPHN, by improving oxygenation and reducing the need for extracorporeal membrane oxygenation therapy (47). However, approximately 40-50% of the infants assigned to NO in these trials failed to respond. Several mechanisms may explain variability in responsiveness to inhaled NO therapy. However, responders did not appear to differ clinically from non-responders. Further studies are necessary to elucidate the potential mechanisms underlying poor responses to inhaled NO and to identify the groups of patients that may receive the maximal benefits from this therapy. Additionally, pharmacological augmentation of inhaled NO responsiveness may also constitute an attractive field for future research. Further studies searching for more selective phosphodiesterases inhibitors, evaluating the effectiveness of augmentation of soluble guanylate cyclase cofactors, or analyzing the effects of antioxidant agents may lead to novel clinical strategies to improve the treatment of PPHN, and to reduce the potential toxicity of inhaled NO.

Recent epidemiological studies have suggested that perinatal factors may be linked with the development of adult disease (61). Thus, adverse environmental events occurring prenatally or early in life are currently receiving progressive attention as predictors of disease in later stages of life (61). During the last years, the chances of survival from PPHN have increased greatly. Recently, it has been reported that young adults that had PPHN showed exaggerated pulmonary vasoconstriction at high altitude, despite having normal pulmonary artery pressure at low altitude (62). In addition, perinatal exposure to hypoxia in the rat, which induces transient pulmonary hypertension, predisposes to augmented pulmonary vasoconstrictor responses to hypoxia (63,64), and increases severity of pulmonary hypertension after later exposure to hypoxia or monocrotaline (65, 66). These data suggest that brief hypoxic exposure during a critical period of lung growth may alter the course of normal pulmonary development, and leaves persistent changes in lung structure and/or function that cause an exaggerated response to adverse stimuli later in life. Therefore, early hypoxic exposition on patients with PPHN might influence the susceptibility of pulmonary vasculature to future adverse stimuli. Consequently, it may be worth to pay attention to those infants who suffered from hypoxia during the perinatal period, critical for lung development, because they may be at risk to develop adult pulmonary

hypertension. Further studies are needed to determine the possible alterations of pulmonary vascular function and/or structure in adult animals subjected to perinatal insults, to understand the mechanisms by which these stimuli may adversely affect pulmonary vessels, and to clarify the links between perinatal adverse environment and altered vasoreactivity.

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Samenvatting

Om de plotselinge overgang van gasuitwisseling via de placenta naar gasuitwisseling via de longen te bewerkstelligen moeten er tijdens de perinatale periode belangrijke structurele en functionele veranderingen plaatsvinden in de longcirculatie. Deze veranderingen resulteren in een ongeveer 10-voudige toename van bloeddoorstroming omdat er een belangrijke afname van de weerstand in de longvaten ontstaat. Als deze aanpassing in vaatweerstand niet optreedt spreken we van een persisterende pulmonale hypertensie van de neonaat (PPHN). PPHN is een klinisch syndroom dat optreedt bij verschillende cardio-pulmonaire afwijkingen die gekenmerkt worden door een blijvende hoge vaatweerstand in de longvaten na de geboorte. Dit resulteert in een rechts-links shunt van bloed over de Ductus Arteriosus of het Foramen Ovale en zal leiden tot ernstige hypoxie.

Omdat het klinisch beeld van PPHN een uiting is van het uitblijven van de postnatale adaptatie in de longcirculatie is het begrijpen van de basale functie en structurele ontwikkeling van de longcirculatie *in utero* van belang om meer inzicht te krijgen in het klinisch beeld van PPHN en in de behandeling ervan. Vanuit deze achtergrond worden in dit proefschrift enkele aspecten van de pathofysiologie en behandeling van PPHN beschreven.
In de hoofdstukken II, III en IV wordt een overzicht van de huidige stand van zaken gegeven en in de hoofdstukken V tot XII worden eigen onderzoeksgegevens gepresenteerd. Omdat PPHN geen duidelijk omschreven ziektebeeld is, maar een klinisch syndroom dat op kan treden bij een diverse groep van afwijkingen met diverse oorzaken is het onderzoek beperkt vanwege het gekozen experimentele model. In het voorliggend onderzoek is gekozen voor een model waarbij is uitgegaan van PPHN veroorzaakt door chronische compressie van de Ductus Arteriosus (hoofdstuk V) en door het bestuderen van de vaatreactiviteit in geïsoleerde longvaten te vergelijken met de vaatreactiviteit in geïsoleerde vaten van de lichaamscirculatie (hoofdstukken VI tot XII).

In **hoofdstuk V** wordt bij foetale lammeren aangetoond dat chronische pulmonale hypertensie veroorzaakt door ductus compressie laat in de zwangerschap kan leiden tot een

afname in productie van eNOS mRNA, eNOS hoeveelheid en eNOS activiteit. Deze bevinding is een bevestiging van eerdere studies die aantoonden dat er in dit experimentele model van PPHN sprake was van een afname van pulmonale vasodilatatie.

In **hoofdstuk VI** wordt de reactie van de pulmonale vaten op hypoxie beschreven. We hebben bij longvaten van pasgeboren biggen aan kunnen tonen dat hypoxie aanleiding geeft tot een passagère contractie, veroorzaakt door een blokkade van endotheel afhankelijke NO productie. Andere endotheel-factoren, zoals b.v. eicosanoieden, waren in dit proces niet betrokken. Echter, een zelfde reactie op hypoxie als in de longarteriën kon ook worden aangetoond in de longvenen, de coronair-arteriën en in de mesenteriaal arteriën ofschoon normaliter alleen de longarteriën met vasoconstrictie reageren op hypoxie. Daarom adviseren we om voorzichtig te zijn met het leggen van verbanden tussen het optreden van een hypoxische pulmonaire vasoconstrictie en de beschreven reactie op hypoxie bij geïsoleerde longarteriën.

Sepsis is een van de belangrijkste factoren die betrokken zijn bij het ontstaan van PPHN. Door sepsis geïnduceerde veranderingen in contractilitiet van longvaten en van vaten in de systeemcirculatie wordt beschreven in de **hoofdstukken VII, VIII,** en **IX.** We bestudeerden de vasculaire respons van long- en mesenteriaal arteriën van biggen op enkele vasoconstrictore agonisten door deze vaten te incuberen met geïnactiveerde groep B *Streptococcus agalactiae* (GBS) en *Escherichia Coli* lipopolysaccharide (LPS). GBS en E. Coli zijn de meest voorkomende verwekkers van sepsis bij pasgeborenen. In deze proefopstelling konden we aantonen dat er in alle vaten sprake was van een verminderde contractile respons op noradrenaline. Dit effect werd nog versterkt door toevoeging van L-arginine (het substraat voor NO synthese), terwijl L-NAME (een NOS-remmer) een tegengesteld effect had. Dit toont aan dat een overproductie van NO verantwoordelijk was voor de verminderde contractile respons. Bovendien toonden we aan dat GBS en LPS een duidelijke toename van iNOS activiteit in de long teweegbrachten. Deze bevindingen wijzen er op dat door GBS en LPS geïnduceerde hyporeactiviteit op noradrenaline veroorzaakt wordt door een toegenomen productie van NO via iNOS inductie.

Pulmonaire hypertensie is de meest uitgesproken circulatoire afwijking die gevonden wordt in iedere proefopzet uitgaande van een sepsis. In ons model vonden wij echter dat als longarteriën geïncubeerd werden met voor pasgeborenen frequent voorkomende pathogenen dat er dan sprake was van een inductie van iNOS en een afgenomen vaatrespons op vasoconstrictieve stoffen. Om een verklaring te vinden voor deze tegenstrijdige bevinding bestudeerden we de respons van longvaten op toevoeging van de thromboxane A_2 analoog U46619, ook weer na incubatie met GBS en LPS. Van thromboxane A_2 is aangetoond dat het verantwoordelijk is voor de vasoconstrictieve respons bij door een sepsis geïnduceerde pulmonaire hypertensie, althans zeker tijdens de eerste fase van het proces. Wij vonden dat met GBS of met LPS geïncubeerde longarteriën geen verminderde reactie vertoonden op U46619. In mesenteriale vaten daarentegen vonden we wel een duidelijk verminderde reactie op door U46619 geïinduceerde vaatconstrictie die weer kon worden opgeheven door NOS remming. Het ontbreken van deze verminderde gevoeligheid van longarteriën op U46619 bij een sepsis zou kunnen bijdragen tot het persisteren van pulmonaire hypertensie, zulks ondanks iNOS inductie in de long.

Om het verschil te kunnen verklaren in vasoconstrictie door thromboxane A_2 (d.w.z. door U46619) en door noradrenaline bestudeerden we, zoals beschreven in **hoofdstuk X**, de interacties tussen deze twee stoffen en de NO/cyclisch GMP weg. Hierbij komen ook de mechanismen ter sprake die betrokken zijn bij de door NO/cyclisch GMP geïnduceerde relaxatie. Wij vonden dat diverse stoffen van de cyclisch GMP weg in staat waren om door noradrenaline gecontraheerde arteriën volledig te relaxeren, terwijl dit veel minder het geval was als de arteriën gecontraheerd waren middels U46619. Dit effect was specifiek voor pulmonale longarteriën bij biggen en kon niet worden aangetoond in longarteriën van de rat of bij andere arteriën van biggen. Bovendien had U46619 geen effect op een door Na-nitroprusside geïnduceerde cyclisch CMP stijging, of op een door phospho-di-esterase geïnduceerde afbraak van cyclisch GMP. Onze bevindingen wijzen in de richting dat U46619 in staat is om de NO/cyclisch GMP weg tot gladde spier relaxatie meer te remmen dan dat er compensatie via cyclisch GMP aanmaak is.

Omdat het doen dalen van de pulmonale vaatweerstand en het tegelijkertijd handhaven van de systeemdruk van essentieel belang is bij de behandeling van pasgeborenen met PPHN, moet het ideale medicament een stof zijn die een selectieve vasodilatatie in de longvaten geeft. Dit zoeken naar een ideaal medicament is al sinds enkele decennia een doel op zich zelf. In **hoofdstuk XI** beschrijven we een vergelijkend onderzoek naar de relaxerende eigenschappen van een 6-tal mogelijk selectief vasodilatatoire stoffen (acetylcholine, Na-nitroprusside, ATP, PGE1, tolazoline en nifedipine) op long- en mesenteriale arteriën bij biggen. Afhankelijk van de onderzochte arterie of van de vooraf gebruikte vasoconstrictor (noradrenaline of U46619), of van beiden, vonden we duidelijke verschillen in relaxerend vermogen van deze stoffen. ATP was het enige medicament dat onafhankelijk van de gebruikte concentratie in alle gevallen een sterker relaxerend effect op longarteriën dan op mesenteriaal arteriën had. Zoals al bekend uit klinisch onderzoek waren met name tolazoline en nifedipine niet selectief in relaxerend effect op arteriën van verschillende orgaansystemen.

Van magnesiumsulfaat ($MgSO_4$) wordt gesuggereerd dat het mogelijk een goed en veilig alternatief medicament voor vaatrelaxatie zou zijn. In **hoofdstuk XII** beschrijven we *in vitro* experimenten om het relaxerend effect van $MgSO_4$ te meten. We vonden dat $MgSO_4$ een sterker relaxerend effect heeft op systeem arterien dan op pulmonaal arteriën en dat het in fysiologische concentraties zelfs het relaxerend effect van NO remt. $MgSO_4$ lijkt geen belangrijke rol te spelen in de reactive processen die betrokken zijn in de gladde spieren van de longvaten. Immers, veranderingen in extracellulaire Mg^{2+} concentraties hebben geen invloed op vasoconstrictieve effecten van U46619 of op de relaxerende effecten van acetylcholine of Na-nitroprusside. Gezien deze bevindingen menen we te mogen concluderen dat $MgSO_4$ *in vitro* geen specifiek relaxerend effect op de longarteriën heeft en suggereren we dat het mogelijk klinisch effect op een ander mechanisme berust.

De opzienbarende ontdekking dat het door endotheelcellen geproduceerde simpele molecuul NO verantwoordelijk is voor vaatverwijding heeft geleid tot veel onderzoek naar een mogelijk specifieke behandeling van PPHN. Als NO wordt toegevoegd aan de inademingslucht zijn er door de zeer korte halfwaarde-tijd geen effecten op de

systeemcirculatie en wordt deze therapie thans veelvuldig toegepast bij de behandeling van PPHN. Een ander gas, koolstof monoxide (CO) heeft een vergelijkbaar effect als NO. In **hoofdstuk XIII** beschrijven we een studie die de vasorelaxerende effecten van NO vergelijkt met die van CO. We vonden dat bij beide gassen het relaxerend effect ontstaat via activatie van guanylaat cyclase in de vaatwand. Echter, het vasorelaxerend effect van CO is slechts een fractie (minder dan 1000-voud) vergeleken met dat van NO. Bovendien nam het relaxerend effect van NO toe met de postnatale leeftijd, terwijl dat van CO afnam. Dit suggereert dat de weg waarlangs de guanylaat cyclase activatie verloopt voor beide gassen een andere is.
Veranderingen in de pO_2 hadden geen invloed op het relaxerend effect van NO. Maar dit effect werd wel versterkt onder invloed van superoxide dismutase, terwijl de effectiviteit van CO niet beïnvloed werd door activiteit van oxidanten. Hieruit mogen we concluderen dat als deze reacties op CO een afspiegeling zijn van het relaxerend vermogen *in vivo*, dat het dan onwaarschijnlijk is dat CO een directe rol speelt in de regulatie van de pulmonale vaattonus.

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