LOCAL MODULATION OF PULMONARY VASCULAR FUNCTION.

A thesis presented for the degree of

Doctor of Philosophy

by

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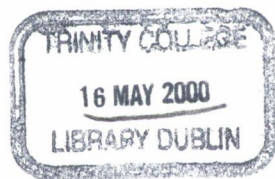
DECLARATION

The experimental and research work presented in this thesis was completed at the Johns Hopkins University, Baltimore, Maryland, USA and was performed entirely by the Author, except for experiments involving the enzyme assays (Methods 3) and immunohistochemistry (Methods 4) which were carried out in association with Randa Zachary and the Northern Blot analysis in association with Leo Otterbein (Methods 5). The work herein has not been submitted as an exercise for a degree to any other university. I agree to the lending or copying of this thesis by the Library of the University of Dublin.

Signed

Sean P. Gaine

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I would like to thank my supervisor, Nicholas A. Flavahan Ph.D.. Nick was an outstanding and inspirational mentor whose door was always open. I would also like to thank his research technicians, Mariesa Hales and Townsend Barnett whose support and friendship made my time in the laboratory so enjoyable.

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Finally, thanks to my wife Therese, for her support and encouragement and to the many friends and colleagues who have given help and advice.

SUMMARY

1. The aim of the present study was to investigate the role of endothelial:smooth muscle interaction in the local modulation of pulmonary vascular function.

2. The main part of the work was carried out in isolated rings of porcine pulmonary artery suspended for isometric tension in an organ chamber preparation. In order to further investigate vascular responses, additional immunohistochemical, biochemical (enzyme assays, western blots) and molecular (Northern blots) methods were employed.

3. The enzyme heme oxygenase-2 (HO-2), which synthesizes carbon monoxide from the breakdown of heme, was localized immunohistochemically to endothelial cells and adventitial nerves of porcine pulmonary arteries. During contraction of distal pulmonary arterial rings with phenylephrine, acetylcholine (10^{-9} to 10^{-6} M) caused relaxation that was only partly inhibited by L-NAME (3×10^{-5}M), the inhibitor of nitric oxide synthase (NOS). The L-NAME resistant response to acetylcholine was not influenced by the inhibitor of cyclooxygenase, indomethacin (10^{-5} M), but was reduced by the heme oxygenase inhibitor SnPP9 (10μM) and also by ODQ (10 μM), an inhibitor of soluble guanylyl cyclase. These results suggest a role for HO and presumably CO in NO-independent relaxation in the distal porcine pulmonary artery.

4. In contrast to the robust staining for constitutive HO-2 in endothelium, we found only faint staining for inducible HO-1 in porcine pulmonary artery smooth muscle. Moreover, the HO antagonist SnPP9 decreased baseline cGMP levels in endothelium-

containing but not in endothelium-denuded arterial rings. In the presence of L-NAME, acetylcholine failed to increase smooth muscle cGMP levels. So although acetylcholine may not activate the heme oxygenase pathway, the basal activity of this enzyme may increase endothelium-dependent relaxation to the agonist perhaps by synergizing with another endothelium-derived mediator, such as EDHF.

5. Heterogeneity in the response to acetylcholine was observed along the porcine pulmonary artery. Acetylcholine evoked a greater relaxation in distal compared with proximal arterial segments. Moreover, while the relaxation observed in proximal arterial rings was abolished by the NOS inhibitor, L-NAME (3×10^{-5}M), there was considerable residual relaxation that was L-NAME independent in the distal artery. While there was no apparent difference in immunohistochemical staining for HO-2 between proximal and distal endothelium, increased responsiveness to exogenous CO was demonstrated in distal compared to proximal arteries. Analysis of responses to the NO-donor, SIN-1 demonstrated similar relaxation of proximal and distal arteries. This apparent increase in sensitivity to the heme oxygenase product, CO, in the distal porcine pulmonary artery may contribute to the observed heterogeneity in endothelium-dependent relaxation to acetylcholine along the pulmonary artery.

6. In isolated porcine pulmonary arteries precontracted with phenylephrine, hypoxia (0% O_2; pO_2 8-12 mmHg; 0-2% O_2 with oxygen electrode) evokes a late phase, endothelium-dependent contractile response. This response is resistant to inhibition of cyclooxygenase or NOS and may be mediated by an endothelium-derived contractile factor (EDCF). Endothelin-1 caused concentration-dependent contraction of arterial

rings that was inhibited by BQ 123 (10^{-6}M) an ET_A receptor antagonist, and relaxation that was abolished by BQ 788 (10^{-7}M) an ET_B receptor antagonist, or by endothelial cell removal. The contractile response to hypoxia was not inhibited by the ET_A receptor antagonist BQ 123 (10^{-6}M), by the ET_B receptor antagonist BQ 788 (10^{-7}M), or by their combination. Therefore, the endothelium-dependent contraction to hypoxia is not mediated by endothelin.

7. Hypoxia caused only relaxation in endothelium-denuded rings. However, when a pulmonary valve leaflet, a rich source of pulmonary endothelial cells, was placed into the lumen of endothelium-denuded rings, hypoxia caused a late phase contractile response that was similar to that observed in arterial rings with native endothelium. These results suggest that hypoxic contraction of pulmonary arteries is mediated by a diffusible contractile factor, distinct from endothelin, released from hypoxic endothelial cells. The pulmonary valve leaflet placed in the lumen of an iliac artery inhibited the hypoxic relaxation observed in vessels from the systemic circulation. Collection of effluent from pulmonary valve leaflets suggests a factor is released by hypoxic endothelial cells with activity in precontracted rings during normoxia and hypoxia. Using valve leaflets sealed in dialysis membranes the molecular weight of the factor appears to be > 25,000.

8. The role of HO as a potential modulator of the hypoxic response was explored in the pulmonary artery. While there is some evidence from the literature to support such a role for HO in the inhibition of hypoxia inducible gene expression, we did not demonstrate a role for HO in the acute endothelium-dependent contraction in our

preparation. In preliminary studies to further explore signaling in hypoxia, a small increase in intracellular calcium was observed in endothelial cells in response to hypoxia, however pretreating pulmonary valve endothelial cells with the calcium chelator, BAPTA-AM prior to transfer experiments did not inhibit the endothelium-dependent hypoxic contraction.

9. The role of heme oxygenase-1 (HO-1) induction *in vivo* on vascular reactivity and the role of HO-1 induction on the response to lipopolysaccharide (LPS) was explored. Recently, intravenous injection of hemoglobin (HGB) into rats was found to be protective from a subsequent lethal dose of LPS and was correlated with induction of the enzyme heme oxygenase-1 (HO-1). To determine whether the hemoglobin mediated induction of HO-1 could influence vasomotor tone the effect of *in vivo* treatment with hemoglobin and/or LPS on vasoconstrictor responses to phenylephrine (PE) was determined in the isolated rat aorta. Induction of HO-1 mRNA expression by LPS and HGB was demonstrated using Northern blot analysis of rat aorta. Pretreatment of the rats with HGB *in vivo* caused vasodepression in organ chamber studies that was inhibited by SnPP9, the inhibitor of HO, suggesting that the vasodepression was mediated by induction of HO-1 and increased production of carbon monoxide. SnPP9 did not, however, prevent the marked decrease in phenylephrine responses in LPS treated vessels.

10. The light dependent effects of the HO inhibitor SNPP9 on vascular responses were explored. The presence of both SnPP9 and light produced a slowly developing relaxation in precontracted arterial rings that was rapidly reversed when the lights were turned off. The relaxation was inhibited by the soluble guanylyl cyclase inhibitor, ODQ

(10 µM) but unaffected by the antioxidant superoxide dismutase (SOD) (150 u/ml) or the NOS inhibitor L-NAME (3×10^{-5} M). These findings indicate that SnPP9, in the presence of light, produces vasorelaxation via a cGMP-dependent, NOS-independent mechanism.

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LIST OF PUBLICATIONS

Part of the work contained in this thesis has been, or will be, published as follows:

1. Heme oxygenase-2: Endothelial and neuronal localization and role in endothelium-derived relaxation. Zakhary R.*, Gaine S.P.*, Dinerman J.L., Ruat M., Flavahan N.A., Snyder S.H., Proceedings of the National Academy of Science USA. 93: 795-798, 1996. *Co-first authors.

2. Hypoxic pulmonary endothelial cells release a diffusible contractile factor distinct from endothelin. Sean P. Gaine, Mariesa Hales, Nicholas A. Flavahan. American Journal of Physiology 274 (*Lung Cell. Mol. Physiol.* 18): L657-664, 1998

3. Alpha1L-adrenoreceptors in canine pulmonary artery. N.A. Flavahan, M.A. Hales, T.D. Aleskowitch, S.P. Gaine, P.M. Vanhoutte, Journal of Cardiovascular Pharmacology, 32: 308-16, 1998

4. Induction of heme oxygenase-1 with hemoglobin depresses vasoreactivity in rat aorta. Sean P. Gaine, Greg Booth, Nicholas A. Flavahan, Augustine M. K. Choi, and Charles M. Wiener. .Journal of Vascular Research 1999 Mar-Apr;36(2):114-9.

5. Pulmonary vascular endothelial responses are altered following cardiopulmonary bypass. Nyhan, D., Gaine S., Hales, M., Zanaboni, P., Simon, B., Flavahan, N., (Journal of Cardiovascular Pharmacology. 1999 Oct;34(4):518-25.

6. Gaine S.P., Zackhary R., Dinerman J.L., Snyder S., Flavahan N.A., Heme oxygenase-2 (HO-2) is present in pulmonary artery endothelial cells and may contribute to NO-independent relaxation. Circulation 92, No 8, I-168, 1995

7. Sean P. Gaine, Randa Zachary, Jay L. Dinerman, Solomon H. Snyder, Nicholas A. Flavahan. Regional differences in EDRF activity in the pulmonary circulation. A possible role for carbon monoxide in distal vessels. Am J Respir Crit Care Med 153: 4, A581, 1996

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10. Carbon monoxide mediates endothelium-dependent relaxation via cGMP in the pulmonary artery. Gaine SP, Hales MA, Flavahan NA. Am J Respir Crit Care Med 1997, 155(4):A636.

12. Selective pulmonary endothelial dysfunction following cardiopulmonary bypass (CPB) in dogs. Nyhan D, Gaine S, Hales M, Zanaboni P, Simon B, Flavahan N. Am J Respir Crit Care Med 1997, 155(4):A785.

Reprints of these papers, where available, can be found in a folder at the back of this thesis.

INTRODUCTION

1. INTRODUCTION.

The pulmonary circulation differs from the systemic circulation in two unique respects. Firstly, the pulmonary circulation is a low pressure circuit whose vascular resistance is only ten percent that of the systemic circulation. Secondly, while systemic arteries dilate in response to hypoxia to improve blood flow to ischaemic tissue, pulmonary arteries constrict, thereby diverting flow to better oxygenated lung units. The mechanisms responsible for the uniqueness of the pulmonary circulation are complex. The focus of this thesis is to explore the local mechanisms responsible for vascular control and that may contribute to the uniqueness of the pulmonary circulation. We explore the role of endothelium-derived relaxing and contracting factors and postulate on two novel mediators that may play a significant role in the local control of pulmonary vascular function.

2. THE ENDOTHELIUM.

The vascular endothelium comprises 10^{11} cells in pulmonary vascular bed of the average adult human (Cruchley 1987). While the presence of endothelium has been known for a long time, it was initially postulated that it functioned as a non-stick 'Teflon' coating preventing circulating cells from adhering to the vascular wall. Similarly, the endothelium was also considered a modified dialysis membrane allowing nutrients from the circulation to diffuse through the wall, but preventing loss of essential intravascular proteins and cells. However, it is now known that endothelial cells have a highly active metabolic function and play a central role in the control of the vasculature (Gryglewski et al. 1988). Vasoactive mediators such as nitric oxide (NO), prostacyclin (PGI_2) and endothelin are generated by endothelial cells, while other mediators such as 5-hydroxytryptamine (5-HT), PGE_1, PGE_2 and $PGF_{2\alpha}$ are

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inactivated. Furthermore, through the action of the endothelial angiotensin converting enzyme (ACE), the precursor angiotensin I is converted to the potent vasoconstrictor, angiotensin II, and the peptide, bradykinin, inactivated (Ryan et al. 1976). Endothelial cells also generate a number of proteins that are crucial to clotting and thrombosis including Von Willebrand's factor, tissue plasminogen activator (tPA) and growth factors such as platelet activating factor (PAF).

Endothelial cells also contain adhesion molecules, termed selectins and integrins, that are involved in the communication between circulating blood cells and the vascular wall. Disruption of endothelial integrity exposes the subendothelial matrix, stimulating neighboring endothelial cells to express P-selectin which facilitates the recruitment of platelets to the damaged vessel. Platelets bind to selectins expressed by activated endothelial cells and decelerate by rolling on the endothelium. Similarly, circulating leukocytes attach to integrins on the activated endothelium and become activated by local chemoattractants inducing them to squeeze between endothelial cells and migrate to sites of inflammation. (Frenette and Wagner 1996; Springer 1995)

2.1 The role of the endothelium in vascular responses.

Robert Furchgott discovered the obligatory role of the endothelium in acetylcholine evoked relaxation in 1980 (Furchgott and Zawadzki 1980). He demonstrated that gentle rubbing of the intimal surface of vascular strips removed the endothelium and eliminated relaxation in response to carbachol and acetylcholine. Furthermore, by developing a sandwich preparation, where the intimal surface of an aortic strip with intact endothelium was placed in direct contact with the intimal surface of a strip with the endothelium removed, Furchgott demonstrated the presence of a labile, diffusible, non-prostanoid substance produced by the intact endothelium

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that caused smooth muscle relaxation in the endothelium-denuded strip. Furchgott thereby confirmed the presence of endothelium-dependent relaxation (Furchgott and Zawadzki 1980). At a symposium in 1986 it was proposed that this endothelium-derived relaxing factor could be nitric oxide (NO) (Ignarro et al. 1987a; Khan and Furchgott 1987). Within two years it had been demonstrated that NO was produced in endothelial cells by a calmodulin-dependent oxygenase enzyme, nitric oxide synthase (NOS) (Palmer et al. 1988; Palmer et al. 1987; Palmer and Moncada 1989). The Noel Prize for Medicine was awarded in 1998 to Furchgott, Ignarro and Murad in honor of these accomplishments.

Our understanding of endothelium-dependent relaxation has come a long way over the past twenty years. We now know that endothelial cells are capable of releasing various relaxing and constricting factors. Theses factors are released in response to hormonal and physical stimuli (Lamontagne et al. 1991; Lamontagne et al. 1992) and act on vascular smooth muscle cells to modulate vasomotor tone (Furchgott and Vanhoutte 1989). Under normal physiologic conditions endothelial cells release predominately relaxing or vasodilator mediators; however, under certain physiologic and pathophysiologic conditions the balance may shift towards release of constricting mediators (Flavahan and Vanhoutte 1995).

2.2 Endothelium-derived relaxing and contracting factors.

2.2.1 Endothelium Derived Relaxing Factors.

Maintaining low pulmonary vascular tone involves complex interaction between endothelium and smooth muscle. The primary endothelium-derived relaxing factors (EDRFs) are nitric oxide (NO) and prostacyclin (PGI_2). *Nitric oxide* (NO) is a free radical gas produced by the enzyme nitric oxide synthase by the conversion of

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arginine to citrulline (Palmer et al. 1988; Palmer and Moncada 1989). NOS is a NADPH-dependent oxygenase which requires tetrahydrobiopterin, FAD, and FMN as cofactors. NOS binds calmodulin in a calcium-dependent manner and can therefore be activated by agonists that increase intracellular calcium. There are three known isoforms of NOS, two of which are constitutively expressed; ecNOS (NOS III) in endothelial cells and neuronal NOS (nNOS; NOS I) in brain and the peripheral nervous system (Bredt et al. 1991; Bredt et al. 1990). Inducible NOS (iNOS; NOS II) is expressed in immune cells such as neutrophils and macrophages, and can be found in most cells following exposure to stressors such as endotoxin. In contrast to the other NOS enzymes, iNOS does not require calcium for activation as it possess an intrinsic calmodulin moiety. While local release of NO by macrophages may be a crucial method of killing infectious organisms, widespread production of NO in response to sepsis may be harmful and result in systemic hypotension and shock (Thiemermann et al. 1993). Expression of iNOS may be inhibited by steroids (Rees et al. 1990b).

NO activates the enzyme soluble guanylate cyclase (sGC) in vascular smooth muscle to produce cyclic GMP mediated relaxation (Dinerman et al. 1993; Ignarro 1991; Martin et al. 1985). Endothelium-dependent relaxation can be divided into NO-dependent and independent. Inhibitors of NOS, such as L-NAME, abolish relaxation in some vascular beds, while residual relaxation, referred to as NO-independent may persist in many (Cowan et al. 1993). However, even in these blood vessels, L-NAME abolishes the increase in cyclic GMP evoked by the agonists, suggesting that the NO-dependent activity is still effectively inhibited (Cowan et al. 1993; Najibi et al. 1994). Therefore, the L-NAME resistant relaxation has been attributed to PGI_2 and an as yet unidentified mediator termed endothelium-dependent hyperpolarizing factor (EDHF).

Nitric Oxide and Endothelium-dependent Relaxation
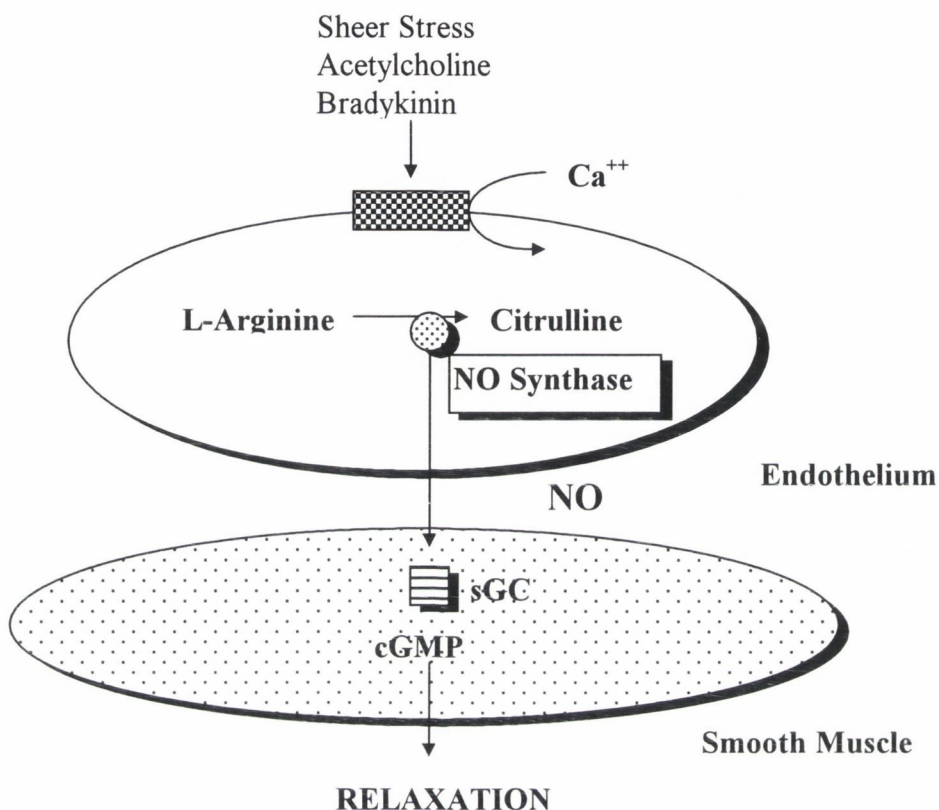
Schematic 1. Endothelium-dependent relaxation via nitric oxide (NO). Abbreviations: sGC; soluble guanylyl cyclase.

In experimental preparations, inhibition of NO or NO synthase causes a small increase in pulmonary artery pressure or in vascular resistance suggesting that tonic release of NO by the pulmonary endothelium accounts for only a small portion of the observed low pulmonary vascular tone. Moreover, inhibition of NO synthase during hypoxia potentiates the vasomotor response suggesting that NO may modulate hypoxic vasoconstriction (Brashers et al. 1988; Graser and Vanhoutte 1991; Johns et al. 1989; Rodman et al. 1990). Of clinical relevance, a decrease in endothelial nitric oxide expression has been demonstrated in patients with pulmonary hypertension (Giaid and Saleh 1995). The clinical use of inhaled nitric oxide is still investigational, however, it

has been demonstrated to produce pulmonary vascular relaxation, without any effect on systemic arterial pressure, in conditions where pulmonary vascular tone is elevated such as primary pulmonary hypertension (PPH) (Sitbon et al. 1995).

Prostacyclin and Endothelium-dependent Relaxation
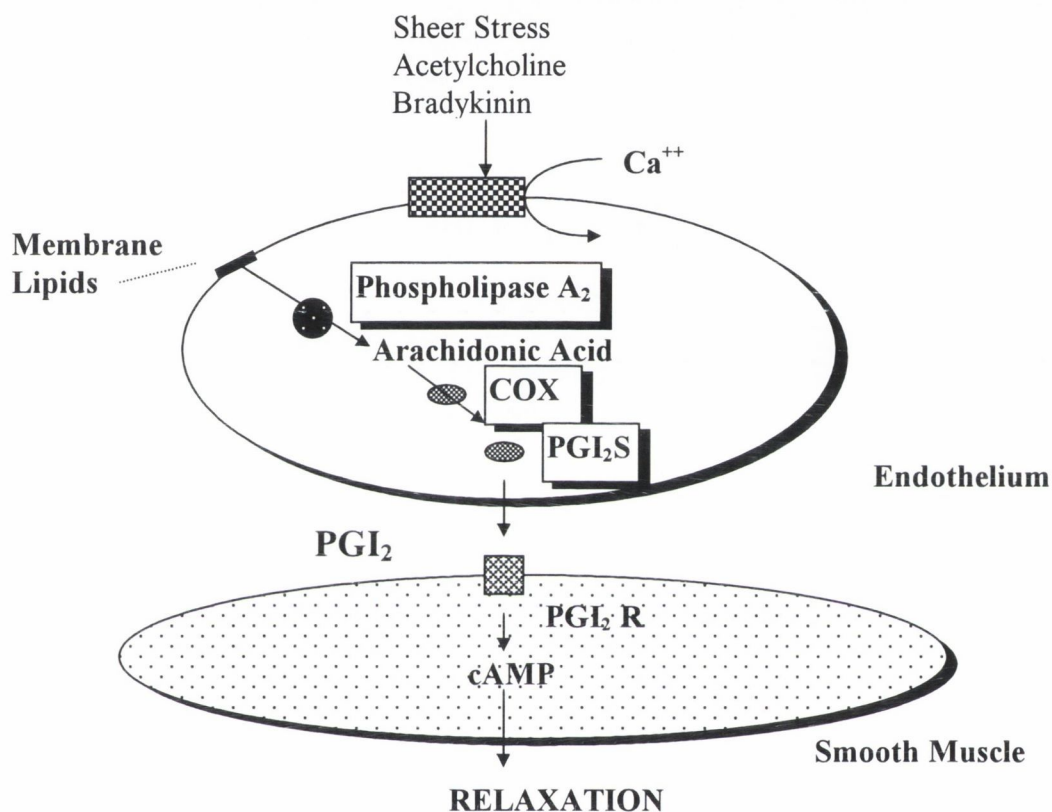
Schematic 2. Endothelium-dependent relaxation via prostacyclin (PGI_2). Abbreviations: COX; cyclooxygenase, PGI_2 R; prostacyclin receptor, PGI_2 S; prostacyclin synthase.

Prostaglandin's were discovered in 1933 by von Euler when a smooth muscle-contracting substance was identified from seminal fluid, hence the name, prostaglandin (von Euler 1934). Some twenty years later it became clear that prostaglandins were a family of unique lipid compounds, with 20-carbon unsaturated carboxylic acids and a cyclopentane ring. The precursor of prostaglandins is arachidonic acid. Arachidonic acid is generated by the calcium-dependent activation of phospholipase A_2 and the

cleavage of membrane lipids. Cyclooxygenase (COX), the enzyme responsible for the conversion of arachidonic acid, to the prostaglandin's exists in constitutive (COX-1) and inducible (COX-2) forms, and is inhibited by aspirin and related compounds (Kujubu and Herschman 1992; Xie et al. 1991).

Prostacyclin (PGI_2), an arachidonic acid metabolite produced by prostacyclin synthase, was discovered in 1976 (Moncada et al. 1976) and demonstrated to inhibit the clumping of platelets. Subsequently it was shown that endothelial cells were the main source of this factor (MacIntyre et al. 1978; Weksler et al. 1977). Prostacyclin, a potent pulmonary vasodilator, causes relaxation of smooth muscle by increasing cyclic AMP (Vanhoutte 1993). While prostacyclin is released by pulmonary vascular endothelium under baseline conditions, it does not appear to be responsible for the low tone pulmonary circulation since inhibition of cyclooxygenase does not cause an increase in vascular resistance (Hales et al. 1978). However, during conditions of high tone, such as during hypoxia, inhibition of cyclooxygenase potentiates vasoconstriction, suggesting that PGI_2 may modulate these responses (Busse et al. 1984; Kovitz et al. 1993). A study in patients with primary and secondary pulmonary hypertension demonstrated an increase in the release of the vasoconstrictor thromboxane A_2 and reduced release of prostacyclin, suggesting that reduced endothelial production of prostacyclin may contribute to or promote progression in pulmonary hypertension (Christman et al. 1992). Patients with severe primary pulmonary hypertension (PPH), a rare usually fatal disease that predominately effects young women, who are treated with a continuous infusion of prostacyclin (epoprostenol, Flolan®) demonstrate improvement in pulmonary artery pressure, exercise tolerance and survival (Barst et al. 1996). Curiously, chronic prostacyclin therapy reduces pulmonary vascular resistance in patients who demonstrate no acute

pulmonary vascular response to vasodilators, which suggests it may function *in vivo* to remodel the pulmonary vascular bed (Fishman 1998; Gaine and Rubin 1998; McLaughlin et al. 1998).

EDHF and Endothelium-dependent Relaxation

Agonist   Calcium ionophore
A23187

AA

p450   ? $\uparrow Ca^{++}$

Endothelium

EDHF

K+

Hyperpolarization

K+

$\downarrow Ca^{++}$

Smooth Muscle

Relaxation
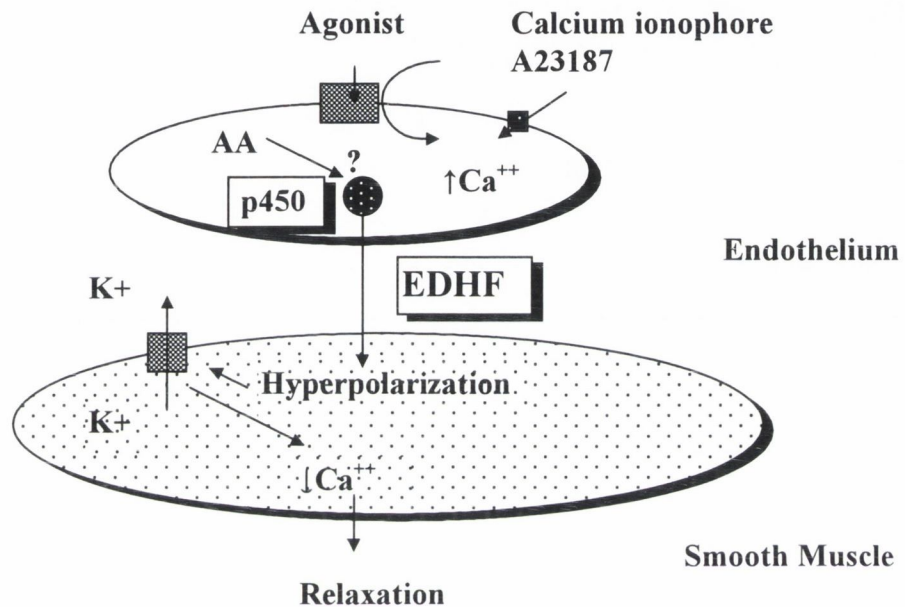
Schematic 3. The production of EDHF by endothelium follows the mobilization of calcium from both intracellular and extracellular pools via phospholipase C-dependent mechanisms. Abbreviations, p450; cytochrome p450, AA; arachidonic acid.

Other potential endothelial-derived relaxing factors include endothelium derived hyperpolarizing factor (***EDHF***). The identity or physiologic role of EDHF is not known, although it may be a cytochrome P450 metabolite of arachidonic acid (Rubanyi and Vanhoutte 1987; Vanhoutte 1993). EDHF is produced by endothelial cells following activation by an agonist. The calcium ionophore A23187 produces endothelium-dependent hyperpolarization in underlying vascular smooth muscle suggesting release and/or production is calcium-dependent (Chen and Suzuki 1990; Nagao and Vanhoutte 1992b). EDHF appears to cause vasodilation by activating at

least two types of potassium channels (K_{ATP} and K_{Ca}) in vascular smooth muscle and causing hyperpolarization of the cell (Nagao and Vanhoutte 1993; Vanhoutte 1993). Whether 'EDHF' is in fact a number of different as yet unidentified agents that act to hyperpolarize vascular smooth muscle is unknown. However, the concept of EDHF was recently challenged by Cohen et al (Cohen et al. 1997) who proposed that L-NAME does not completely inhibit NOS, and that the L-NAME-sensitive and resistant components of endothelium-dependent relaxation are mediated by NO. This proposal by Cohen et al that responses previously thought to be EDHF are mediated by NO is fundamentally flawed and will be addressed more fully in the discussion section as it has a direct relevance to our results.

2.2.2. Endothelium Derived Contracting Factors

Endothelin in Endothelium-dependent Contraction and Relaxation
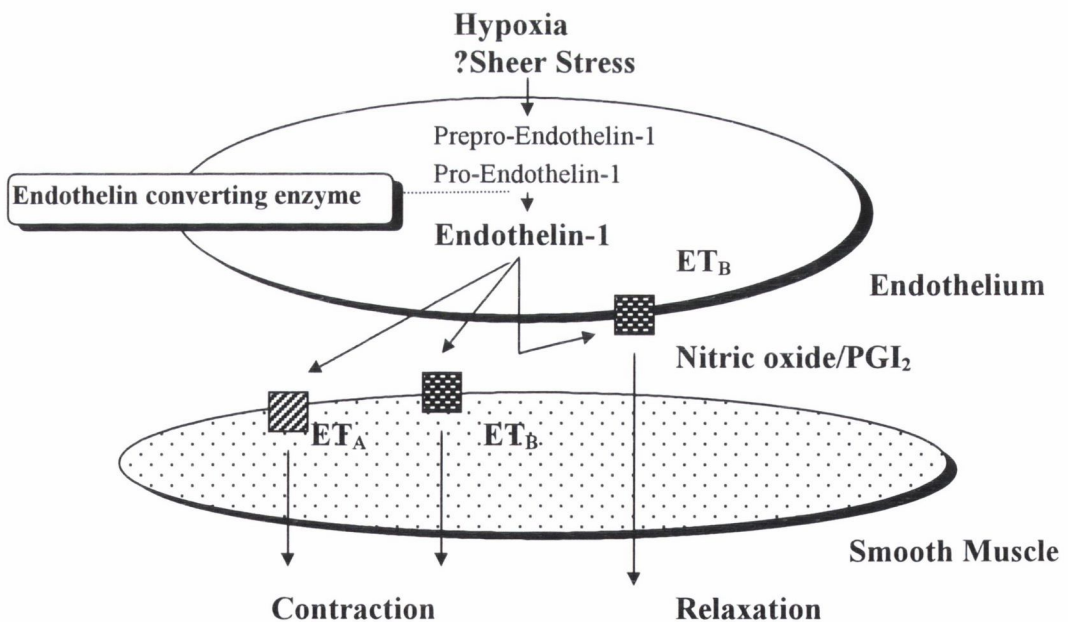
Schematic 4. Endothelin evokes both endothelium-dependent contraction and relaxation.

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The constricting factors produced by the endothelium (EDCFs) include endothelin, superoxide anion and the cyclooxygenase-dependent mediators, thromboxane (TXA_2) and prostaglandin H_2 (Flavahan and Vanhoutte 1995). *Endothelin-1* is a 21 amino acid peptide (2,429K) that is a potent constrictor of pulmonary vessels (Yanagisawa et al. 1988). Endothelins (ET-1, 2 and 3) are synthesized by many different cell types, although endothelin-1 (ET-1) appears to be produced exclusively by endothelial cells (Arai et al. 1990; Yanagisawa et al. 1988). There are also three known endothelin receptors, two of which have been cloned with approximately 60% homology (ET_A and ET_B). ET_A and ET_B are located on vascular smooth muscle and mediate contraction (Arai et al. 1990; Shetty et al. 1993; Sumner et al. 1992), whereas ET_B receptors are also located on endothelial cells mediating relaxation via increased NO and/or prostacyclin production (Hirata et al. 1993; Sakuri T et al. 1990). Evidence is emerging for a heterogeneous population of ET_B-like receptors in pulmonary resistance arteries, including an atypical ET_B receptor (MacLean et al. 1998). A putative ET_C receptor, selective for ET-3, has been cloned (Karne et al. 1993), and may contribute to vascular smooth muscle contraction.

Table 1. Endothelin Receptors.

Receptor	Rank Order of Potency	Selective Agonists	Selective Antagonists
ET_A	ET-1>ET2>ET-3		BQ123
ET_B	ET-1=ET2=ET-3	Sarafotoxin	BQ788
ET_C	ET-3>ET-1		

Superoxide anion produced by the endothelium mediates smooth muscle contraction directly and indirectly by inactivating NO by reacting to form the toxic peroxynitrite (Gryglewski et al. 1986; Katusic and Vanhoutte 1989; Rubanyi and Vanhoutte 1986; Squadrito and Pryor 1995). While it was originally proposed that

superoxide was generated by cyclooxygenase in endothelial cells, it is now appreciated that there are a number of mechanisms capable of producing reactive oxygen species, including nitric oxide synthase (NOS) (Pou et al. 1992), xanthine oxidase (Terada et al. 1991) and NADH oxidoreductase (Mohazzab et al. 1994). The cyclooxygenase products *thromboxane* (Hamburg et al. 1975) and *prostaglandin H_2* cause smooth muscle contraction via the same receptors and can directly cause platelet aggregation (Ito et al. 1991; Tod et al. 1986). Elevated levels of thromboxane metabolites have been observed in patients with pulmonary hypertension (Christman et al. 1992). Recently, attention has focused on a new combined thromboxane synthase and receptor antagonist, Terbogrel (Boehringer Ingelheim Pharmaceuticals, Inc), for use in treating individuals with pulmonary hypertension. By inhibiting both the production of thromboxane and its receptor, any increase in prostaglandin H_2 will be antagonized, and more endogenous prostacyclin will be produced, at least in theory .

It has been suggested that a metabolite of cytochrome P450, *20-hydroxyeicosatetraenoic acid* (20-HETE) may also be an EDCF (Escalante et al. 1989; Ma et al. 1993; Schwartzman et al. 1989). It has been demonstrated to cause vasoconstriction by inhibiting potassium channel activity, depolarizing vascular smooth muscle and increasing intracellular calcium concentration, and has been postulated as being involved in modulating the myogenic response to changes in transmural pressure. It may also potentially play a role in endothelium-dependent contraction in response to hypoxia.

2.3 Endothelial cell activation and signal transduction.

A diverse range of stimuli activate the endothelium and result in the release of vasoactive mediators. Circulating hormones (e.g. angiotensin, epinephrine), autocoids

(e.g. histamine, bradykinin), mediators released from circulating cells (e.g. serotonin, ADP), factors released from autonomic (e.g. acetylcholine, norepinephrine) and sensory nerves (e.g. substance P) activate a wide variety of endothelial cell membrane-bound receptors. The endothelium also generates a number of endothelial cell activators itself (e.g. endothelin, ADP, bradykinin) (Flavahan and Vanhoutte 1995; Furchgott and Vanhoutte 1989). Selected agonists elicit release of different relaxing factors and the response to each agonist may vary considerably between blood vessels, and indeed along each vessel (Zellers and Vanhoutte 1989). These membrane bound receptors are linked to a variety of subcellular signaling mechanisms. Increased intracellular calcium is the final common pathway for the release of the EDRF's, NO, PGI_2 and EDHF. Lower concentrations of calcium are required to stimulate NO than prostacyclin or EDHF (Parsaee et al. 1992). It appears that while influx of extracellular calcium is involved in NOS activation, phospholipase A_2 may respond to release of intracellular stores (Luckhoff et al. 1988). EDHF release appears to have a lower sensitivity to calcium, as higher concentrations of the calcium ionophore, A23187, are required to generate an EDHF response (Chen and Suzuki 1990). Alternatively, the vascular smooth muscle may be less sensitive to the actions of EDHF.

There are at least four major pathways for calcium entry into endothelial cells. The principal pathway appears to be membrane-bound receptor operated channels coupled to G-proteins. These in turn activate phospholipases such as phospholipase $C\beta$ (Berridge 1993). Phospholipase C leads to production of second messengers such as IP_3 and IP_4. IP_3 elevates calcium by release from intracellular stores, while IP_4 increases extracellular calcium influx through membrane-bound calcium-permeable channels (Berridge 1993). There is significant heterogeneity in the nature of the G-protein that couples the membrane-bound receptors to phospholipase C (Flavahan et

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al. 1989; Flavahan and Vanhoutte 1995). As a result endothelial cells from different arteries respond differently to the same agonist bound to surface receptors (Flavahan and Vanhoutte 1995). There is also a distinct set of membrane receptors that are not bound to G-proteins and are stimulated by growth factors (Ullrich and Schlessinger 1990). These membrane bound receptors have inherent tyrosine kinase activity which activates a distinct form of phospholipase ($C\gamma$) also resulting in generation of IP3 and IP4 and increased intracellular calcium (Ku et al. 1993).

Endothelial cells also can respond to their local environment with increased intracellular calcium. This is accomplished by stretch activated, or mechanosensitive non-selective cation channels that allow an influx of calcium in response to cell stretching (Lansman et al. 1987). In addition endothelial cell also contain sodium/calcium exchangers and "leak" channels (Adams et al. 1993). A shear stress sensitive K-channel has also been described in endothelium which leads to activation of mediator release (Ohno et al. 1993). However, unlike vascular smooth muscle, endothelial cells are believed to lack voltage operated calcium channels (VOCC), although exceptions have
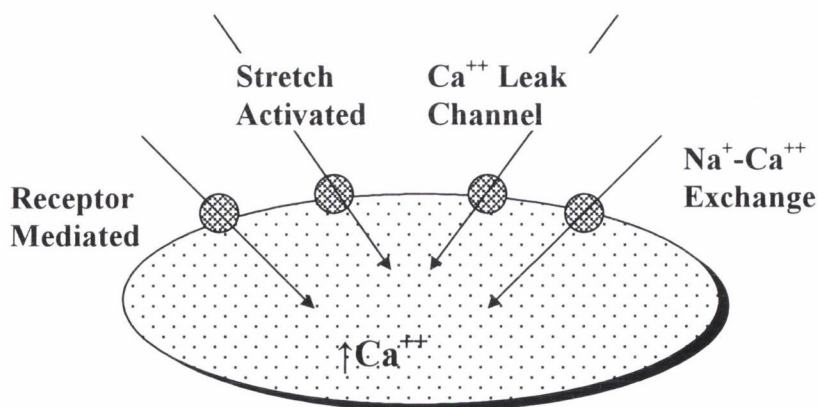
Schematic 5. Mechanisms for calcium entry in endothelial cells.

been described (Adams et al. 1993). As a result the resting membrane potential (RMP) is an important regulator of transmembrane calcium influx (Adams et al. 1989). In cultured endothelial cells the membrane potential is directly related to the log of the extracellular potassium concentration. Stimuli which cause hyperpolarization of the RMP thereby increase the electrochemical gradient for calcium entry (Adams et al. 1989).

3. HYPOXIC PULMONARY VASOCONSTRICTION.

Acute reduction in alveolar oxygen tension (P_AO_2) causes pulmonary vasoconstriction within minutes. On a local level hypoxic pulmonary vasoconstriction diverts blood to better ventilated regions, thereby reducing shunt and maintaining efficient ventilation:perfusion matching. However, global hypoxic pulmonary vasoconstriction is not beneficial and can lead to smooth muscle hypertrophy and fixed pulmonary vascular disease (Gaine et al. 1997). The acute vasoconstrictor response to hypoxia is seen in isolated perfused lungs (Wiener et al. 1991) and in excised pulmonary arterial rings (Kovitz et al. 1993) suggesting local regulation, rather than a requirement for neural reflexes or systemically released mediators. It appears that alveolar hypoxia stimulates the precapillary arteries (Jamieson 1964; Staub 1961) which are the predominant site of the observed increased vascular resistance (Marshall and Marshall 1983) (Isawa et al. 1978). Nevertheless, constriction is also seen in larger pulmonary vessels and has been demonstrated *in vivo* by high resolution CT scanning of animals during hypoxia (Herold et al. 1992). The mechanism of hypoxic pulmonary vasoconstriction remains elusive despite years of directed research. Search for a locally produced acute hypoxic mediator has not proved fruitful and has led investigators to explore intrinsic responses unique to pulmonary smooth muscle

(Madden et al. 1992; Murray et al. 1990). Recent studies have demonstrated that some potassium channels in pulmonary vascular smooth muscle are inactivated or inhibited by hypoxia (Yuan et al. 1993). Inhibition of smooth muscle cell potassium channels results in membrane depolarization, influx of calcium and cell shortening (Murray et al. 1990; Post et al. 1992). Further evidence that a potassium channel is responsible for hypoxic vasoconstriction derives from the demonstration of a hypoxia-sensing potassium channel in the carotid body (Prabhakar et al. 1995). Inactivation of these channels may in part explain the acute vasconstrictor response to hypoxia. Furthermore, dysfunctional voltage gated potassium channels may play a role clinically in the development of primary pulmonary hypertension (Yuan et al. 1998a). Pulmonary artery smooth cells obtained from patients with pulmonary hypertension at the time of lung transplantation demonstrate dysfunctional $K_{V1.5}$ channel activity resulting in higher resting intracellular calcium concentrations, presumably accounting for the increased tone observed *in vivo* (Yuan et al. 1998b).

3.1 The role of endothelium in hypoxic responses.

The role of endothelium in mediating or modulating hypoxic pulmonary vasoconstriction has not been clearly defined. Early studies demonstrated that hypoxia caused immediate contraction of isolated pulmonary and systemic arteries that was entirely dependent on the presence of the endothelium (Holden and McCall 1984; Rubanyi and Vanhoutte 1985). Subsequent studies demonstrated that this contraction was mediated by inhibition of the basal production of the endothelium-derived dilator, NO (Graser and Vanhoutte 1991; Johns et al. 1989; Kovitz et al. 1993). Because hypoxic constriction in isolated lungs did not involve this mechanism (Archer et al. 1989; Brashers et al. 1988), the role of the endothelium has been questioned.

Although modulation of endothelium-derived dilators may not mediate hypoxic pulmonary constriction, altered activity of endothelium-derived constrictor mediators could play an important role in initiating or amplifying smooth muscle constriction in response to hypoxia. Hypoxia for example has been demonstrated to increase endothelin production in isolated resistance arteries (Rakugi et al. 1990), in intact lungs (Shirakami et al. 1991), and may (Kourembanas et al. 1991) or may not (Markewitz et al. 1995) increase endothelin-1 production from cultured endothelial cells. There has generally been agreement that endothelin receptor antagonists attenuate the pulmonary hypertension and vascular remodeling associated with chronic hypoxia (Bonvallet et al. 1994; Chen et al. 1995; DiCarlo et al. 1995). However the role of endothelin in acute hypoxic vasoconstriction is controversial. The ET_A antagonist, BQ 123, attenuated acute hypoxic vasoconstriction in an *in vivo* rat study (Oprail et al. 1995) although this has not been a consistent finding (Takeoka et al. 1995). In isolated canine or rat arteries, blockade of ET_A receptors with BQ 123 did not alter the contractile response to hypoxia (Douglas et al. 1993; Ishizaki et al. 1995; Wong et al. 1993). However, these and other studies of isolated arteries have focused on immediate and transient, endothelium-dependent contractions to hypoxia that are mediated by inhibition of dilator mediators rather than the release of an EDCF (Kovitz et al. 1993).

Kovitz et al have demonstrated a late phase, endothelium-dependent contraction to hypoxia in porcine isolated pulmonary arteries that was not mediated by inhibition of dilator mediators. Based in part on a theoretical analysis, they concluded that this hypoxic contraction was mediated by an endothelium-derived contractile factor (Kovitz et al. 1993). A similar mechanism was proposed for a endothelium-dependent slowly developing contraction in isolated rat pulmonary arterial rings in

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response to hypoxia (Leach et al. 1994). Leach et al have proposed a novel role for the endothelium in modulating the hypoxic response. They determined that smooth muscle calcium also increases in response to hypoxia, but levels off and is stable while smooth muscle contraction continues to increase. They have suggested that the endothelium may play a role in producing a factor that sensitizes the contractile apparatus to calcium producing an increase in tone without a further increase in calcium. The nature of the endothelium-derived factor involved is unknown (Robertson et al. 1995). We sought to further analyze the mechanism underlying this late-phase hypoxic contraction in the porcine model and to determine if the mediator was endothelin.

3.2 Endothelial cell calcium and hypoxia.

There are few published studies to date on the effects of hypoxia on endothelial cell calcium and the results have been conflicting. One study found that exposure of human umbilical vein endothelial cell monolayers to hypoxia (10 mmHg O_2) for two hours increased $\{Ca^{2+}\}_i$ due to an influx of extracellular calcium to values similar to that seen following agonist stimulation (Arnould et al. 1992). Archer et al found that acute hypoxia (50 mmHg O_2 for three min) in bovine pulmonary artery endothelial cells, transiently increased $\{Ca^{2+}\}_i$ due to release of calcium from the endoplasmic reticulum (Archer and Cowan 1993). By contrast, Stevens et al found that hypoxia (35 mmHg O_2), depolarized cultured bovine pulmonary artery endothelial cells, causing a decrease in calcium influx (Stevens et al. 1994). The reason for these conflicting results may reflect the use of cultured cells from different locations (Adams et al. 1989). Cultured endothelial cells are known to differ significantly from freshly dispersed cells in their expression of calcium and potassium channels.

4. HEME OXYGENASE IN THE LOCAL MODULATION OF PULMONARY VASCULAR FUNCTION.

4.1 Emerging roles for heme oxygenase.

While the role of the gas NO is well established as a signaling molecule, little is known about the role of endogenous CO produced by the enzyme heme oxygenase (HO). Heme oxygenase was first described in 1968, as the enzyme responsible for the degradation of heme to biliverdin (Tenhunen et al. 1968; Tenhunen et al. 1969). Two isoforms were described in 1986, HO-1, and HO-2 (Maines 1988). Biliverdin is subsequently converted to bilirubin by biliverdin reductase.

There is considerable variability in the tissue distribution of the isoforms of HO. HO-1, found in greatest quantities in the liver and spleen, is readily inducible in response to cellular stress such as exposure to heavy metals, heme products, UV radiation, endotoxin, and heat shock (Applegate et al. 1991; Choi and Alam 1996; Keyse et al. 1990; Keyse and Tyrrell 1989). HO-2 is a constitutive non-inducible enzyme expressed in greatest concentrations in the brain and testis. Both are microsomal membrane bound enzymes that require three oxygen's, and six reducing equivalents to produce biliverdin, iron and *carbon monoxide* (CO) from each heme (Maines 1988). The electrons are provided by closely associated cytochrome P-450 reductase (NADPH). Attention has focused on the protective anti-oxidant properties of bilirubin (Otterbein et al. 1995; Stocker et al. 1987) and on the induction of ferritin following the release of free iron (Balla et al. 1992; White and Munro 1988) as the mediators of the postulated beneficial effects of HO in response to cellular stress (Balla et al. 1992; Camhi et al. 1995a; Choi and Alam 1996) (Lee et al. 1996; Prestera et al. 1995).

Heme Oxygenase and Heme Degradation

Heme Proteins

Heme   NADPH

Heme oxygenase   **P450** $3\ O_2$
6 Electrons

NADP

Iron   **Biliverdin**   **CO**

Biliverdin Reductase

Bilirubin
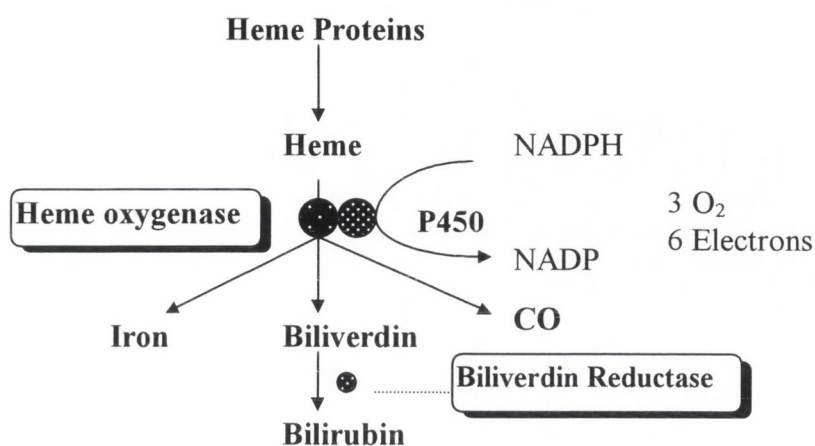
Schematic 6. Function of the enzyme heme oxygenase in the degradation of heme. Electrons are provided by the closely associated cytochrome p-450 reductase.

Both forms of HO are inhibited by metal protophyrins, such as SnPP9 and ZnPP9 (Marks 1994; Posselt et al. 1986; Sassa 1987), although there has been some controversy about their specificity (Zakhary et al. 1996; Zygmunt et al. 1994).

The enzyme heme oxygenase shares many similarities with nitric oxide synthase. Both enzymes exist in constitutive (ecNOS and HO-2) and inducible (iNOS and HO-1) forms. They also both produce a toxic gas (NO and CO) that share some physiochemical properties. CO, like NO, binds to the iron atom of the soluble guanylyl cyclase heme moiety, activating it and increasing cGMP (Brune and Ullrich 1987; Furchgott and Jothianandan 1991; Graser et al. 1990; Vedernikov et al. 1989). Furthermore, exogenous CO inhibits platelet aggregation (Brune and Ullrich 1987) and has been shown to relax blood vessels (Furchgott and Jothianandan 1991). However, while constitutive ecNOS is activated by an increase in intracellular calcium, no role for calcium has yet been described for the activation of HO.

Recent evidence has suggested a role for CO as a neuronal messenger (Bredt et al. 1990; Bredt and Snyder 1994a; Verma et al. 1993). Solomon H. Snyder and his group have demonstrated discrete localization's of HO-2 in rat brain (Verma et al. 1993). The HO-2 was demonstrated in regions where nNOS was absent suggesting perhaps a parallel function to NO. Inhibition of HO with ZnPP9 decreased the level of cGMP in HO-2 rich regions. Subsequent neuronal work has supported a role for HO (Shinomura et al. 1994) and perhaps CO in long term potentiation (LTP) (Ikegaya et al. 1994), olfaction (Ingi and Ronnett 1995), as a neuroendocrine modulator (Lamar et al. 1996; Pozzoli et al. 1994) and in spatial learning (Bing et al. 1995). Endogenous CO from peripheral nerves has also been suggested to mediate smooth muscle relaxation in the human gut, lower esophageal sphincter of the cat and the anal sphincter of the oppossum (Farrugia et al. 1993; Ny et al. 1996; Ny et al. 1995; Rattan and Chakder 1993). HO-2 has also been localized to the glomus cells of the carotid body, where CO may modulate carotid body reactivity to hypoxia (Prabhakar et al. 1995). Nevertheless, some authors have disagreed and have suggested that HO may have a metabolic role in nerve function rather than function as a novel neurotransmitter (Vollerthun et al. 1995).

While the role of nitric oxide in endothelium-dependent relaxation is well established (Bredt and Snyder 1994a; Ignarro 1991; Moncada et al. 1988), little is known about the role of endogenously produced CO in the control of vascular tone (Christodoulides et al. 1995; Suematsu et al. 1995). Some preliminary work suggests a role for CO in the control of vascular tone mediated by the autonomic nervous system (Johnson et al. 1995). Metal porphyrins (ZnDPBG and ZnPP9), inhibitors of HO, when given to instrumented rats have been shown to increase mean arterial resistance but have no effect following pretreatment with inhibitors of autonomic nervous

function (chlorisondamine or prazosin to inhibit ganglionic or α_1-adrenoceptor function, respectively). Furthermore, treatment with the heme oxygenase substrates heme-L-arginate or heme-L-lysinate, to stimulate formation of heme oxygenase products, lowered blood pressure in awake spontaneously hypertensive rats (SHR) by approximately 35 mmHg. This effect was blunted by pretreatment with an inhibitor of heme oxygenase, zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG). The heme oxygenase product Biliverdin did not lower blood pressure in SHR, and the vasodepressive actions of heme-L-lysinate were unaffected by pretreatment with deferoxamine to chelate free iron. (Johnson et al. 1996). However, the pressor effect of ZnDPBG may rely on inhibition of carbon monoxide production in the nucleus tractus solitarii in the brain rather than directly on blood vessels (Johnson et al. 1997). The low tone sinusoidal circulation in the rat liver has also been shown to be affected by administration of ZnPP9 (1 μM), resulting in a 30% increase in vascular resistance and a decrease in measured baseline CO production (Suematsu et al. 1995). The role of HO in the pulmonary circulation was unknown and was explored in this thesis.

4.2 Heme oxygenase, carbon monoxide and hypoxic sensing.

Cells use molecular oxygen (O_2) as the final electron acceptor in the mitochondrial electron transfer chain to produce ATP. Decreases in oxygen tension therefore have a profound effect on cellular metabolism. A number of important genes, termed hypoxia-inducible genes, are induced in response to hypoxia, directing oxygen deprived cells into alternative metabolic pathways to conserve energy when resources are limited (Kourembanas et al. 1990; Kourembanas et al. 1991; Wang and Semenza 1993). Other genes, such as vascular endothelial growth factor (VEGF) direct the growth of new blood vessels to deliver more blood to tissues in the setting of hypoxia

(Liu et al. 1995; Tuder et al. 1994). The gene erythropoetin (EPO) is also induced in response to hypoxia and evokes an increase in red blood cell production in order to improve oxygen delivery to tissues. A novel transcription factor, hypoxia inducible factor (HIF-1), regulates the increased hypoxic expression of these genes as well as endothelin and heme oxygenase (Semenza et al. 1994; Semenza et al. 1996; Wang et al. 1995a; Wang et al. 1995b; Wang and Semenza 1995). HIF has been shown to increase in the lung when rats or mice are exposed to reduced ambient O_2 concentrations for 30 to 60 min, consistent with the proposed role of HIF in coordinating adaptive transcriptional responses (Wiener et al. 1996). However, the mechanisms by which cells sense decreased oxygen and initiate these responses is largely unknown.

The current theory that the oxygen sensor is a probably a hemoprotein is supported by a number of important observations (Wang and Semenza 1996). Firstly, divalent cations (Co^{++}, Ni^{++} and Mn^{++}) induce EPO-gene expression. Divalent cations displace iron (Fe^{++}) in the heme moiety of hemoproteins thereby shifting it to the deoxy configuration similar to the configuration evoked by a reduction in cellular oxygen. Secondly, hypoxia-inducible gene expression (e.g. EPO, VEGF and endothelin) is inhibited by CO, which has a higher affinity for heme than molecular O_2. This suggests that the hemoprotein sensing mechanism that is turned on when oxygen tension falls, can be returned to the 'relaxed' state by binding CO (Wang and Semenza 1996).

HO-1, and CO, may therefore play a unique role in modulating the cellular response to hypoxia and indeed there is evidence that it does (Kourembanas and Bernfield 1994). Kourembanus and her group demonstrated that hypoxia induced HO-1 in vascular smooth muscle resulting in increased CO production and the generation of cGMP (Morita et al. 1995).

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The Proposed Role of HO and CO in Regulating Hypoxic Responses

$\downarrow O_2$

Hypoxic Sensor $\xrightarrow{?}$ Hypoxic Vasomotor Responses

− ↗   ↘ +

CO   HIF

↗   ↖   ↙ +   ↘ +

HO-2   HO-1   VEGF EPO, etc.
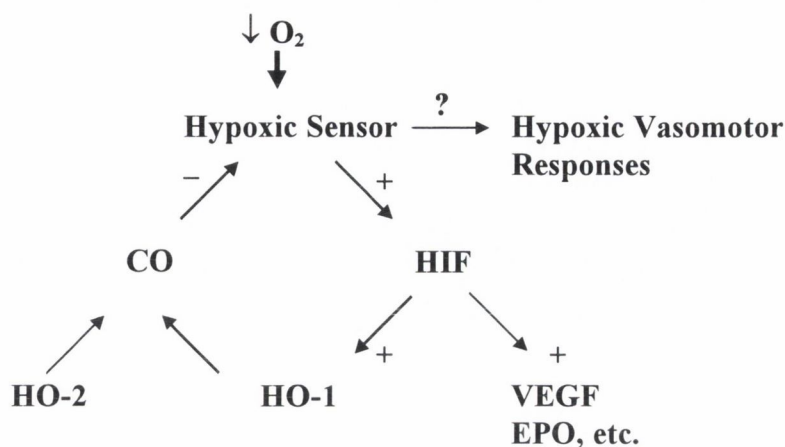
Schematic 7. The hypoxic sensor, in response to low oxygen tension, turns on HIF which leads to induction of specific genes such as HO-1, VEGF and EPO . However, increased HO-1 production will result in increased CO. The hypoxic sensor may then be switched off by binding with CO. Hypoxic vasomotor responses include hypoxic pulmonary vasoconstriction in the pulmonary vascular bed and hypoxic vasodilation in the systemic circulation.

HO-1 expression decreased after twelve hours of sustained hypoxia. However, when the production of CO was inhibited by the HO inhibitor ZnPP9, the expression of HO-1 remained elevated. This suggests that CO can modulate the HO-1 response to hypoxia. So, when cells become hypoxic HIF turns on the expression of important enzymes such as HO-1. HO-1 can then modulate the cellular response to hypoxia by switching off oxygen sensitive genes by the production of the endogenous oxygen mimic, CO. Indeed, in further support of this modulating role for HO and CO in cellular response to hypoxia, it has also been shown that smooth muscle CO, generated by induction of HO-1, inhibits the production of the endothelial genes, endothelin and VEGF, in co-cultured endothelial cells (Morita and Kourembanas 1995). Whether

endogenous CO plays a role in modulating vascular responses to hypoxia has not been determined.

4.3 HO-1 induction and stress responses.

Along with a possible role in endothelium-dependent relaxation and hypoxic responses, the enzyme heme oxygenase may also be involved in modulating vascular responses to septic shock. One of the hallmarks of septic shock induced by endotoxin or its active component, lipopolysacharide (LPS), is hypotension and the loss of responsiveness to vasoconstrictors. The etiology of the depressed vasomotor response is unclear, but has been attributed to the induction of nitric oxide synthase by oxidant stress (Loegering et al. 1995) and increased production of NO (Beasley et al. 1990; Griffiths et al. 1995; Scott et al. 1996; Takakura et al. 1994; Umans et al. 1993; Weigert et al. 1995), although other mechanisms have also been suggested including the induction of COX-2 and the production of prostacyclin (Wu et al. 1994; Yamanaka et al. 1993). Recently, intravenous injection of hemoglobin was found to be protective from a subsequent lethal dose of LPS in rats and was correlated with induction of the enzyme HO-1 (Otterbein et al. 1995). It has been suggested that the antioxidant effects of the bilirubin produced by HO-1 induction may be responsible for the protection. However, induction of HO-1 and increased levels of bilirubin will be paralleled by an increase in CO. While the increased CO production may also prove to be beneficial in sepsis by modulating the induction of key stress enzymes (Morita and Kourembanas 1995) or perhaps by antagonizing the effects of NO at the soluble guanylyl cyclase receptor (CO as partial agonist) (Furchgott and Jothianandan 1991), augmented CO production may also be potentially harmful by stimulating

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vasorelaxation via guanylate cyclase (Furchgott and Jothianandan 1991). Whether sufficient CO is produced following enzyme induction to produce relaxation or to impair vasoconstrictor responses is not known. We therefore sought to determine whether the *in vivo* induction of HO-1 by the pretreatment of rats with hemoglobin would impair vasoconstriction to phenylephrine (PE) in isolated rat aorta and/or protect against the detrimental effects of LPS on vascular responsiveness *in vitro*.

METHODS

1. ORGAN CHAMBER STUDIES

1.1 Porcine blood vessel preparation

Male pigs (~25kg) were anesthetized with ketamine (700 mg IM) followed by pentobarbital sodium (12.5mg/kg iv). The pigs were then killed by exsanguination through the femoral arteries. The heart, lungs and iliac arteries were removed and transported to the laboratory in modified Krebs-Ringer bicarbonate solution (4°C). Intrapulmonary lobar and segmental arteries, representing branches of generations 1-8, were isolated and cleared of adherent connective tissue. Rings were obtained by dividing the vessel into 5 mm long segments. The internal diameter of the arterial rings was measured on the flat unstretched vessel, and the size was classified as proximal (8-12 mm ID) or distal/small (2-4 mm ID) (Schematic 8). In some rings, the endothelium was removed by gently rubbing the intimal surface with a cotton swab and was confirmed during the course of each experiment by the loss of the relaxant response to acetylcholine (10^{-6}M) or bradykinin (10^{-7}M).

The arterial rings were suspended between two stainless steel stirrups in organ chambers (Schematic 10) filled with 25 ml of modified Krebs-Ringer bicarbonate solution (in mM): 118.3 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 25.0 $NaHCO_3$, 11.1 glucose, and 0.016 Ca-EDTA (control solution). Chambers were maintained at 37°C, pH 7.4, and unless otherwise stated, gassed with 16% O_2-5% CO_2 -balance N_2. One of the stirrups was anchored in the chamber, and the other was connected to a strain gauge (model FT03, Grass, Quincy, MA) for the measurement of isometric force (Grass polygraph model 7E)(Schematic 10).
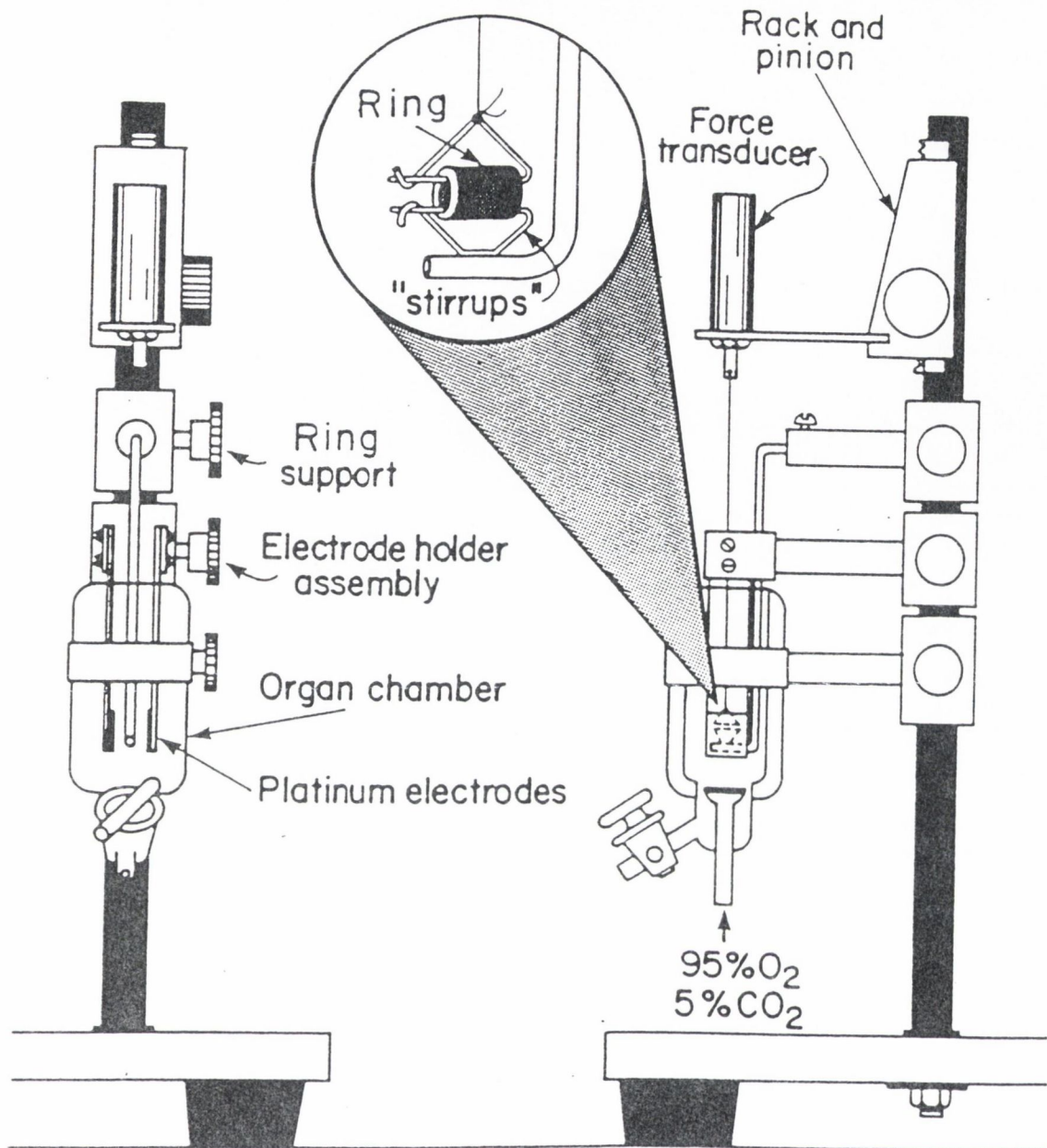
Schematic 10. Organ chamber set-up.

The arterial rings were suspended between two stainless steel stirrups in organ chambers filled with 25 ml of modified Krebs-Ringer bicarbonate solution. Chambers were maintained at $37^\circ C$, pH 7.4, and unless otherwise stated, gassed with 16% O_2-5% CO_2 -balance N_2. One of the stirrups was anchored in the chamber, and the other was connected to a strain gauge (model FT03, Grass, Quincy, MA) for the measurement of isometric force (Grass polygraph model 7E).

0   1   2   3   4   5   6 7   8

PA
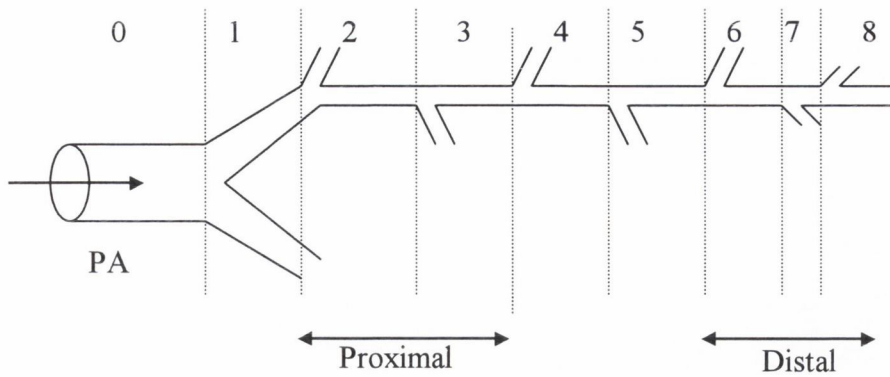
Proximal   Distal

Schematic 8. Intrapulmonary lobar and segmental arteries, representing branches of generations 1-8, were isolated and cleared of adherent connective tissue. Arterial rings were classified as proximal or distal based on their internal diameter measured on the flat unstretched vessel.

Arterial rings were stretched at 10-min intervals in increments of 0.5-1g to reach optimal resting tone. Optimal resting tone was the minimal level of stretch that allowed for the largest contractile response to KCl (20mM). This was previously determined to be 2.5g for small arteries and 5g for other arteries (Kovitz et al. 1993). Once stretched to optimal tone the contractile response to 60mM KCl was determined. KCl was then removed from the organ chambers and tone allowed to return to prestimulation levels. The KCl maximum (achieved using 60mM) was determined to be equivalent to the phenylephrine (PE) maximum achieved with 10^{-4} M (Table 2). PE is more difficult to wash-out than KCl and repeat PE can lead to receptor desensitization. As a result, we use the initial maximum response to KCl as a measure of the expected responsiveness to PE. Therefore, the measured EC_{50} level of tension for KCL (EC_{50KCl}) is used as the estimated EC_{50} level of tension for PE (Kovitz et al. 1993).

Table 2. Comparison of KCl Max with PE Max in Proximal Pulmonary Artery.

Pulmonary Artery	n	Maximal Response to KCL in grams	Maximal Response to PE in grams	
Endothelium	5	11.2 ± 1.3	$12.1 \pm .9$	NS
No Endothelium	5	8.5 ± 1.9	9.4 ± 1.3	NS

Values are means $\pm$ SE; n, no. of animal from which blood vessels were taken. NS, no statistically significant difference (p >0.05).

1.2 Evaluation of endothelium-dependent relaxation in the porcine pulmonary artery.

Following washout of KCl (60mM) and the return of tone to pre-stimulation levels, antagonists were added to treatment rings prior to contraction with phenylephrine (PE). In order to evaluate endothelium-dependent relaxation, rings were precontracted with PE to 50% of their maximal response to KCl. Concentration-response curves to dilators were then determined by increasing the concentration in half-log increments once the response to the previous concentration had stabilized. Endothelium-dependent relaxation to acetylcholine increases along the porcine pulmonary artery from proximal to distal (Kovitz et al. 1993; Zellers and Vanhoutte 1989). Therefore, 'bracketed' controls were used in experiments evaluating endothelium-dependent relaxation in the distal pulmonary artery. Treatment rings were bracketed on each side by control rings from immediately proximal and distal arterial segments (Schematic 9). The control response was taken as the average of the two bracketed controls expressed as a percent of the phenylephrine precontraction. If bracketed controls differed by greater than 10% the experiment was excluded. Antagonist-treated rings and bracketed controls were studied in parallel and only one exposure to agonist was studied in each ring.

Treatment Ring
↓

Small
Pulmonary Artery

Bracketed Controls
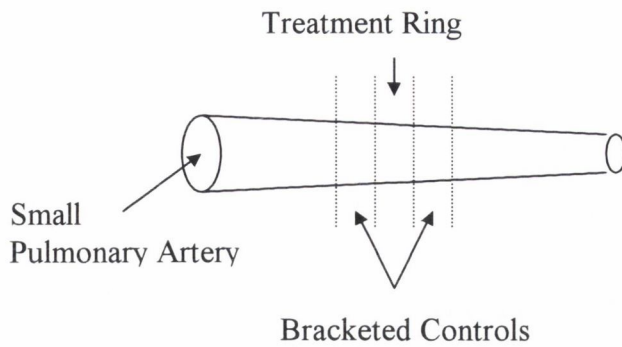
Schematic 9. Bracketed controls were used in experiments evaluating endothelium-dependent relaxation in the distal pulmonary artery.

Experiments involving porphyrins were performed in a darkened room because of their light sensitivity. In other experiments endothelium-independent relaxation was evaluated; after contraction with phenylephrine (to EC_{50KCl} level of tension) responses to the NO donor, SIN-1, or to CO were determined.

1.3 Endothelin-1 concentration response in proximal porcine pulmonary arteries.

When analyzing the influence of endothelin-1 in the proximal pulmonary artery, endothelium-intact and denuded rings were incubated for 30 min in the presence and absence of the endothelin receptor antagonists, BQ 123 ($10^{-6}M$)(ET_A) and/or BQ 788 ($3x10^{-7}M$)(ET_B) before exposing the arteries to endothelin. Concentration-response curves to endothelin were determined under quiescent conditions by increasing the concentration of endothelin in half-log increments (10^{-10}- $3x10^{-7}M$) once the response to the previous concentration had stabilized.

In order to facilitate analysis of endothelin-1 induced relaxation, the response of endothelium-intact and denuded rings to endothelin ($3x10^{-9}M$) was determined following precontraction with PE (EC_{50KCl}). In some rings the effect of ET_A and ET_B receptor inhibition was determined by incubation of the antagonists 30 min prior to

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PE. The endothelin receptor antagonists had no effect on baseline tone and did not influence the contractile response to PE. Control and antagonist-treated rings were studied in parallel and only one exposure to endothelin-1 was studied in each ring.

1.4 Evaluation of arterial responses to hypoxia in the proximal porcine pulmonary artery.
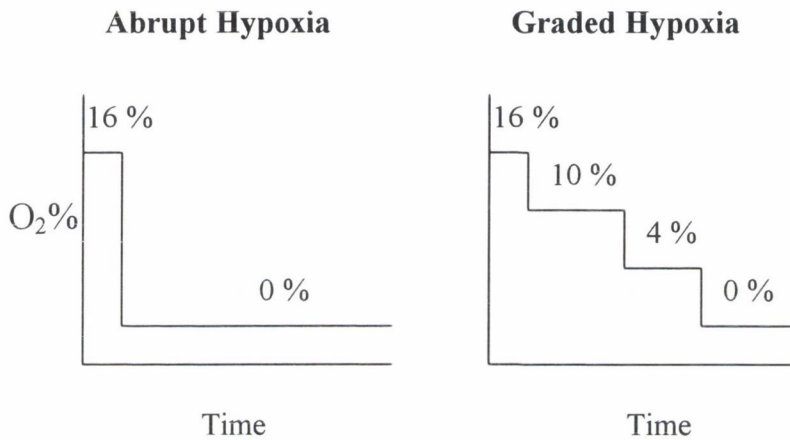
Schematic 12 Two protocols were used to evaluate hypoxic responses. Oxygen was either abruptly decreased from 16% to 0% O_2, or decreased in a graded fashion through 10%, 4% and 0% O_2.

To evaluate arterial responses to hypoxia, rings were first precontracted with PE (EC_{50KCl}). Once the response stabilized, the oxygen tension was either abruptly decreased from 16% O_2 to 0% O_2 or decreased in a stepwise manner from normoxia (16% O_2) through hypoxia (10% , 4% and 0% O_2) (Schematic 12). 16% O_2 was chosen to produce the tissue oxygen tension most consistent with *in vivo* conditions (Kovitz et al. 1993). Each level of oxygen was maintained for 20-40 min to allow stabilization of the response. The oxygen tension in the organ baths was determined using a blood-gas analyzer (model BMS 3Mk2, Radiometer, Copenhagen, Denmark) or an oxygen electrode. At 16% O_2, the pO_2 was 120-127 mmHg (16% with oxygen

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electrode); at 10% O_2, pO_2 was 77-81 mmHg (10% with oxygen electrode); at 4% O_2, pO_2 was 40-43 mmHg (4% with oxygen electrode); and at 0% O_2, pO_2 was 8-12 mmHg (0% with oxygen electrode).

In order to determine the role of endothelin in the proximal porcine pulmonary arterial response to hypoxia, ET_A (BQ 123 10^{-6}M) and/or ET_B (BQ 788 3×10^{-7}M) receptor antagonists were given 30 min prior to precontraction with PE and subsequent exposure to either graded or abrupt hypoxia.

1.5 Endothelial transfer experiments in pulmonary and systemic arterial rings.

The pulmonary valve has three semilunar leaflets
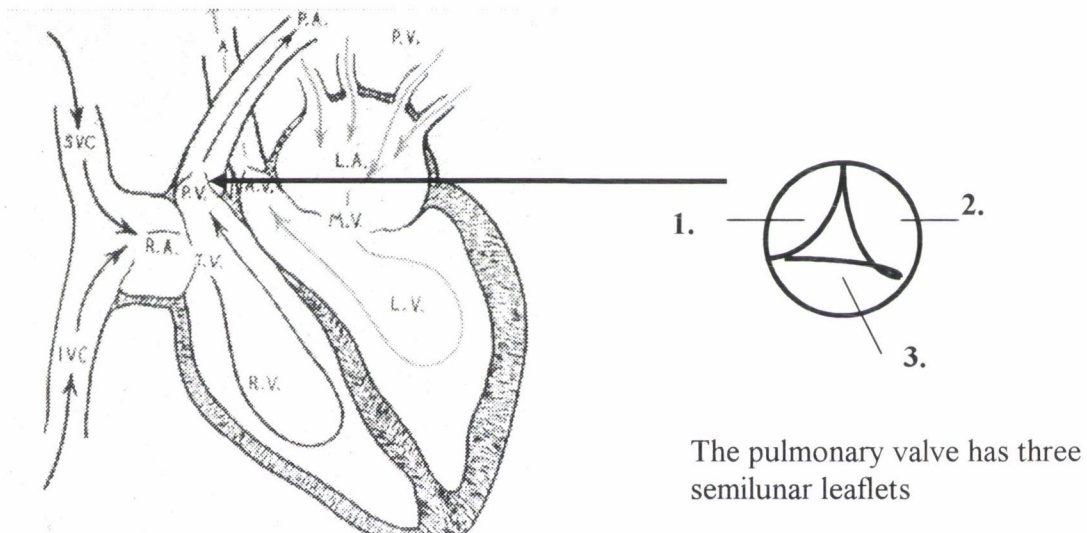
Schematic 13. The pulmonary valve leaflets were dissected from the pulmonary artery outflow tract.. Abbreviations: IVC, inferior venae cava, RA, right atrium, RV, right ventricle, PA, pulmonary artery, TV, tricuspid valve and PV pumonary valve

To determine if the endothelium-derived mediator of hypoxic contraction was a diffusible EDCF, transfer experiments were performed using pulmonary valve leaflets as a source of endothelial cells. Valve leaflets were dissected from the pulmonary outflow tract of the porcine heart (Schematic 13) and stored at 4°C in modified Krebs-Ringer bicarbonate solution until required in the experimental protocol. Valve leaflets were from the same animals as the arterial rings used in each experiment.

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Following the contraction of proximal pulmonary arterial rings to the EC_{KCl50} level of tension the response to acetylcholine ($10^{-6}M$) or bradykinin ($10^{-7}M$) was determined in endothelium-denuded rings in order to confirm the removal of endothelium. The phenylephrine was then removed from the chambers and the tension of the rings allowed to return to baseline. The passive tension (5g) was then relaxed by 2 g, the organ baths lowered and a pulmonary valve leaflet placed in the lumen of the endothelium-denuded arterial rings (Schematic 11). Control rings were treated in the same manner except no leaflet was inserted. The organ chambers were then raised and the passive tension returned to 5g. Arterial rings were then contracted with phenylephrine (EC_{50KCl}) and exposed to hypoxia. In some experiments the effect of inhibition of nitric oxide synthase (NOS) and cyclooxygenase on the response to hypoxia was determined by incubating rings with L-NAME ($3X10^{-5}M$) and indomethacin ($10^{-5}M$) 20 min prior to PE. In order to determine the molecular weight of the endothelium-derived factor, in some experiments the valve leaflets were placed in dialysis membrane bags of known molecular weight cut off (MWCO) prior to being placed in the arterial lumen and the response to hypoxia observed.

The effect of the pulmonary valve leaflet on systemic arterial responses to hypoxia was evaluated by placing the valve in the lumen of endothelium-denuded porcine iliac arteries. Iliac arteries were chosen for the evaluation of systemic arterial responses to hypoxia because they are similar in size to the proximal pulmonary artery.

In order to determine the influence of the valve leaflet on the reactivity of pulmonary artery rings, phenylephrine concentration response curves were generated following placement of the valve. In some experiments valve leaflets were first incubated in distilled water ($4^{o}C$ for 4 hours) to damage the endothelium and to

determine whether stearic effects of the valve might effect vascular reactivity. Furthermore, in some cases L-NAME ($3X10^{-5}M$) was added prior to valve placement and the PE concentration response to evaluate the effect of basal release of NO from the valve leaflet.

To analyze the influence of intracellular calcium in mediating the endothelial response to hypoxia, pulmonary valve leaflets were incubated with the cell permeant calcium chelator, BAPTA-AM ($75\mu M$). Each valve was carefully mounted in an individual modified organ chamber filled with 20 ml of modified Krebs-Ringer bicarbonate solution maintained at $37^{\circ}C$, pH 7.4, and gassed with 16% O_2-5% CO_2 - balance N_2. BAPTA-AM ($75\mu M$) or carrier DMSO (0.1%v/v) were added to the organ chamber 45 min before removing the valves. Valves were washed (x3) with modified Krebs-Ringer bicarbonate solution prior to being placed in the lumen of endothelial-denuded arterial rings in transfer experiments.

1.6 Preparation of effluent from hypoxic endothelium during normoxic and hypoxic conditions

Porcine hearts were obtained from an abattoir and transported to the laboratory in modified Krebs-Ringer bicarbonate solution ($4^{\circ}C$). On each day of experiment eighteen pulmonary valve leaflets were obtained from six porcine hearts and were mounted in a modified organ chamber with 18mls of modified Krebs-Ringer bicarbonate solution (1 ml per valve). The chamber was maintained at $37^{\circ}C$, pH 7.4, and gassed with 16% O_2-5% CO_2 -balance N_2. After 45 min the Krebs-Ringer bicarbonate solution was removed and aliquoted in 1 ml ependorf tubes (normoxic effluent) and stored at $-70^{\circ}C$. The Krebs-Ringer bicarbonate solution was then replaced, and gassed with 0% O_2-5% CO_2 -balance N_2. After a further 45 min the Krebs-Ringer bicarbonate solution was removed and also placed in 1 ml aliquots

(hypoxic effluent) and stored at -70°C. The effect of the effluent on pulmonary arterial rings was subsequently determined.

1.7 Rat blood vessel preparation

Pathogen-free male Sprague-Dawley rats (200-225g) were used in experiments to determine the effect of HO-1 induction on vascular reactivity and response to LPS. Rats were briefly anesthetized with isoflurane (2% vol/vol) before rat hemoglobin (100-300 mg/kg iv) was administered intravenously. Hemoglobin is a potent inducer of HO-1. 16 hours later, rats were injected with either sterile saline or a lethal dose of LPS (40mg/kg)(*Escherichia coli* serotype 0127:B8). Administration of LPS at > 20mg/kg in rats produces 100% mortality within 10 h of injection (Clerch and Massaro 1993; Otterbein et al. 1995). Therefore, four hours after the LPS or sterile saline the animals were sacrificed by decapitation and the thoracic aorta isolated and cleared of adherent connective tissue with the aid of a dissecting microscope. Aortic rings were obtained by dividing the vessel into 5 mm long segments. The arterial rings were suspended between two stainless steel stirrups in organ chambers filled with 25 ml of modified Krebs-Ringer bicarbonate solution and gassed with 16% O_2-5% CO_2 -balance N_2. Arterial rings were stretched at 10-min intervals to reach optimal resting tone, previously determined to be 5gr for the aorta. Once stretched to optimal tone the contractile response to 60mM KCl was determined. KCl was then removed from the organ chambers and tone allowed to return to prestimulation levels.

Concentration-response curves to phenylephrine were determined under quiescent conditions by increasing the concentration of phenylephrine in half-log increments (10^{-8}-10^{-4}M) once the response to the previous concentration had stabilized. The effect of pretreatment *in vivo* with hemoglobin on vascular reactivity,

and its effect on the response to LPS were evaluated. In some rings the effect of the HO inhibitor SnPP9 (30 μM) was determined by pre-incubation 30 min prior to phenylephrine.

2. FLUORESCENCE MICROSCOPY

2.1 Preparation of endothelial cells for calcium imaging.

An attempt was initially made to use front surface (endothelium) imaging of pulmonary artery segments to measure endothelial cell calcium while the tension in the vascular segment was being recorded. While we developed a number of techniques to load the endothelium independently of the underlying smooth muscle (McNeil 1989) we were unable to overcome the problem of porcine pulmonary artery autofluorescence, despite using alternative probes of different wavelengths, such as FURA Red, and calcium-green. We subsequently focused on freshly isolated endothelial cells and valve leaflets to investigate endothelial calcium responses. We chose to avoid endothelial cell culturing on account of the impact culturing can have on ion channel expression (Adams et al. 1993).

Endothelial cells were freshly isolated from the porcine pulmonary artery. To explore differences in endothelial cell calcium responses along the porcine pulmonary artery, lobar and segmental arteries were isolated and divided into proximal (12-8 mm ID) and distal (5-1 mm ID) segments based upon internal diameter measurements. Isolation of endothelial cells was performed under sterile conditions. The arterial segments were opened longitudinally and incubated for ten minutes at room temperature with the intimal surface down in 2 ml of 0.25% collagenase in MEM (minimal essential media). The intimal surface of the arterial segment were then carefully rinsed with 30 ml MEM. The MEM buffer containing the dispersed

endothelial cells were centrifuged (1500 rpm at 4^OC for five min) to harvest the endothelial cells. The cells were resuspended in 10 ml MEM and recentrifuged to remove any residual collagenase from the incubation medium. The cells were resuspended in 0.4 ml MEM and then plated on to quartz coverslips, inside a six-well culture dish (75 μl per coverslip) and placed in a cell culture incubator (37^OC, 5% CO_2, balance air) for 3 hours to enable the cells to attach. At that time 3 mls of fresh MEM were added and the cells incubated overnight. The cells were analyzed within 24 hours.

In separate experiments pulmonary valve leaflets were used as a source of freshly isolated endothelial cells. Valve leaflets were dissected from the pulmonary outflow tract of the porcine heart and stored at 4^oC in modified Krebs-Ringer bicarbonate solution until required in the experimental protocol (Methods 1.5). Endothelial cells were identified using the endothelium-specific marker Di-I acetylated LDL (10 μM). Endothelial cells on quartz coverslips, or pulmonary valve leaflets were incubated with Di-L acLDL for 45 min at room temperature in a sealed chamber in 3mls Krebs gassed with 16% O_2, 5% CO_2, balance N_2. After incubation the cells were washed in filtered Krebs and mounted on an inverted microscope.

2.2 Organ chamber for measurement of intracellular calcium during hypoxia.

A number of organ chamber set-ups for mounting on an inverted microscope were explored. In order to determine the effect of hypoxia on intracellular calcium in endothelial cells, a modified organ chamber was designed (Schematic 14). The chamber was constructed of stainless steel and mounted on a brass water jacket to maintain the temperature at 37^oC. The chamber holds 10mls of Krebs that was gassed

with either 16% or 0% oxygen and 5% CO_2 - N_2 balance. During hypoxia (0% O_2), oxygen was measured at 3 $\pm$ 1% with an oxygen electrode. Quartz coverslips containing dispersed endothelial cells or valve leaflets were mounted horizontally at the base of the chamber. In experiments designed to explore endothelial cell calcium responses to different agonists a separate organ chamber was used. The endothelial
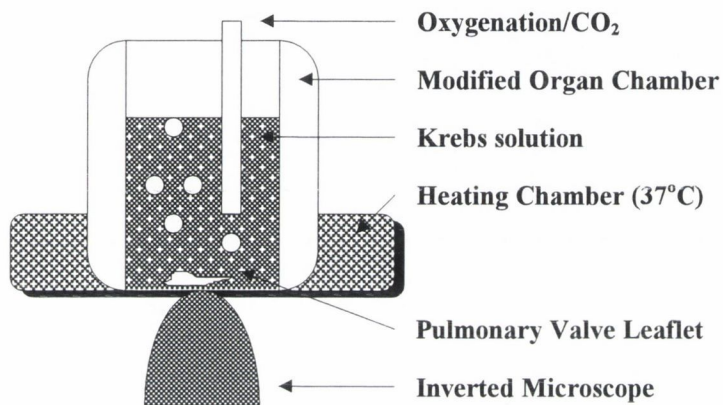
Schematic 14. Modified organ chamber to measure endothelial cell calcium responses to hypoxia in the pulmonary valve leaflet.

cells were maintained at 37°C, using a temperature controlled perfusion chamber and continuously superfused (4 ml/min) with buffer solution. Drugs were introduced into the superfusate.

2.3 Fluorescence imaging.

Endothelial cells, incubated overnight and attached to quartz coverslips, or freshly isolated on pulmonary valves leaflets, were washed in Krebs-Ringer bicarbonate solution. The endothelial cells were then incubated with FURA-2AM (5 μM), the membrane permeant ester of FURA-2, in the dark for 45 min at room temperature in a sealed chamber gassed with 16% O_2, 5% CO_2, balance N_2. After incubation the cells were gently rinsed three times with filtered Krebs to remove residual extracellular

FURA-2AM. The quartz coverslips with adherent endothelial cells, were incorporated onto the stage of the fluorescent inverted microscope.

The endothelial cells were analyzed using a PTI Deltascan spectrofluorometer coupled via fiberoptic cable to a Nikon inverted epifluorescence microscope. Dye-stained specimens were alternately excited at different wavelengths (340 and 380 nm, using monochromators /chopper) and fluorescence detected at an emission wavelength of 510 nm. The ratio of 340 to 380 (R340/380) was used as an indicator of $\{Ca^{++}\}_i$. Absolute $\{Ca^{++}\}_i$ was not calculated because the dissociation constant of FURA -2 for Ca^{++} is different from that obtained *in vitro* (Karaki 1989). A 20x phase/fluor objective (numerical aperture, 0.75; Nikon, Diaphot) was used for valve preparations, while a 40x oil/fluor objective was used for isolated endothelial cell preparations.

3. ENZYME ASSAY

3.1 Heme oxygenase activity

Measurement of HO activity was based on the method of Sierra and Nutter (Sierra and Nutter 1992). Forebrains of male Spargue-Dawley rats were homogenized in 10 mM potassium phosphate buffer (KPO4⁻) pH 7.5, and centrifuged for one hour at 14,000 x g at 4°C. The reaction mixture consisted of 20.3 μM ^{14}C heme, 2mM NADPH, and 5-20 μg of total protein. The reaction was carried out at 37°C for 30 min. ^{14}C heme conversion into ^{14}C bilirubin was assayed in 14K rat brain supernatant. The reaction was stopped by the addition of excess non-radioactive heme and bilirubin as carriers followed by placement in an ice water bath. Two microlitres of the reaction mixture were spotted onto a silica gel thin-layer chromatography sheet (Kodak). The chromatogram was developed using a chloroform:acetic acid (20:1) solvent. The spots corresponding to heme and bilirubin were cut and placed in 10 ml scintillation fluid.

The radioactivity associated with each sample was quantitiated in a Beckman scintillation counter to determine the conversion of ^{14}C heme into ^{14}C bilirubin. The results were expressed as picomoles of bilirubin formed/mg protein/hour.

3.2 NOS activity

Fresh bovine aorta were denuded of endothelial cells by scraping, and the endothelial cell homogenized in 100 ml of ice cold buffer [50mM Tris.HCl, pH 7.4/ 1mM EDTA/1mM EGTA/PIC 3] and centrifuged at 20,000 x g for 15 min (Bredt and Snyder 1989). The supernatant was passed over a 0.75ml column of Dowex AG50WX-8 (Na^+ form) to remove endogenous arginine. Incubations were initiated by addition of 50 µL of homogenate to buffer containing 10mM NADPH (50 µl), 10mM $CaCl_2$ (50 µl). All lights were turned off to permit introduction of the inhibitory porphyrins. The reaction mixture was preincubated for 10 min with a porphyrin before adding 200 µM [^3H]-arginine (1µCi/ml) (50 µl). [^3H] citrulline production was quantified after the separation from [3H] arginine using thin layer chromatography in a Beckman scintillation counter.

3.3 Soluble guanylyl cyclase activity

Cerebella from male Sprague-Dawley rats were cut at 0.5mm intervals in both the sagittal and coronal planes using a McIlwain tissue chopper (Bredt and Snyder 1994b). The slices were dispersed in Krebs-Henseleit buffer containing 118 mM NaCl, 4.7 mM KCl, 2 mm CaCl2, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 11mM glucose. After one hour of preincubation, 20 µL aliquots of gravity packed slices were transfered to prewarmed 5ml minivials (Beckman) containing 250 µl of Krebs-Henseleit buffer (equilibrated with 95% O_2/5% CO_2). All lights were turned off to permit introduction of the porphyrins. Porphyrins were incubated for 20 min before

adding 30µM sodium nitroprusside (SNP). After a 3 min exposure to SNP slices were inactivated by boiling for 5 min in 1 ml of 50mM Tris.HCl pH 8.4/5mM EDTA. Following sonication, cGMP was determined by radioimmunoassay as outlined below (Methods 6).

4. IMMUNOHISTOCHEMISTRY

4.1 Immunohistochemical analysis of ecNOS and HO-2 expression in the pulmonary artery.

Pulmonary artery proximal and distal segments were dissected and fixed in 4% parafomaldehyde in 0.1 M phosphate buffer (pH 7.3) for 4-6 hours at 4°C. The tissue was cryoprotected by 30% sucrose in the phosphate buffer. Tissues were then placed in embedding medium (Tissue-Tek, Miles) (Prabhakar et al. 1995). Cryostat sections, 8µm thick, were mounted on gelatin coated slides. Slides were thawed for 30 min at room temperature and exposed to Tris-buffered saline (TBS)/0.3% H_2O_2 in absolute methanol for 20 min to block endogenous peroxidases, and permeabilized in 0.4% Triton X-100 for 30 min before being blocked for 1 hr in 5% instant milk in TBS. Slides were then incubated in primary antibody (8 µg/ml). The antiserum to HO-2 was developed from a peptide based on amino acids 247-258 of HO-2. For preadsorption experiments, primary antibody was incubated with a 20 -fold excess of HO-2 synthetic peptide for 24 hours at 4°C. Immunostaining was developed with avidin-biotin using diaminobenzidine as a chromogen. (Vectastain ABC kit, Vector Laboratories).

5. WESTERN AND NORTHERN BLOTS

5.1 Preparation of Endothelial Cell Membranes

Fresh endothelial cells were obtained for analysis from either porcine pulmonary valve leaflets or scraping of fresh bovine aorta. The endothelial cell

scrapings or valve leaflets were placed in buffer (50 mM Tris-HCl, 2 mM $MgCl_2$, 1 mM EDTA, 0.1 mM PMSF, 1mg/ml leupeptin, 1mg/ml antipain) (solution A), and then homogenized (30 strokes, ground-glass homogenizer, 4^oC). The homogenate was centrifuged at 1400 x g for 10 min, and the supernatant collected and centrifuged at 40,000 x g for 25 min. The resulting pellet was suspended in solution A and centrifuged a second time at 40,000 x g for 25 min. The pellet was resuspended in solution A and aliquots frozen (-70^oC) until used. Protein concentration was determined using a BCA Protein Assay kit from Pierce (Rockford, ILL).

5.2 Preparation of Vascular Smooth Muscle Membranes

Porcine pulmonary arteries and rat aortas were carefully cleared of connective tissue, cut into rings (~5 mm long) and denuded of endothelium by inserting a cotton swab into the lumen of the rings. The rings were frozen in liquid nitrogen and stored at -70^oC. The frozen tissue was then pulverized in a stainless steel homogenizer, which had been cooled in dry ice and then was resuspended in 200 μl of lamlees buffer and boiled for 5 min. The samples were then sonicated (machine power 3, 80% for 3 min) and centrifuged at 1400 x g for 10 min. The supernatant was then collected and centrifuged at 40,000 x g for 25 min. The resulting pellet was suspended in solution A and centrifuged a second time at 40,000 x g for 25 min. The pellet was then resuspended in solution A and aliquots frozen (-70^oC) until used. Protein concentration was determined using a BCA Protein Assay kit from Pierce (Rockford, ILL).

5.3 Western Blot Analysis

For identification of HO-1 and HO-2 using specific antisera, arterial proteins (30 μg) were transferred from SDS-PAGE gels (11% acrylamide) to nitrocellulose or

PVDF membranes (Immobilon-P, Millipore) electrophoretically (200 V, 2.5 hr, Bio-Rad Transblot). After transfer, excess protein binding sites on the membrane were blocked using 3 mM NaN_3, 0.1% Tween-20, 3% albumin in TBS (2 hr., 37°C). The membranes were rinsed (2 x 15 min, 0.2% SDS, 2% NP-40 in TBS) and then incubated with antisera in 3 mM NaN_3, 0.05% Tween-20, 2% NP-40, 1% albumin in TBS overnight (4°C). Antibody binding was detected using ECL enhanced chemilumisence (Pierce) or ^{125}I-protein A (10^6 cpm/ml) (2 hr, 4°C), and labelled proteins were identified by autoradiography (-70°C). Three experiments were performed in duplicate. HO-1 antisera were affinity-purified, rabbit polyclonal antibodies raised against HO-1. The antiserum to HO-2 was developed from a peptide based on amino acids 247-258 of HO-2 . in the Snyder laboratory and previously used to evaluate the role of HO-2 in the carotid body and central nervous system (Prabhakar et al. 1995) (Verma et al. 1993). The specificity of these antisera are retained in porcine cells. HO-1 and HO-2 protein antibodies were used at a dilution of 1:1000

5.3 RNA Extraction and Northern Blot Analysis

Total RNA was isolated by the STAT-60 RNAzol method with homogenization of tissues in RNAzol lysis buffer followed by chloroform extraction (Tel-test "B" Inc., Friendswood, TX). Total RNA (10 μg) was electrophoresed in a 1% agarose gel and then transferred to Gene Screen Plus nylon membrane (Dupont, Boston, MA) by capillary action (Otterbein et al. 1995). The nylon membranes were prehybridized in hybridization buffer (1% bovine serum albumin [BSA], 7% sodium dodecyl sulfate [SDS], 0.5 M phosphate buffer, pH 7.0, 1.0 mM ethylenediamine tetraacetic acid [EDTA] at 65°C for 2 h followed by hybridization in hybridization buffer containing ^{32}P-labelled rat HO-1 complementary deoxyribonucleic acid (cDNA)

at 65°C for 24 h. Nylon membranes were then washed twice in wash buffer A (0.5% BSA; 5% SDS, 40 mM phosphate buffer pH 7.0, 1 mM EDTA) for 15 min each at 65°C followed by washes in buffer B (1% SDS, 40 mM phosphate buffer, pH 7.0, 1.0 mM EDTA) for 15 min three times each at 65°C. Ethidium bromide staining of the gel was used to confirm the integrity of the RNA. To further control for variation in either the amount of RNA in different samples or loading errors, blots were hybridized with an oligonucleotide probe corresponding to 18S rRNA after stripping off the HO-1 probe. Three experiments were performed in duplicate. A full-length rat HO-1 cDNA, generously provided by Dr. S. Shibahara of Sendai University, Japan, was subcloned into pBluescript vector (Otterbein et al. 1995). A *HindIII/EcoRI* digestion was performed to cut the 0.9 kb HO-1 cDNA insert out of the pBluescript vector. A 24 base pair oligonucleotide (5'ACG GTA TCT GAT CGT CTT CGA ACC 3') complementary to the 18S rRNA was synthesized using a DNA synthesizer (Applied Biosystems; Foster City, CA). HO-1 cDNA was labeled with 32P-CTP using the random primer (Boehringer Mannheim, Germany).

6. cGMP RADIOIMMUNOASSAY.

6.1 Determination of cGMP levels in arterial strips following acetylcholine.

Arterial strips: Porcine pulmonary artery strips were prepared as described previously. Arterial strips were mounted in modified organ chambers in 10 ml of krebs buffer and gassed with 16% O_2 5% CO_2 balance N_2 at 37°C. All rings were incubated with L-NAME (3×10^{-5}M) while some rings were treated with SnPP9 (30 µM). Following 30 min incubation with L-NAME $\pm$ SnPP9, arterial rings were treated with acetylcholine (1 µM). At precise time intervals (0, 15, 30 45 sec) after addition of

acetylcholine, the arterial rings were rapidly removed from the organ chamber and frozen in liquid nitrogen (-196°C). The rings were stored at -70°C for subsequent determination of cGMP content using radioimmunoassay.

Extraction procedure: Frozen arterial rings were pulverized in a stainless steel homogenizer, which had been cooled in dry ice, and the frozen fine powder quickly transfered to an eppendorf tube with a flat bladded spatula. 1ml of ice-cold 6% trichloroacetic acid (TCA) was then added and the sample vortexed. Samples were then homogenized by sonication for one minute and centrifuged @ 3400 rpm for 10 min at 4°C. The supernatent was removed and placed in 15 ml conical, stoppered tube and 10 ml of H_2O saturated ether added while the pellets were stored at -70°C for subsequent determination of protein concentration, as outlined below. The stoppered tube, containing the supernatant, was vortexed at room temperature for 60 sec, the ether and aqueous phases were allowed to separate and the ether layer was removed by aspiration. This procedure was repeated two additional times. A 500 µL aliquot of the sample was then lyophilized and stored at -70°C.

cGMP assay protocol: Before radioimmunoassay, the samples were resuspended in 500µL of 0.05 M assay buffer (acetate buffer, pH 5.8 with sodium 0.01% (w/v) sodium azide) vortexed and assayed for cGMP utilizing a radioimmunoassay kit (Amersham). Duplicate 150 µl aliquots were added to 100 µL assay buffer for a dilution of 1:1.75. The samples were then acetylated (using 1 volume acetic anhydride and 2 volumes triethylamine), incubated for 1 hour at room temperature with 100 µl of the antiserum complex and then challenged with antigen (100 µl succinylated cyclic GMP-[125I] tyrosine methyl ester). The samples were incubated at 4°C for 18 hr. Amerlex-M second antibody reagent was added and the

antibody bound fraction separated by centifugation. (1,500g for 10 min) and the supernatant discarded. The radioactivity of the samples was measured using a gamma counter (Gamma Trac 1191, Tm Analytic). All samples were done in duplicate along side the standard curve (1-64 fmol).

Measurement of the protein concentration in the arterial ring: 1 ml of 1N NaOH was added to the residual pellet and re-sonicated for 15 sec. Then 250 μL was transfered to 4.75 ml of homogenization buffer for a 1: 20 dilution. 100 μL was then used in the protein assay. Protein concentration was determined using a BCA Protein Assay kit from Pierce (Rockford, ILL). Results were calculated as fentomoles of cGMP per mg of protein and the duplicates averaged.

7. STATISTICAL ANALYSIS

Statistical evaluation of the data was performed using Student's t test for paired or unpaired analysis. When more than two means were compared, analysis of variance was performed. If a significant F value was found, Scheffe's test for multiple comparisons was employed to identify differences among groups. Values were considered to be statistically significant when $P < 0.05$. Results were expressed as means $\pm$ SE. When mentioned in the text, n refers to the number of animals from which blood vessels were taken. Unless otherwise stated, each experiment includes a treatment and control group from the same animal. Therefore, n refers to all groups described in the results and depicted in the figure.

EC_X, or the concentration of agonist causing X% of the maximal response, were determined by regression analysis of the linear portion of concentration-effect

curves. Antagonist dissociation constants, K_B, were determined using the formula: $K_B = [B]/(CR-1)$, where [B] is the concentration of antagonist, and CR the ratio of agonist-concentrations producing equal responses in the presence and absence of the antagonist.

8. DRUGS AND SOLUTIONS

8.1 Drugs and solutions used

Acetylcholine (Calbiochem)

Di-L acetylated LDL (Molecular probes)

BAPTA-AM (Molecular Probes)

Bradykinin (Calbiochem)

BQ123 (American Peptide Co.)

BQ788 (American Peptide Co.)

Carbon monoxide (See below)

Cycloheximide (Sigma)

Endothelin-1 (Peninsula labs)

FURA AM (Molecular Probes)

Hemin (Sigma)

Indomethacin (Sigma)

L-NAME (Sigma)

ODQ (Torcis Cookson)

PP9 (Porphyrin Products)

Phenylephrine hydrochloride(Sigma)

$PGF_{2\alpha}$ (Cayman Chemical Co.)

Rauwolscine (Sigma)

Prazosin, HCl (Sigma)

SK&F 94836 (Smith Klein and Beecham)

SnPP9 (Porphyrin Products)

SIN-1 (3-morpholinosydnonimine) (RBI, Interactive)

SOD/Superoxide dismutase (Sigma)

Zaprinast (Sigma)

ZnPP9 (Porphyrin Products)

Drugs were dissolved in distilled water and kept on ice during the course of the experiment with the exception of: endothelin and bradykinin, which were dissolved in acetic acid followed by dilution in distilled water (final chamber concentration for acetic acid of 0.001% v/v); BQ 123, which was dissolved in DMSO followed by dilution in distilled water (final chamber concentration of DMSO 0.01% v/v); BQ 788, which was dissolved in methanol followed by dilution in distilled water (final chamber concentration of methanol of 0.04% v/v); BAPTA-AM, dissolved in DMSO (final chamber concentration of DMSO 0.1% v/v) These concentrations of solvents had no effect on hypoxic reactivity or on responses to constrictor or dilator agonists. Stock solutions of porphyrins were prepared in subdued light by dissolving the solid in 30% final volume 0.1 N NaOH. When fully dissolved, the pH was titrated using HCl for a final pH of 7.2-7.8. All chemicals were the highest purity available. Stock solutions were prepared each day. All concentrations are expressed as the final molar concentration in the organ chamber (M).

8.2 Supersaturated solution of carbon monoxide.

Distilled water (20ml) in an air tight glass tube was gassed with 100% CO at room temperature for 20 min in a fume cupboard. The glass tube was maintained at atmospheric pressure by means of an exhaust line. For administration in organ chamber experiments, the tube was inverted and the saturated solution drawn off using a 19 gauge needle. The concentration of CO [CO] was calculated from the solubility of CO in H_2O (approximately 1 mmol/l H_2O)(Karlsson et al. 1985).

RESULTS

1. ENDOTHELIUM-DEPENDENT RELAXATION. A ROLE FOR HEME OXYGENASE, AND CARBON MONOXIDE.

Rationale: The enzyme heme oxygenase has many similarities with nitric oxide synthase. Both enzymes exist in constitutive (ecNOS and HO-2) and inducible (iNOS and HO-1) forms. They both also produce a toxic gas (NO and CO) that can activate soluble guanylyl cyclase in vascular smooth muscle and increase cGMP. While the role of NO in endothelium-derived relaxation is well established, little is known about endogenously produced CO. We explored the role of constitutive HO-2 and CO in vascular reactivity by determining the expression of the enzyme in vascular tissue and evaluating the effect of enzyme inhibitors on endothelium-dependent relaxation in the pulmonary artery.

1.1 HO-2 and CO in the porcine pulmonary artery.

1.1.1. Localization of heme oxygenase in blood vessels.

Microsomes were prepared from rat testes and porcine aortic endothelial cells. Western blot analysis employed HO-2 antisera at a dilution of 1:1000 and were developed by enhanced chemiluminescence. A single 36-kDa band that corresponds to the known molecular mass of HO-2 was demonstrated in both the testes and endothelial cell preparation (Figure 1c). Preincubation with antigenic peptide abolished immunoreactivity. These experiments were comppleted on three separate animals.

To evaluate whether carbon monoxide (CO) could play a role in vascular reactivity the expression of the constitutive CO producing enzyme, HO-2, was determined in a number of blood vessels from the systemic and pulmonary vascular bed. Immunohistochemical analysis of arterial sections using a polyclonal antibody to the enzyme revealed distinct staining along the endothelium in pulmonary, mesenteric,

and basilar arteries (Figure 1a). Preabsorption of the antiserum with immunizing peptide abolished immunoreactivity. Staining for HO-2 was not demonstrated in the underlying vascular smooth muscle. Staining was completed on tissue obtained from three separate animals and the images depicted are representative of the observed expression in all animals. While all vessels stained positively the only artery evaluated that consistently did not have robust endothelial staining was the porcine coronary artery.

Staining was also demonstrated in the adventitial layer of the pulmonary artery sections in a punctate pattern. Staining for neurofilament protein was coextensive with the advential staining for HO-2, suggesting the presence of the enzyme in pulmonary vascular nerve fibers (Figure 1b).

The HO-2 antibody did not recognize the inducible form of the enzyme, HO-1. With antisera selective for HO-1 (StressGen Biotechnologies, Victoria, BC), we observed faint staining of smooth muscle cells. This reflected low enzyme abundance rather than decreased affinity of the antisera for HO-1, since in previous experiments the same antibody produced robust staining of spleen. Preincubation of the antibody with the immunizing peptide abolished immunoreactivity. We observed no HO-1 staining in advential nerves.

These results indicate that the CO producing enzyme, HO-2, is present in blood vessels and localized to the endothelium and nerve fibers. We sought therefore to determine whether it might play a role in endothelium-dependent relaxation along the pulmonary artery.

1.1.2. NO-dependent and independent relaxation in small porcine pulmonary arteries.

Acetylcholine (1nM-1μM) concentration response curves were generated in small (2-4 mm ID) porcine pulmonary arterial rings pre-contracted with phenylephrine to 50% of the maximal response to KCl (EC_{50KCl}). The maximum relaxation to acetylcholine was 95.1 ± 2.4 % (n=10). Endothelial removal abolished this relaxation and resulted in contraction to acetylcholine (1 μM) of 11.3 ± 2.6% (n=6)(Figure 2).

In order to determine the contribution of mechanisms other than nitric oxide to endothelium-dependent relaxation, acetylcholine concentration response curves were generated in the presence of the nitric oxide synthase (NOS) inhibitor L^{ω}-nitro-L-arginine methyl ester (L-NAME). While L-NAME ($3x10^{-5}$M) given 20 min prior to precontraction with PE (EC_{50KCl}) had no significant effect on baseline tone nor an effect on the maximal response to PE (Kovitz et al. 1993), it did increase the potency of PE in endothelium-intact rings indicating the inhibition of the basal release of NO from endothelium ($logED_{50}$ value of -6.63 ± 0.09 versus -6.85 ± 0.08, n=12, $p < 0.05$; in small endothelium-intact rings with and without L-NAME, respectively n=6, $P < 0.05$). Therefore, all arterial rings were precontracted to the same level (EC_{50KCl}) but with different concentrations of PE. In the presence of L-NAME, the maximum relaxation to acetylcholine was decreased to 49.4 ± 3.6% ($p < 0.05$) (n=16), consistent with a significant L-NAME-resistant component to endothelium-dependent relaxation (Figure 2). The rationale behind the concentration of L-NAME used ($3x10^{-5}$M) is outlined in the discussion.

To determine whether a cyclooxygenase product, such as prostacyclin, was responsible for the L-NAME resistant relaxation, the effect of the cyclooxygenase inhibitor, indomethacin (10^{-5}M), was evaluated in the presence of L-NAME ($3x10^{-5}$M).

Indomethacin had no significant effect on baseline tone nor on the response to PE. Concentration response curves to acetylcholine (1nM-1µM) were generated in arterial rings precontracted with PE (EC_{50KCl}). Indomethacin did not inhibit L-NAME-resistant relaxation. The maximum relaxation to acetylcholine in the presence of L-NAME was $53.9 \pm 9\%$ and $44.1 \pm 9.3\%$ in control and indomethacin treated rings, respectively (n=4)(p > 0.05;NS)(Figure 3).

These results suggest that in small porcine pulmonary arteries acetylcholine evokes significant L-NAME-resistant relaxation that is not explained by a cyclooxygenase product, such as prostacyclin.

1.1.3. The selectivity of heme oxygenase inhibitors. Enzyme assay experiments.

Evaluating the role of HO-2 and CO in endothelium-dependent relaxation requires selective enzyme inhibitors. Protoporphyrins substituted with various metals such as zinc (Zn), tin (Sn) and copper (Cu), have been widely used as heme oxygenase inhibitors. However, protoporphyrins can also inhibit other heme-dependent enzymes that are important in endothelium-dependent relaxation (including NOS and sGC). In order to determine the specificity of the protoporphyrins, SnPP9 and ZnPP9, their inhibitory effect on the enzymes ecNOS, soluble guanylyl cyclase (sGC) and HO-2 were evaluated and compared with the non-inhibitory protoporphyrin, PP9 (Table 3.).

Table 3. Selectivity of heme oxygenase inhibitors

	IC_{50} µM	% inhibition at 100 µM	
Drug	**HO-2 (n=3)**	**ecNOS (n=3)**	**sGC (n=3)**
SnPP9	7.5 ± 0.8	35 ± 12	30 ± 6.0
ZnPP9	7.0 ± 0.6	50 ± 8.2	45 ± 4.9
CuPP9	50 ± 4.3	Not studied	55 ± 5.3
PP9	> 100 µM	30 ± 6.2	No inhibition*

Results are expressed as percentage of control activity for independent determinations in three animals (n=3), each assayed in triplicate. (*) At 100mM PP9 produced a 25% augmentation in sGC activity (p<0.05).

The IC_{50} of SnPP9 for HO-2 was $7.5 \pm 0.8\mu M$ while the IC_{50} for ecNOS and sGC were greater than 100 μM. The IC_{50} of ZnPP9 (7.0 ± 0.6 μM) for HO-2 was similar to SnPP9, but ZnPP9 was somewhat less specific producing greater inhibition of ecNOS and sGC at 100μM than SnPP9. Furthermore, ZnPP9 also precipitated in physiological/krebs buffer. The protoporphyrin PP9 was a useful negative control, with an IC_{50} exceeding 100μM for HO-2, and with $30 \pm 6.2\%$ inhibition of ecNOS at 100 μM. Moreover, PP9 did not inhibit sGC. At 100 but not at 1 or 10 μM, we observed a 25% (p < 0.05) augmentation of sGC activity. PP9 has previously been reported to activate sGC (Ignarro et al. 1984).

These results suggest that the metal protoporphyrins, SnPP9 and ZnPP9, are relatively selective inhibitors of HO.

1.1.4. Heme oxygenase inhibitors and endothelium-dependent relaxation.

In order to determine whether HO-2 contributed to endothelium-dependent relaxation, the effect of the protoporphyrin inhibitors on acetylcholine evoked relaxation was evaluated. Because of the light sensitivity of porphyrins these studies were performed in the dark. For contractility studies SnPP9 was chosen over ZnPP9.

Arterial rings were precontracted with PE (EC_{50KCl}) before being incubated for 30 min in the presence and absence of the heme oxygenase antagonist, SnPP9 (1-100 μM). Thirty minutes was determined from preliminary experiments to be the minimal

incubation time required for maximum inhibition of endothelium-dependent relaxation by the porphyrins. SnPP9 (1-100 μM) had no effect on the phenylephrine precontraction. Concentration response curves to acetylcholine were then generated. The maximal relaxation to acetylcholine, 94.5 ± 4.4%, was not affected by SnPP9 (10μM), 100 ± 0% (p>0.05;NS)(n=4)(Figure 4). A higher concentration of SnPP9 (100 μM) appeared to decrease the maximal relaxation to acetylcholine from 94.8 ± 4.5% in controls to 80.9 ± 7.7% however the difference did not reach statistical significance (p > 0.05; NS) (n=4) (Figure 5).

The effect of the heme oxygenase inhibition on L-NAME-resistant relaxation was also evaluated. The maximum relaxation to acetylcholine in arterial rings treated with L-NAME (3×10^{-5} M), 44 ± 11%, was not inhibited by SnPP9 (1 μM), 40.9 ± 6.9% (p>0.05;NS)(n=5)(Figure 6). However, higher concentrations of SnPP9 did inhibit L-NAME-resistant relaxation. Maximum relaxation in control rings, 45.5 ± 5.8%, was inhibited by SnPP9 (10 μM) to 25.5 ± 6.4% (p < 0.05) (n=7) (Figure 7). While SnPP9 (100 μM) inhibited submaximal relaxation to acetylcholine (ach 10^{-7}M; 44.7 ± 4.3, versus 23.2 ± 6.9, (p <0.05)) with a rightward shift in the concentration effect curve, maximal relaxation in control, 58.7 ± 1.8% and SnPP9 (100 μM) treated rings, 55.9 ± 5.1%, were not significantly different (n=5)(p> 0.05;NS)(Figure 8).

The porphyrin PP9 does not inhibit heme oxygenase, and was therefore used to determine the specificity of the observed inhibition of L-NAME-resistant relaxation. Maximal relaxation to acetylcholine, 71.7 ± 1.7%, was not inhibited by PP9 (10 μM) 71.4 ± 10.2% (n=3)(p>0.05;NS) (Figure 9).

1.1.5. Correlation between enzyme assay inhibition by SnPP9 and the effect on vascular reactivity.

The effects of SnPP9 on vascular reactivity (Results 1.1.4) were compared with the enzyme assay studies of the selectivity of SnPP9 (Results 1.1.3). The inhibitory effect of SnPP9 on endothelium-dependent relaxation was assessed at the $IC_{30(Ach)}$ level of response to acetylcholine (that is the concentration of acetylcholine producing 30% relaxation in control arteries and arteries treated with L-NAME), which was interpolated from concentration-effect curves (n=4-7)(Schematic 15). The inhibitory effect at 30% relaxation to acetylcholine was chosen as it was applicable across all experimental protocols (i.e. all acetylcholine concentration response curves in all experimental protocols, with or without antagonists, relaxed by more than 30%). The $IC_{50(SnPP9)}$ was then calculated from the inhibitory effect at 30% relaxation to acetylcholine of SnPP9 (1, 10, 100 μM). The $IC_{50(SnPP9)}$ was determined to be 7.6 μM for SnPP9 in L-NAME treated rings. Higher concentrations of SnPP9 were needed to inhibit the NO-dependent component of relaxation corresponding to an $IC_{50(SnPP9)}$ in the absence of L-NAME of 85 μM. (Figure 10). The potency of SnPP9 in reversing L-NAME-resistant or NO-independent relaxation closely resembled its potency in inhibiting HO-2 activity in the enzyme assay experiments (IC_{50} 7.5 μM)(Figure 11). Higher concentrations of SnPP9 were needed to inhibit NO-dependent relaxation, similar to the enzyme assay inhibition of ecNOS ($35 \pm 12\%$ inhibition at SnPP9 100 μM) and soluble guanylyl cyclase ($30 \pm 6\%$ inhibition at SnPP9 100 μM) .

The inhibitory effect of SnPP9 assessed at the $IC_{30(Ach)}$ level.
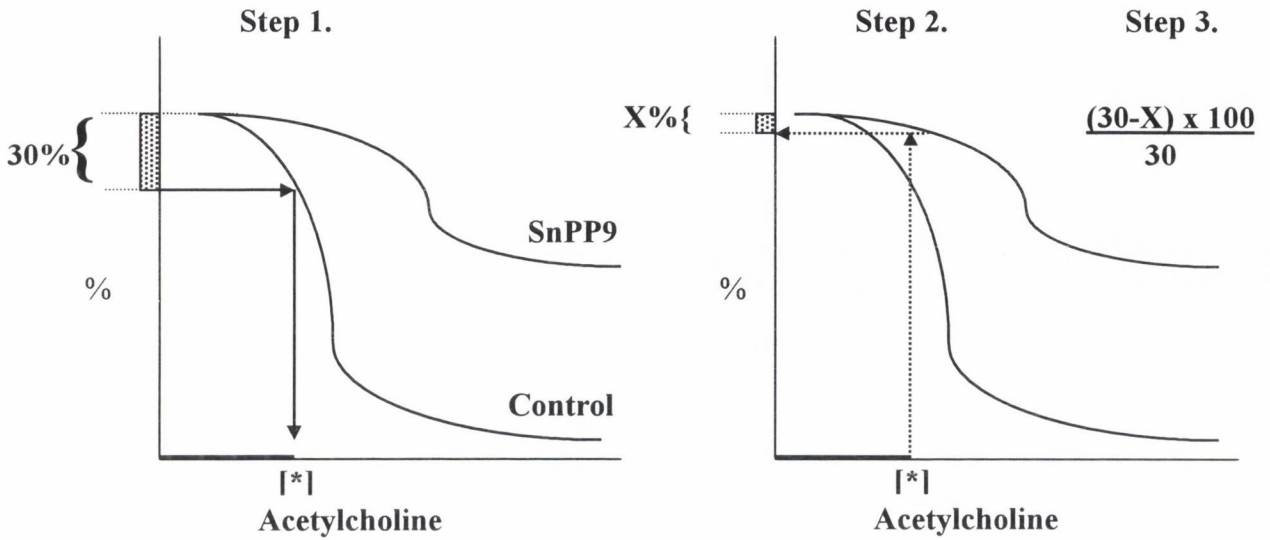
Schematic 15. The inhibitory effect of SnPP9 on endothelium-dependent relaxation was assessed at the $IC_{30(Ach)}$ level of response to acetylcholine. **Step1**. determination of the concentration of Ach (*) producing 30% relaxation in precontracted control arterial rings. **Step 2** determination of the percent relaxation produced by the same concentration of Ach in SnPP9 treated rings. **Step 3** calculation of the percent inhibition by SnPP9 of relaxation. $(30-X)x100/30=$% inhibition of relaxation at $IC_{30(Ach}$.

Of note, SnPP9 (100 μM) demonstrated greater inhibition of endothelium-dependent relaxation (54.9 $\pm$ 15.5 %) when the effects were measured at lower concentrations of acetylcholine i.e. SnPP9 (100 μM) inhibited the submaximal acetylcholine evoked relaxation but had no significant effect on maximal relaxation. This suggests there may be an effect of HO-2 at lower concentrations of acetylcholine and lower concentrations of endothelium-derived NO not obvious when NO concentrations increase. Alternatively, higher concentration of porphyrins may stimulate guanylyl cyclase counteracting their inhibitory effect on HO. While this has not been described with SnPP9 it can be observed with PP9 (Ignarro et al. 1984).

The close correlation between the inhibitory effect of SnPP9 in enzyme assay and vascular reactivity studies suggests SnPP9 was indeed acting via inhibition of HO, further supporting a role for HO in endothelium-derived relaxation.

1.1.6. Inhibition of soluble guanylyl cyclase and NO-independent relaxation.

In order to confirm the presence of a soluble guanylyl cyclase-dependent component to L-NAME-resistant relaxation in the porcine pulmonary artery, the inhibitory effect of the specific sGC antagonist, ODQ, was evaluated. Arterial rings were precontracted with phenylephrine (EC_{50KCl}) and concentration response curves to acetylcholine were generated in the presence or absence of ODQ (10 μM). The maximum relaxation to acetylcholine in controls, $57.6 \pm 7.4\%$, was inhibited by ODQ, to $34.2 \pm 2.2\%$ (n=4; $p<0.05$). These results confirm that a soluble guanylyl cyclase activator is involved in L-NAME-resistant relaxation in the porcine pulmonary artery. The similar degree of inhibition of NO-independent relaxation demonstrated with SnPP9 further supports the hypothesis that the mediator is CO (Figure 12).

1.1.7. cGMP levels and NO-independent responses.

To further explore the mechanism of action for HO in endothelium-dependent relaxation cGMP levels were determined in the porcine pulmonary artery. Levels were measured in endothelium-intact and denuded arterial rings in the presence of L-NAME under baseline conditions and following acetylcholine.

Following incubation of endothelium-intact arterial rings with SnPP9 (30μM), cGMP levels were reduced by 23.9 %, $p < 0.05$ (n=6). Removal of the endothelium reduced baseline cGMP by 42.9 % (n=3). This reduction in cGMP levels by SnPP9 and

removal of the endothelium are in keeping with the observation from that SnPP9 at 30μM should reduce HO-2 activity by between 40 and 50 % (Figure 11). Meanwhile, SnPP9 had no effect on endothelium-denuded rings (Table 4a).

Table 4a. Effect of SnPP9 and endothelial removal on smooth muscle cGMP in the presence of L-NAME

% Change in cGMP from Control Paired Treatment	fmol/gr	%Change	n	P Value
Endothelium -intact	5.5 ± 1.7	-23.9%	(n=6)	<0.05*
Endothelium-intact and SnPP9	4.2 ± 1.2			
Endothelium-intact	6.3 ± 1.8	-42.9%	(n=3)	<0.05*
Endothelium-removed	3.6 ± 0.9			
Endothelium-removed	6.1 ± 0.2	+6.5%	(n=4)	>0.05NS
Endothelium-removed and SnPP9	6.5 ± 1.9			

Table 4b. Effect of acetylcholine on cGMP levels in the presence of L-NAME

cGMP levels following Acetylcholine (1 μM) Paired Treatment	fmol/gr	%Change	n	P Value
Endothelium-intact no acetylcholine	5.7 ± 1.3	2%	(n=5)	>0.05NS
Endothelium-intact 15 sec	5.8 ± 1.4			
Endothelium-intact no acetylcholine	4.0 ± 1.4	38.2%	(n=6)	>0.05NS
Endothelium-intact 30 sec	5.6 ± 1.9			

cGMP levels were also determined in endothelium-intact small porcine pulmonary artery rings at timed intervals following acetylcholine (1μM). Following acetylcholine (1μM), cGMP levels were not significantly elevated from control levels at 15 and 30 seconds (Table 4b). These results suggest that basal CO release by endothelial HO-2

may contribute to cGMP levels in vascular smooth muscle but that levels do not increase following endothelial activation with acetylcholine.

1.2 Regional differences in endothelium-dependent relaxation in the porcine pulmonary artery.

The results outlined above suggest that HO-2 may play a role in endothelium-dependent relaxation in small porcine pulmonary arteries. However, large porcine pulmonary arteries do not have as pronounced a response to acetylcholine as smaller vessels (Kovitz et al. 1993; Zellers and Vanhoutte 1989). We therefore sought to explore the mechanisms responsible for this difference in vascular reactivity along the porcine pulmonary artery and to determine whether HO-2 and CO might contribute to the observed heterogeneity.

1.2.1. Heterogeneity in response to acetylcholine along the pulmonary artery.

Concentration response curve to acetylcholine (1nM-1μM) were performed on arterial rings from both the proximal and distal porcine pulmonary artery pre-contracted with PE to EC_{50KCl}. The maximum relaxation of proximal arterial rings to acetylcholine was $20.6 \pm 9.2\%$ (n=3) (Figure 13) compared with $95.1 \pm 2.4\%$ (n=10) in distal rings (Figure 2). In the presence of the nitric oxide synthase inhibitor L-NAME (3×10^{-5}M) the proximal endothelium-dependent relaxation ($20.6 \pm 9.2\%$) was abolished and arterial rings contracted by $4.5 \pm 5.5\%$ (n=3)(Figure 13). However, in distal rings, L-NAME uncovered a significant NO-independent relaxation, with arterial rings relaxing by $49.4 \pm 3.6\%$ ($p < 0.05$) (n=16). Endothelial removal abolished relaxation to acetylcholine (acetylcholine 1 μM) and converted the response to

contraction, with proximal arterial rings contracting by 9.3 ± 5.3 % (n=7) and distal arterial rings contracting by 11.3 ± 2.6%(n=6) (Figure 14 and 15).

Therefore, while endothelium-dependent relaxation evoked by acetylcholine was entirely NO-dependent in the proximal porcine pulmonary artery, distal rings had a significant NO-independent response. The heterogeneity in responses to acetylcholine along the porcine pulmonary artery may be characterized by differences in endothelial cell activation, production of endothelium-derived mediators or the responsiveness of the underlying smooth muscle. In the previous section we saw how NO-independent and cyclooxygenase-independent responses were present in the distal pulmonary artery. Using immunohistochemical analysis of the expression of ecNOS and HO-2 along the pulmonary artery, measuring intracellular calcium responses to endothelial cell activation and by determining the response of vascular smooth muscle to the NO donor, SIN-1, and CO along the pulmonary artery we explored the mechanisms responsible for the observed heterogeneity and sought to determine whether HO-2 and CO might contribute to the observed differences along the pulmonary artery.

1.2.2. Nitric oxide synthase and HO-2 expression along the pulmonary artery.

Immunohistochemical analysis of proximal and distal pulmonary artery sections using a polyclonal antibody for the enzyme endothelial nitric oxide synthase (ecNOS) demonstrated distinct staining along the vascular endothelium. Staining was more prominent in the proximal than distal pulmonary artery. The image depicted is representative of that obtained in experiments from three separate animals. The staining was completed only on porcine pulmonary artery segments. Preabsorption of

the antiserum with immunizing peptide abolished immunoreactivity (Figure 16; B and D).

Immunohistochemical analysis of pulmonary artery sections using a polyclonal antibody to the enzyme, HO-2, revealed similar staining of the endothelium between proximal and distal arterial segments (Schematic 8; Generations 1-3 and 6-8 respectively). (Figure16; A and C). While these described differences in HO-2 and ecNOS staining along the porcine pulmonary artery under similar conditions for the same animal were striking and reproducible, they are qualitative rather than quantitative differences. To further explore the differences would require a western blot analysis and/or densitometry. Isolating sufficient cells from the distal pulmonary artery for western blot analysis proved problematic and was not pursued. We acknowledge the quantitative limitations in our immunohistochemical methods and images.

1.2.3. Exogenous carbon monoxide and nitric oxide donors along the pulmonary artery.

Regional differences in NO-dependent relaxation in the pulmonary artery could result from either heterogeneity in the endothelial cell response to activators or differences in smooth muscle responses to NO. NO primarily mediates smooth muscle relaxation via activation of soluble guanylyl cyclase. SIN-1 is a NO donor that was used to determine the sensitivity of the smooth muscle to NO in the proximal and distal pulmonary artery. Exogenous CO also activates soluble guanylyl cyclase although it is 100 fold less potent than NO (Furchgott and Jothianandan 1991).

Concentration response curves were generated with the NO donor, SIN-1 in both endothelium-intact and denuded proximal and distal pulmonary artery rings in the

presence of L-NAME (3×10^{-5}M) following precontraction with phenylephrine (EC_{50KCl}). There was no significant difference in responsiveness, with the EC_{50KCl} measured at 7.0 ± 0.2 µM, for proximal and 7.0 ± 0.2 µM, ($p > 0.05$; NS) for distal arteries, and maximum relaxation of $101.3 \pm 6.1\%$ and 89.9 ± 0.4 % respectively, ($n=3$)($p > 0.05$;NS)(Figure 17).

In other experiments the effect of exogenous carbon monoxide (25, 50, 100 µM) and the soluble guanylyl cyclase inhibitor ODQ (10µM) were determined. Maximum relaxation in distal arterial rings was $48.8 \pm 5.5\%$, while proximal rings were less responsive relaxing by $28.8 \pm 5\%$ of the phenylephrine precontraction ($p < 0.05$)($n=3$). Following pretreatment with ODQ (10µM) the relaxation to CO was abolished in both distal and proximal rings and resulted in a contraction of $7.7 \pm 1.9\%$ and $2.7 \pm 2.7\%$ respectively. Endothelial cell removal did not affect responses to CO (Figure 18 and 19).

1.3 Summary

We demonstrated significant heterogeneity in the response to acetylcholine along the porcine pulmonary artery. In proximal arterial segments the response to acetylcholine is smaller than distal segments and entirely inhibited by L-NAME. Nevertheless, the smooth muscle response to the NO-donor SIN-1 is similar in proximal and distal segments. While this would initially suggest that less NO is released by proximal pulmonary artery endothelial cells in response to acetylcholine, we demonstrated increased NOS expression on immunohistochemical staining in the proximal artery. These results would therefore imply a difference in signaling and release of NO between proximal and distal segments in response to endothelial activators such as acetylcholine. The distal pulmonary artery on the other hand has a

greater response to acetylcholine and a significant portion is not mediated by either NO or a cyclooxygenase product. Using a selective inhibitor, we determined that the enzyme HO-2 and presumably its product CO, may play a role in endothelium-dependent relaxation in the distal porcine pulmonary artery. While HO-2 is present in both proximal and distal pulmonary artery endothelial cells, it does not appear to have any significant impact on relaxation to acetylcholine in the proximal segments. Indeed, the proximal pulmonary artery appears to be less responsive to exogenous CO than distal segments. Finally, in the presence of L-NAME, acetylcholine did not increase cGMP levels in the distal pulmonary artery smooth muscle, therefore, the mechanism by which HO-2 influences endothelium-dependent relaxation remains to be elucidated.

2. HYPOXIC PULMONARY VASOCONSTRICTION.

Rationale: The mechanisms responsible for hypoxic pulmonary vasoconstriction (HPV) are complex and controversial. The proximal porcine pulmonary artery has an contractile response to hypoxia that is endothelium-dependent and not mediated by either inhibition of NO or a cyclooxygenase product (Kovitz et al. 1993). The proximal porcine pulmonary artery therefore provides a useful model to explore endothelial:smooth muscle interaction during hypoxia and to attempt to discover the nature of the endothelium-dependent contracting factor (EDCF).

2.1 The hypoxic response in the proximal porcine pulmonary artery.

During a contractile response to phenylephrine, moderate hypoxia (10% O_2) caused graded relaxations in proximal arteries that were similar in rings with and without endothelium. A further reduction in oxygen tension (4% O_2) caused transient contractions in rings with endothelium (equivalent to $17.1 \pm 12\%$ of the normoxic

contraction to PE, n=7) followed by graded relaxation that was similar to that observed in rings without endothelium. In rings with endothelium, more severe hypoxia (0% O_2) caused an initial transient contraction (equivalent to $21.7 \pm 7.6\%$ of the normoxic contraction to PE, n=7), followed by a slowly developing contractile response (equivalent to $91.7 \pm 11\%$ of the contraction to PE, n=7). In endothelium-denuded rings, anoxia (0% O_2) did not cause any significant change in tension (Figure 20).

2.2 Endothelin in the proximal pulmonary artery and its role in the hypoxic response.

Endothelin is an endothelium-derived contractile factor that may be released in response to hypoxia. ET produces a characteristic slowly developing contraction not unlike that observed following hypoxia in the proximal porcine pulmonary artery. Prior to evaluating the effect of endothelin receptor antagonists on the endothelium-dependent hypoxic response, endothelin responses were characterized in the proximal pulmonary artery.

2.2.1. Endothelin-1 concentration response in proximal pulmonary arteries.

Under quiescent conditions, endothelin-1 caused concentration-dependent contraction of pulmonary arterial rings with endothelium. Endothelium removal increased the response to endothelin-1, causing an upward shift in the concentration-effect curve (Figure 21 and 22). The response to endothelin-1 was inhibited by the ET_A receptor antagonist BQ 123 ($10^{-6}M$) which caused a rightward shift of the concentration-effect curve ($p<0.05$)(n=5), with no change in the maximum response ($p>0.05$; NS)(n=5), in rings with and without endothelium (Figure 21 and 22). In endothelium-denuded rings, contractions to endothelin-1 were not significantly influenced by the ET_B antagonist BQ 788 ($3 \times 10^{-7}M$), either in the presence or absence

of BQ 123 (10^{-6}M) (p>0.05; NS)(n=5)(Figure 21 and 22). However, in endothelium-intact arterial rings, the addition of BQ 788 (3×10^{-7}M) significantly enhanced the response to endothelin both in the presence and absence of BQ 123 (10^{-6}M) (p<0.05)(n=5)(Figure 21).

2.2.2. Endothelium-dependent relaxation and endothelin-1.

In order to determine the role of endothelin in proximal pulmonary artery relaxation, arterial rings with endothelium were contracted with phenylephrine ($EC_{50(KCl)}$). Endothelin-1 (3×10^{-9}M) caused a biphasic response consisting of an initial relaxation followed by a sustained contraction. The relaxation was abolished by the ET_B receptor antagonist, BQ 788 (3×10^{-7}M) or by removal of the endothelium (p<0.05)(n=3)(Figure 23). The contraction to endothelin was increased by BQ 788 or endothelium removal and was abolished by the ET_A receptor antagonist BQ 123 (10^{-6}M)(p<0.05)(n=3). BQ 788 (3×10^{-7}M) had no effect on the response to endothelin in endothelium-denuded rings.

2.2.3. Calculation of the -Kb for BQ 123.

BQ 123 caused a non-parallel shift in the concentration-effect curve, being more potent at low compared to high levels of tension. For example, in endothelium-denuded rings BQ 123 caused a significantly greater shift in the curve at the EC_{30KCl} level compared to the EC_{100KCl} level of tension. The concentration ratio (CR) was 1.3 ± 0.2 (20-fold shift) and 0.55 ± 0.14 (3.5-fold shift), for the EC_{30KCl} and EC_{100KCl} , respectively n=5, p<0.05. The EC_{30KCl} and EC_{100KCl} levels are equivalent to approximately 20% and 70% of the

maximal response to endothelin-1. The $-\log K_B$ for BQ 123 calculated at the EC_{50KCl} level was 6.99 ± 0.17 (n=5) (endothelium-denuded rings).

2.2.4. Effect of an ET_A and ET_B receptor antagonist on hypoxic contraction.

During a contraction to phenylephrine (EC_{50KCl}), moderate hypoxia (10%-4% O_2) caused graded relaxation that was similar in rings with and without endothelium. Severe hypoxia (0% O_2) caused a transient increase, followed by a slowly developing and sustained increase in tension in endothelium-containing rings but no significant change in tension in denuded rings (Figure 20). The endothelin receptor antagonists BQ 123 (10^{-6}M) and/or BQ 788 (3×10^{-7}M) did not affect the vascular responses to moderate or severe hypoxia (Figure 24a,b,c).

2.2.5. Activity of endothelin-1 (10^{-8}M) and the ET_A receptor antagonist BQ 123 (10^{-6} M) during hypoxia.

To determine whether severe hypoxia (0% O_2) had any effect on the activity of endothelin-1 and the ET_A antagonist, BQ 123, endothelin-1 (10^{-8}M) was given to precontracted endothelium-denuded arterial rings during severe hypoxia (0% O_2) in the presence and absence of BQ 123 (10^{-6}M). Endothelin-1 (10^{-8}M), given during the plateau phase of the hypoxic relaxation produced a contraction ($138.5 \pm 11.5\%$, n=2) that was similar in magnitude to that observed during normoxia ($129.5 \pm 14.5\%$ n=4). Administration of the ET_A receptor antagonist BQ 123 (10^{-6}M) 30 minutes prior to beginning the PE contraction, inhibited ET-1 (10^{-8}M) given during the plateau phase of the hypoxic relaxation (0% O_2)(Graded 16, 10, 4, 0% protocol). Contraction to during hypoxia was similar ($71.7 \pm 11.1\%$ (n=2)) to that seen during normoxia ($76.1 \pm 21.5\%$) (n=4)(No Figure)

2.3 Demonstration of a novel EDCF in the porcine pulmonary artery

The cardiac valve leaflet, is a rich source of endothelial cells. Structurally, it consists of a layer of fibrous tissue lined on either side by a single layer of endothelium. Aside from the endothelium, valve leaflets demonstrate occasional fibroblast cells on histological examination. The valve leaflet can be used to evaluate endothelial function and has the advantage of being a native intact layer of cells unlike cultured endothelium. We developed a unique method to evaluate endothelial:smooth muscle cell interaction. By being able to remove and restore the endothelium to arterial rings in organ chambers the respective roles of the endothelium and smooth muscle in the control of vascular tone could be explored.

2.3.1. Demonstration of EDRF activity in pulmonary valve.

During contraction to $ED_{(50KCl)}$ with phenylephrine, bradykinin $(3x10^{-7}M)$ caused relaxation of endothelium-containing rings but no change in tension of endothelium-denuded arterial rings. The placement of a pulmonary valve leaflets, which is a rich source of endothelial cells, into the lumen of the endothelium-denuded arterial ring restored a vasodilator response to bradykinin (n=3) (Representative tracing; Figure 25). This suggests that the pulmonary valve leaflet is a suitable source of fresh endothelium for analyzing production of endothelium-derived mediators. However the endothelium-dependent relaxation observed is relatively small; this might either indicate that the production of EDRF from the valve endothelium is low or that the preparation does not allow for the ideal transfer of a labile EDRF such as NO.

2.3.2. Effect of a pulmonary valve leaflet on endothelium-denuded hypoxic responses.

In endothelium intact proximal pulmonary artery rings, *abrupt exposure* to anoxia (16 to 0% O_2) caused a transient contraction followed by a slowly developing

sustained contraction similar to that observed following graded exposure to hypoxia (16, 10, 4 and 0% O_2) However, in endothelium-denuded arterial rings, abrupt hypoxia caused relaxation of phenylephrine-induced contraction. The presence of a pulmonary valve leaflet in endothelium-denuded rings converted the hypoxic (0% O_2) relaxation into a slowly developing sustained contractile response (Representative tracings. Figure 27). This response was similar to the endothelium-dependent response observed in pulmonary arteries with native endothelial cells. These experiments were replectated in five separate animals.

The effect of the valve leaflet on *graded hypoxia* was also evaluated. During a contractile response to phenylephrine, moderate hypoxia (10% and 4% O_2) caused transient contractions in proximal arteries with endothelium that were followed by relaxation. Moderate hypoxia (10% and 4% O_2) produced relaxation without the initial transient contraction in endothelium-denuded rings containing the valve leaflets. In rings without endothelium or valve leaflets, moderate hypoxia caused relaxation that was greater in magnitude to that observed in rings with either endothelium or valve leaflets.

As oxygen tension was further decreased, severe hypoxia (0% O_2) caused an initial contraction that was followed by a slowly developing contractile response in rings with endothelium and in endothelium-denuded rings following pulmonary valve leaflet placement. However, in endothelium-denuded rings, severe hypoxia (0% O_2) did not cause any significant further change in tension (Figure 28).

The graded hypoxia protocol was also completed in the presence of indomethacin (10^{-5}M) and L-NAME (3×10^{-5}M), to inhibit cyclooxygenase and NOS respectively. The initial transient contraction on exposure to hypoxia (10%, 4% and 0% O_2) seen in endothelium-intact rings in the absence of L-NAME or indomethacin

was abolished. The magnitude of late phase hypoxic (0% O_2) contraction was the same in endothelium-denuded arterial rings containing the valve leaflet as that observed in rings with native endothelium. (Figure 29). These results demonstrate that the valve leaflet produces endothelium-dependent contraction through the release of contractile mediators rather than through the inhibition of vasodilator mediators.

2.3.3. Effect of pulmonary valve endothelium on systemic vasculature hypoxic responses.

During a contractile response to phenylephrine, porcine iliac arteries with and without endothelium, completely relaxed upon abrupt exposure to severe hypoxia (0% O_2) in the presence of L-NAME and indomethacin, $103 \pm 0.3\%$. Placement of a pulmonary valve leaflet in endothelium-denuded porcine iliac arterial rings resulted in a significant decrease in the hypoxic relaxation to $46.8 \pm 16.5\%$ ($p<0.05$)(n=3)(Figure 30).

2.3.4. Effect of the valve leaflet on contractility of the arterial ring to phenylephrine.

The placement of an untreated valve leaflet in the lumen of an endothelium-denuded ring, depressed the response to phenylephrine, causing a parallel rightward shift in the concentration effect curve, without affecting the maximum response. The inhibitory effect of the valve was not influenced by L-NAME + indomethacin or by prior incubation of the valve leaflet in distilled water (4°C for 4 hours) to damage the endothelium (Figure 26). Therefore it appears that the valve has minor stearic effects on contractility.

2.4. Characterization of the hypoxic endothelial contractile factor.

A number of experiments were designed and executed to better define the nature of the diffusible, endothelium-derived hypoxic factor, released by the pulmonary valve endothelium.

2.4.1. Use of a dialysis preparation to determine the molecular weight of the hypoxic factor.

Abrupt exposure to anoxia (0% O_2) caused relaxation of phenylephrine-induced contraction in endothelium-denuded proximal pulmonary artery rings. Following reoxygenation and washout of phenylephrine, a pulmonary valve leaflet was placed in a dialysis bag with a 25K molecular weight cut off (MWCO), and placed into the lumen of an endothelium-denuded ring. The rings were then contracted with phenylephrine (EC_{50KCl}) before again being exposed to hypoxia (0% O_2). The arterial ring containing the valve and dialysis bag failed to contract in response to hypoxia. The valve was then removed, the oxygen returned to baseline (16% O_2) and the phenylephrine washed out over one hour. The valve leaflet was then removed from the dialysis bag and reinserted into the ring lumen. The arterial ring was again contracted with phenylephrine (EC_{50KCl}) and re-exposed to hypoxia (0% O_2). The arterial ring now contracted in response to hypoxia. These results would suggest that in response to hypoxia the valve leaflets produce either a labile substance or a factor with a molecular weight of over 25K that produces smooth muscle contraction (Figure 31).

2.4.2. Activity of effluent from the hypoxic endothelium.

The activity of the effluent obtained from pulmonary valve leaflets under hypoxic (0% oxygen) and normoxic conditions (16% oxygen) was evaluated. In these

experiments, following precontraction with PE, the absence of endothelium was first confirmed by the lack of relaxation to either acetylcholine (10^{-6}M) or bradykinin (10^{-7}M) and by the lack of contraction in response to abrupt hypoxia (16 to 0% O_2 protocol). The phenylephrine was then washed out over one hour and the rings re-contracted with phenylephrine.

After exposing precontracted endothelium-denuded proximal pulmonary artery rings to *hypoxia* (16 to 0% oxygen) for 20 minutes, 1 ml of the effluent obtained during either normoxic or hypoxia exposure of pulmonary valves leaflets was added. Twenty minutes was chosen as the point at which the arterial rings have reached maximal relaxation in response to hypoxia and at which point endothelium-intact proximal pulmonary arterial rings begin the late phase hypoxic contraction. While the normoxic solution had no effect on hypoxic tone, the solution obtained during hypoxia caused a slowly developing contraction that was qualitatively similar to that seen in endothelium-intact rings (effluent obtained from three out of six collections; the other three collections had no effect on tone). Following reoxygenation and phenylephrine washout, the rings were again contracted to the EC_{50KCl} and re-exposed to hypoxia. The endothelium-denuded rings that had contracted in response to the valve effluent solution now relaxed (Figure 32).

During *normoxia* endothelium-denuded arterial rings were contracted to 10% of the KCl maximum with phenylephrine (EC_{10}). The EC_{10KCl} was chosen as the tension most consistent with the tone achieved during hypoxic relaxation by proximal segments (Kovitz et al. 1993). 1 ml of either the normoxic or hypoxic effluent was then added. While the normoxic effluent had no effect on tone, the hypoxic effluent caused a slowly developing contraction (effluent obtained from two out of three collections). The effluent that produced a contraction during normoxia were from the same batch that

produced contraction during hypoxic conditions. Similarly, the effluent with no effect in normoxia had no effect either during hypoxia.

While these results suggest the presence of a stable factor produced by endothelium in response to hypoxia, contraction was not reproduced consistently with all collected effluent. The factors influencing the inconsistency between experiments was not determined (Figure 32).

2.5. The role of calcium in hypoxia mediated endothelium-dependent contractile responses.

While considerable attention has focused on changes in intracellular calcium in pulmonary artery smooth muscle in response to hypoxia (Post et al. 1992; Yuan et al. 1993), little is known about the effects of hypoxia on endothelial cell calcium (Archer and Cowan 1993; Arnould et al. 1992). We evaluated the effects of hypoxia on intracellular calcium using spectrofluorometric analysis of FURA-2AM labeled endothelial cells and by chelation of intracellular calcium in organ chamber experiments.

2.5.1. Effect of hypoxia on intracellular calcium using the fluorescent dye FURA-2AM

Pulmonary valve endothelial cells were loaded with the cell permeant calcium dye, FURA-2AM. Continuous infusion of bradykinin (10^{-6}M) resulted in a biphasic increase in intracellular calcium (n=3) (Figure 33a). Exposure to anoxia (0% oxygen) resulted in a slow rise in fluorescence consistent with a rise in intracellular calcium (n=2). Reoxygenation led to a prompt and complete reversal in the fluorescent ratio (Figure 33b). No conclusions can be drawn from these however, because insufficient experiments were performed.

2.5.2. Effect of chelation of endothelial intracellular calcium on the hypoxic response, using BAPTA-AM.

Pulmonary valve leaflets were pre-incubated for 45 min with the cell permeant chelator, BAPTA-AM (75 μM). The valve leaflets were then washed in warm krebs to remove residual extracellular BAPTA-AM. The valve leaflets were then placed into the lumen of endothelium-denuded arterial rings. The arterial rings were then contracted with phenylephrine and abruptly exposed to hypoxia (16 to 0% O_2). The valve leaflets restored the late-phase slowly developing contraction to hypoxia (0% O_2) and were not significantly different from control valve leaflet responses (n=4) (Figure 34).

2.6 Evaluating the role of heme oxygenase in acute hypoxic responses.

Current opinion favors a heme based oxygen sensor in cells that becomes activated in the absence of oxygen, but returns to relaxed state when bound to oxygen. Indeed, both exogenous and endogenous CO, presumably acting as oxygen mimics and binding to the heme sensor, have been demonstrated to inhibit a number of hypoxia-inducible genes. Furthermore, exogenous CO has also been shown to inhibit hypoxic pulmonary vasoconstriction and carotid body responses to hypoxia. However, whether endogenous CO production by HO modulates in vitro response to hypoxia is not known and was evaluated by determining whether HO inhibition by SnPP9 would effect the endothelium-dependent contraction or endothelium-independent relaxation in response to hypoxia in the proximal porcine pulmonary artery.

2.6.1. Effect of heme oxygenase inhibition on the hypoxic response in pulmonary artery rings.

Proximal pulmonary arterial rings were precontracted with PE (EC_{50KCl}) in the presence and absence of the heme oxygenase inhibitor, SnPP9 (30 μM) and then exposed to graded hypoxia (16% to 10% to 4% and 0% O_2). Control and SnPP9 treated arterial rings demonstrated a similar response, relaxing to hypoxia (10% and 4% O_2) followed by an initial transient contraction and subsequent slowly developing sustained contraction to anoxia (0% O_2) (n=3). Therefore the HO inhibitor SnPP9 had no effect on endothelium-dependent contraction to hypoxia nor on the endothelium-independent relaxation. These results suggest that either HO plays no role in modulating acute hypoxic vasoconstriction or that the heme sensor is not involved in acute hypoxic vasoconstriction.

3. INDUCTION OF VASCULAR HO-1 AND THE EFFECT ON VASCULAR REACTIVITY.

Rationale: It has been proposed that, through its antioxidant properties, HO-1 induction *in vivo* protects rats from the lethal effects of LPS. Alternatively however, increased CO production could play a beneficial role if it were to act as a partial agonist and thereby inhibit the vasodepressor effects of NO produced by increased iNOS expression. On the other hand, HO-1 transcription is also induced by LPS and increased CO production could potentially contribute to the depressed vasoreactivity seen in septic shock. We sought to determine whether the *in vivo* induction of HO-1 by the pretreatment of rats with hemoglobin would (i.) impair vasoconstriction to

phenylephrine (PE) in isolated rat aorta and (ii.) protect against the detrimental effects of LPS on vascular responsiveness *in vitro*.

3.1. Effect of LPS and hemoglobin on KCl evoked contraction.

Rats treated with LPS *in vivo* had a decrease in the maximal vasoconstrictor response of the thoracic aorta to KCl (60 mM) *in vitro* decreasing it from 2.43 ± 0.11 g in control aorta to 1.83 ± 0.16 g (n=4 , p<0.05). Rats treated with hemoglobin *in vivo* had no effect on the response to KCl and it did not alter the effect of LPS on the vasoconstrictor response (Figure 35).

3.2. Effect of LPS and hemoglobin on phenylephrine evoked contraction.

Rats treated with hemoglobin had a significant decrease in the PE_{max} of the thoracic aorta (2.85 ± 0.22 vs 2.07 ± 0.26 g, n=4, p<0.05) without a change in EC_{50} (Table 5, Figure 36). LPS caused a greater reduction in PE_{max} (to 0.86 ± 0.22 g, n=4, p< 0.05) than hemoglobin, also without a change in EC_{50} (Table 5, Figure 36). There was no apparent additional vasodepressor effect of hemoglobin in LPS treated vessels.

3.3. Northern blot analysis of HO-1 in rat aorta following hemoglobin and LPS.

Northern blot analysis demonstrated that intravenous injection of hemoglobin 16 hours prior to sacrifice and of LPS 4 hours prior to sacrifice caused a marked increase in expression of HO-1 mRNA in rat aorta (Figure 37). The blot dipicted is representative of three separate experiments completed on tissue from three separate animals. Similar results were obtained in all three animals. Three separate animals were used for LPS experiments and three for hemoglobin experiments.

3.4. Effect of a heme oxygenase inhibitor on vasodepressor effects of hemoglobin and LPS.

To determine whether the vasodepressor effect of hemoglobin or LPS on the PE concentration response was due to induction of HO, vessels were treated with the HO inhibitor tin protoporphyin (SnPP9 30 µM) 30 minutes before obtaining concentration responses to PE. SnPP9 alone had no effect on the concentration response to PE in control animals (Table 6, Figure 38). However, SnPP9 abolished the vasodepressor effect of hemoglobin suggesting that induction of HO was responsible for the effect of hemoglobin on the PE concentration response (Table 6, Figure 38). The inhibitory effect of LPS on responses to PE were unaffected by SnPP9 suggesting that induction of HO could not explain the effect of LPS on the concentration response to PE.

Table 5. The effect of Hemoglobin and LPS on PE_{max} and PE_{EC50}

VESSEL	PE_{max}	EC_{50}	n
Control	2.85 ± 0.22	-6.91 ± 0.17	4
Hemoglobin	2.07 ± 0.26 *	-6.76 ± 0.20	4
LPS	0.86 ± 0.24 *,/	-6.76 ± 0.10	4
Hemoglobin + LPS	0.45 ± 0.07 *,/	-6.56 ± 0.15	4

(* = $p < 0.05$ vs. control, / = $p < 0.05$ vs. hemoglobin)

Table 6. The effect of SnPP9 on PE_{max} and PE_{EC50}.

VESSEL	PE_{max}	EC_{50}	n
Control	2.27 ± 0.73	-6.85 ± 0.15	4
SnPP9	2.24 ± 0.64	-6.75 ± 0.15	4
Hemoglobin + SnPP9	2.33 ± 0.37	-6.98 ± 0.25	4
LPS + SnPP9	0.21 ± 0.05 *	-6.25 ± 0.14 *	4

(*$p < 0.05$ vs. control)

4. VASCULAR RESPONSES TO LIGHT. A ROLE FOR CO?

Rationale: While the phenomena of light induced relaxation of blood vessels has been known for over thirty years (Furchgott et al. 1961), little is known about the mechanisms involved. The phenomena however has some clinical relevance. Premature infants in neonatal intensive care units have been observed to open their ductus arteriosis in response to light on the chest (Rosenfeld et al. 1986). Furthermore, protoporphyrins are also used clinically in these same neonates to treat the hyperbilirubinemia of prematurity. The combination of light and porphyrins is potentially dangerous. Indeed, protoporphyrins are known to be light sensitizers and have been used clinically to direct cancer therapy (Land 1984). Moreover, light dependent effects of protoporphyrins have been described *in vitro* (Zygmunt et al. 1994) and experiments involving protoporphyrins are performed in the dark on account of their sensitivity to light. We therefore sought to explore the effect of light on vascular tone and to describe the effects in the presence of the porphyrin, SnPP9.

4.1 Effect of light on porphyrin treated arterial rings.

Light dependent effects of SnPP9 were explored in pulmonary artery rings. In the presence of L-NAME ($3x10^{-5}$M), endothelium-denuded arterial rings were treated with SnPP9 (10 µM). In the presence of light more phenylephrine was added to SnPP9 treated rings to achieve the ED_{30KCl}. When the lights were turned off again the SnPP9 treated arterial rings contracted rapidly ($180 \pm 12.4\%$ of the precontraction) while there was no change in tone in control rings ($2.9 \pm 2.6\%$ (n=4)(p>0.05;NS) (Figure 39). When the lights were switched back on the contraction rapidly reversed in SnPP9

treated rings. Light dependent responses with SnPP9 were observed in rings with or without endothelium.

The soluble guanylyl cyclase inhibitor, ODQ (10 μM) completely inhibited the effect of light in SnPP9 (10 μM) treated arterial rings (n=3). The antioxidant superoxide dismutase (SOD) (150 u/ml) had no effect on the response in SnPP9 treated rings (n=3). These results suggest that the porphyrin, SnPP9, when exposed to light can evoke relaxation in arterial rings and that neither the endothelium nor nitric oxide synthase are necessary for the response. The relaxation appears to be mediated by activation of soluble guanylyl cyclase. The nature of the mediator and whether it is similar to the mediator responsible for photorelaxation in the absence of porphyrins is not known.
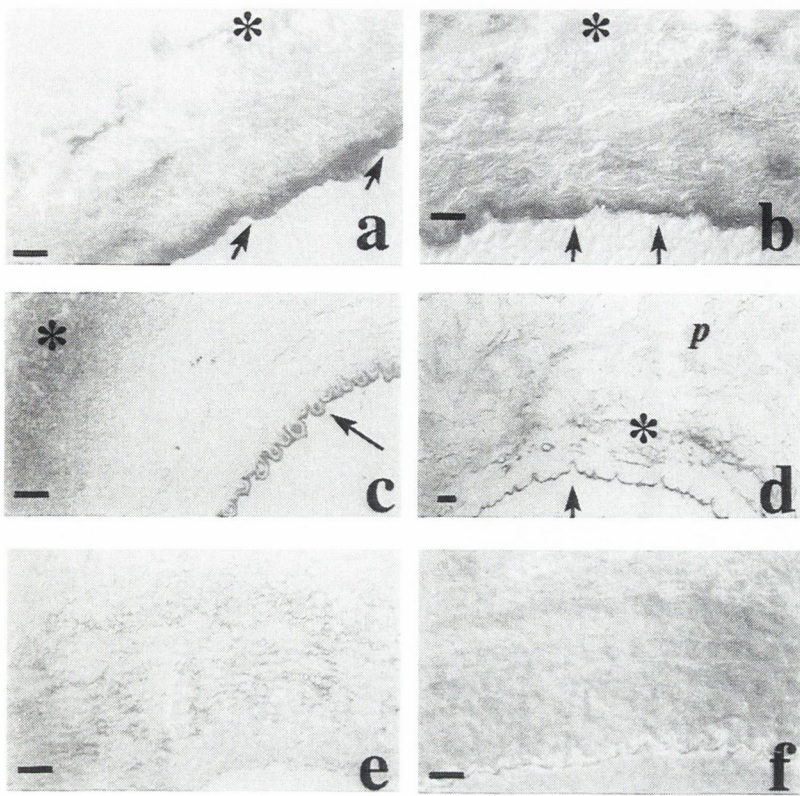
1a. Localization of HO-2 in blood vessels.

Localization of HO-2 in blood vessels using immunohistochemistry as described in the methods. These figures are representative of results from three separate animals. The arrow points to staining along the vascular endothelium facing the lumen; the asterisk denotes the tunica adventitia (Bar = 100 μM). The following porcine arteries were stained with HO-2 antiserum: *a*, distal pulmonary; *b*, proximal pulmonary; *c*, mesenteric; *d*, basilar. Diffuse staining was observed in the smooth muscle of the distal pulmonary artery stained with commercial HO-1 antiserum (*e*). Preabsorption of HO-2 antiserum with the immunizing peptide abolished immunoreactivity (*f*).

B
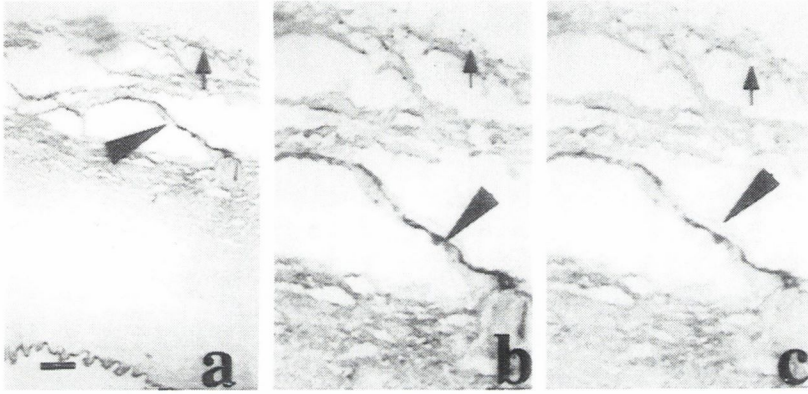
1b. Localization of HO-2 in blood vessels.

Localization of HO-2 to varicose nerve fibers in the adventitial layer of the distal porcine pulmonary artery using immunohistochemistry as described in methods. This figure is representative of results from three separate animals. Arrowheads denote staining of nerve fibers with HO-2 while arrows identify punctate staining of nerve terminals or fibers cut transversely (*a* and *b*). To confirm neuronal staining, a serial section was stained with antiserum generated against neurofilament (*c*) (Bar = 100 µM).
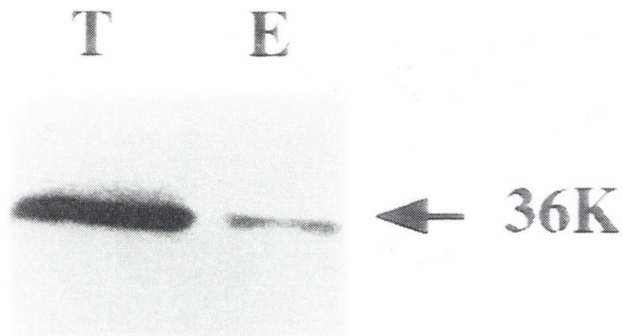
1c. Localization of HO-2 in blood vessels.

Microsomes were prepared from rat testes (T) and porcine aortic endothelial cells (E). This blot is representative of results from three separate animals. Twenty micrograms of total protein was loaded per lane, subjected to SDS/PAGE, and transferred to nitrocellulose. Western blots employed HO-2 antisera at a dilution of 1:1000 and were developed by enhanced chemiluminescence. Preincubation with antigenic peptide abolished immunoreactivity.
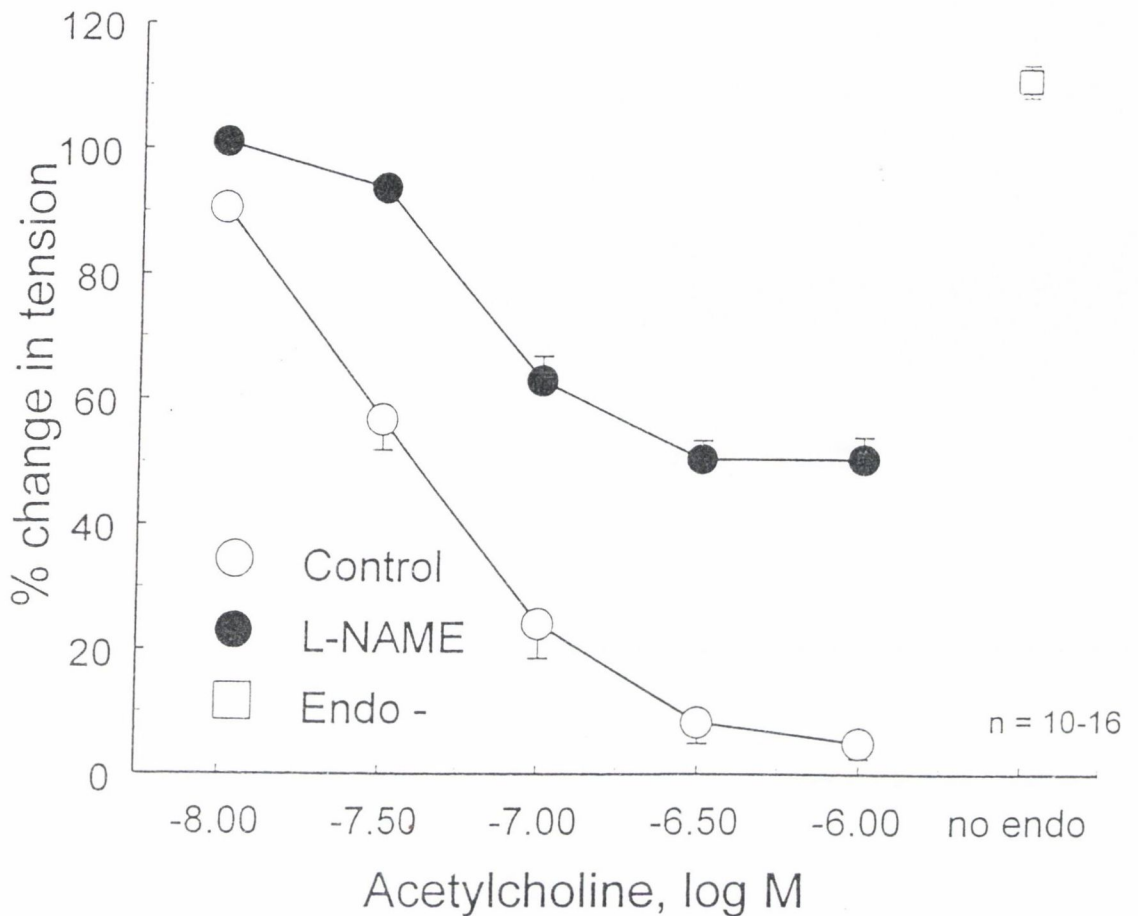
2. NO-dependent and independent responses to acetylcholine in small porcine pulmonary arteries.

Vasodilator response to acetylcholine (10^{-9}-10^{-6} M) in small porcine pulmonary arteries in the presence (•) and absence (○) of L-NAME (3×10^{-5} M) and following endothelial removal (□). The maximum relaxation to acetylcholine in the absence of L-NAME was 95.1 ± 2.4 % (n=16). Endothelial removal abolished relaxation to acetylcholine resulting in contraction, 11.3 ± 2.6% (p<0.05)(n=10). In the presence of L-NAME arterial rings relaxed by 49.4 ± 3.6% (16)(p < 0.05). Results are expressed as a percent change in phenylephrine contraction and are presented as means $\pm$ SE.
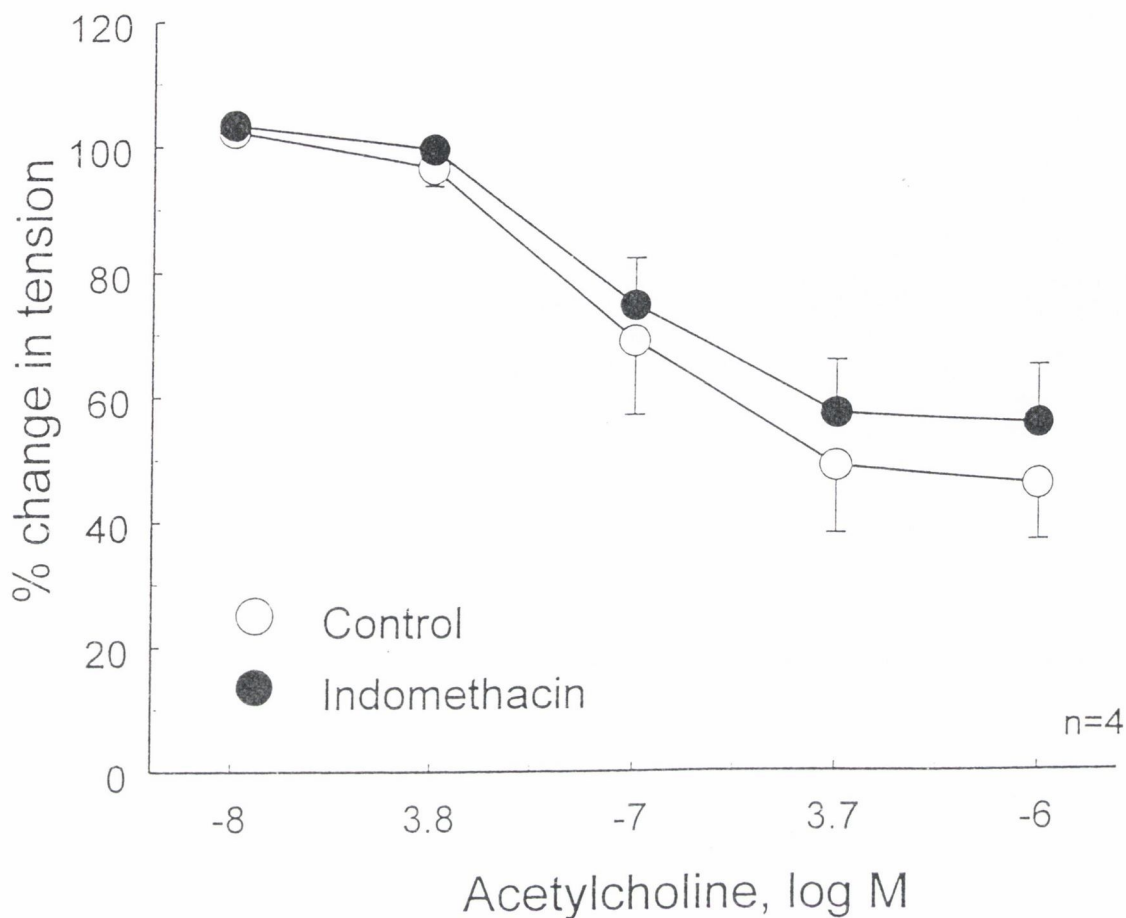
3. Effect of cyclooxygenase inhibition on NO-independent relaxation in small porcine pulmonary arteries.

Vasodilator response to acetylcholine (10^{-9}-10^{-6} M) in small porcine pulmonary arteries pretreated with L-NAME (3×10^{-5}M) in the presence (●) and absence (○) of indomethacin (10^{-5}M). The was no significant difference in the maximum relaxation in L-NAME treated rings, $53.9 \pm 9\%$ versus those treated with L-NAME and indomethacin, $44.1 \pm 9.3\%$ (n=4)(p>0.05;NS). Results are expressed as a percent change in phenylephrine contraction and are presented as means $\pm$ SE.
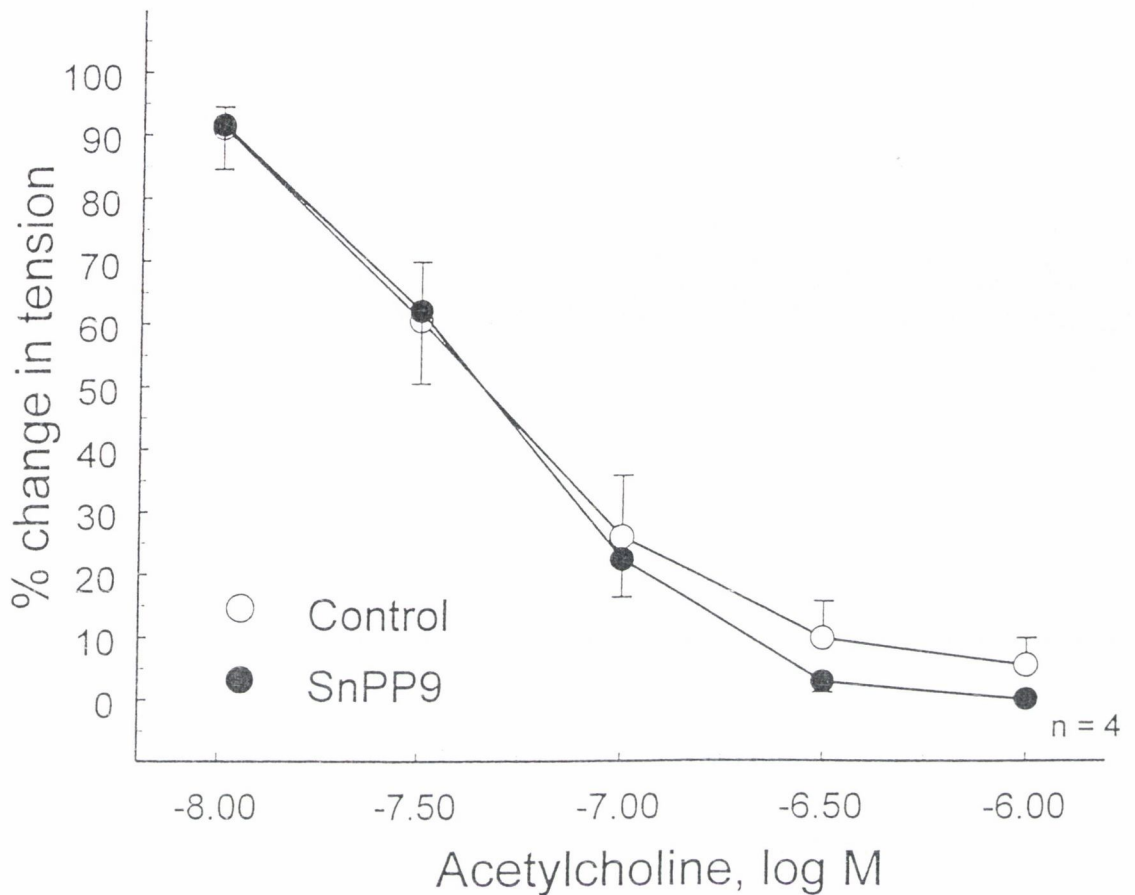
4. Heme oxygenase inhibition (SnPP9 10 μM) and NO-dependent relaxation.

The heme oxygenase inhibitor SnPP9 (10 μM) had no significant effect on vasorelaxation evoked by acetylcholine (10^{-9}-10^{-6} M) in small porcine pulmonary arteries. The maximum relaxation to acetylcholine, was 94.5 ± 4.4% in controls (○), and 100 ± 0% (n=4)(p>0.05;NS) in rings treated with SnPP9 (●). Results are expressed as a percent change in phenylephrine contraction and are presented as means ± SE.
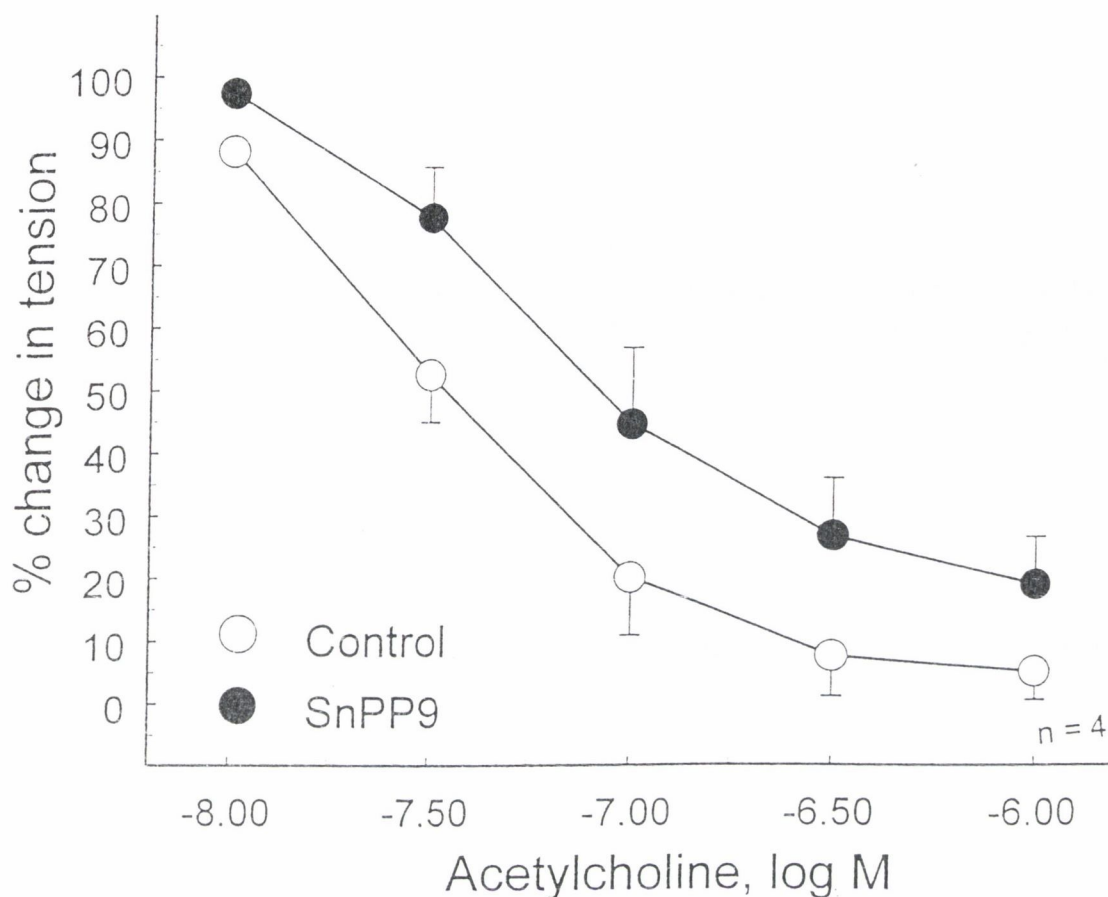
5. Heme oxygenase inhibition (SnPP9 100 μM) and NO-dependent relaxation.

The heme oxygenase inhibitor SnPP9 (100 μM) had no significant effect on the maximum relaxation evoked by acetylcholine (10^{-9}-10^{-6} M) in small porcine pulmonary arteries. The maximum relaxation to acetylcholine, was $94.8 \pm 4.5\%$ in controls (○), and $80.9 \pm 7.7\%$ in the presence of SnPP9 (●)(n=4)(p>0.05;NS). Results are expressed as a percent change in phenylephrine contraction and are presented as means $\pm$ SE.
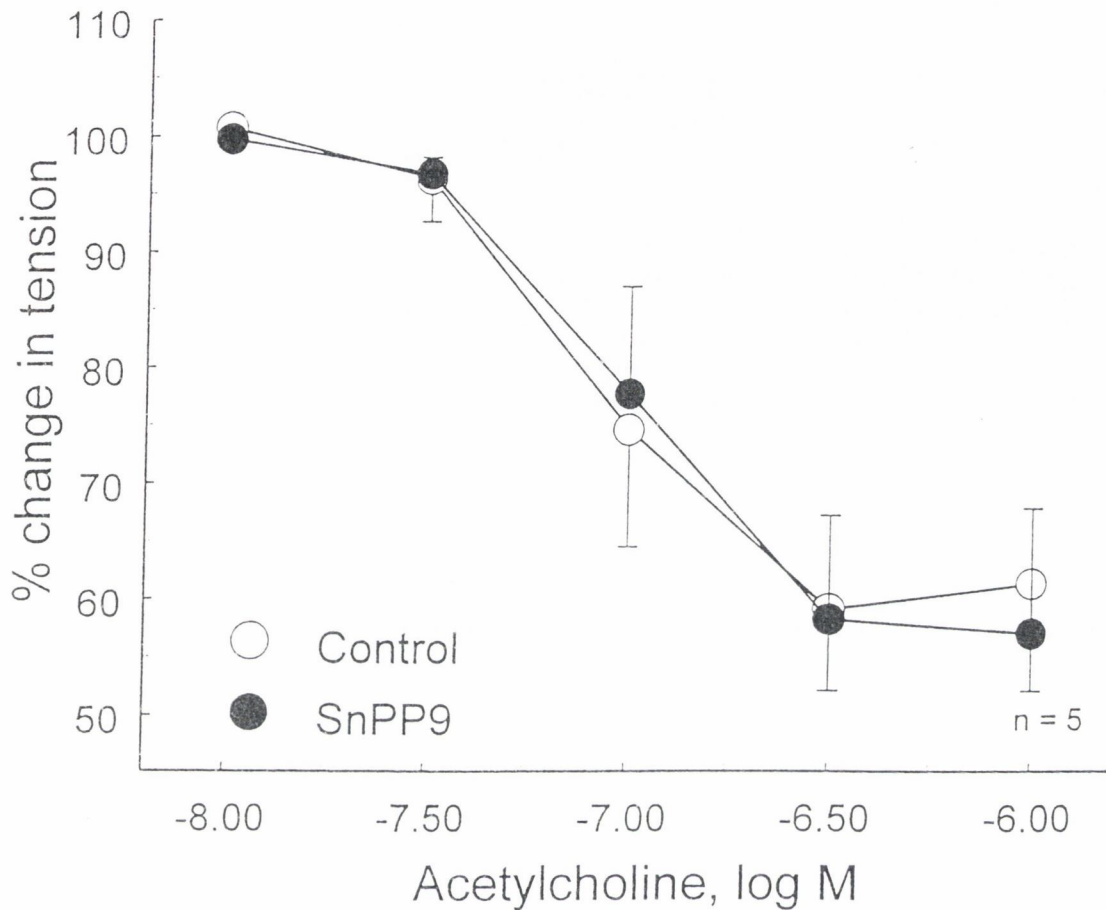
6. Heme oxygenase inhibition (SnPP9 1 μM) and NO-independent relaxation.

The effect of heme oxygenase inhibition on NO-independent (presence of L-NAME 3×10^{-5} M) vasorelaxation evoked by acetylcholine (10^{-9}-10^{-6}M) in small porcine pulmonary arteries. The maximum relaxation to acetylcholine in controls (○), $44 \pm 11\%$, was not significantly different in the presence of SnPP9 (1 μM) (●), $40.9 \pm 6.9\%$ (n=5)(p>0.05;NS). Results are expressed as a percent change in phenylephrine contraction and are presented as means $\pm$ SE.
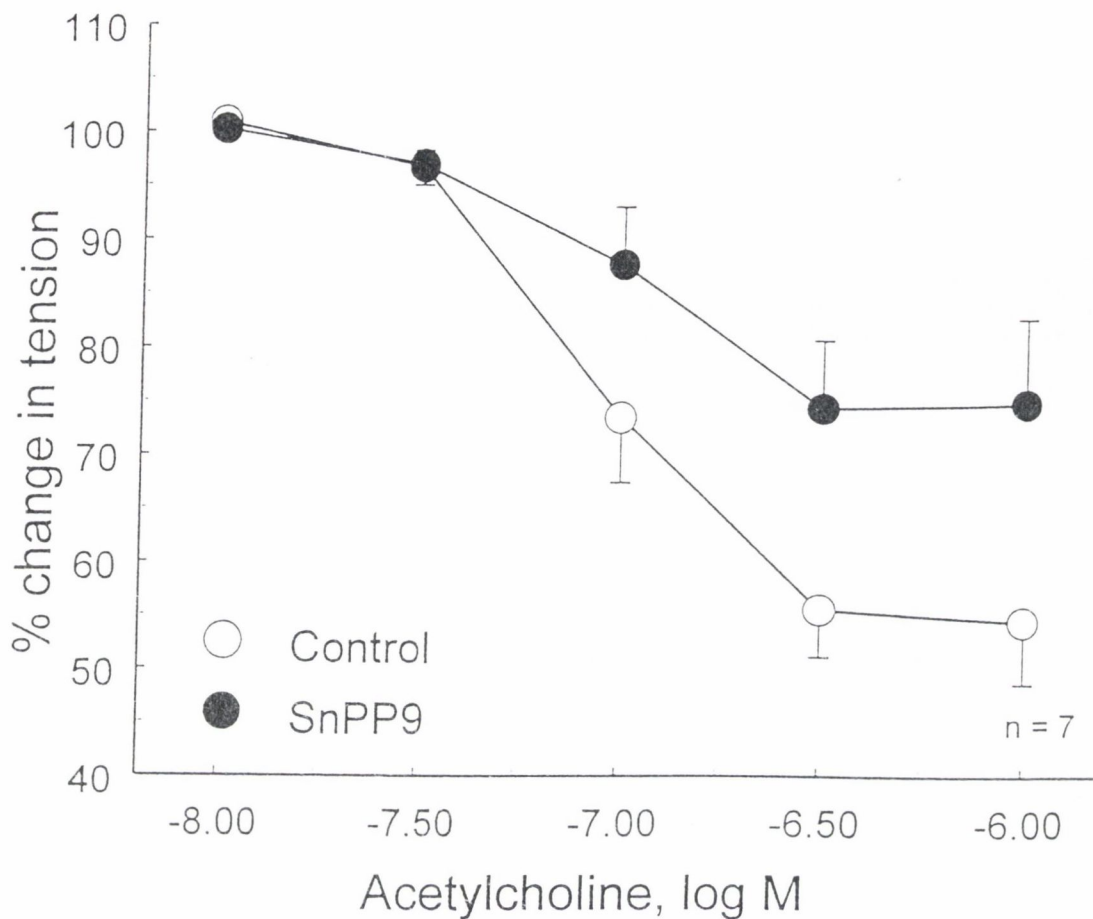
7. Heme oxygenase inhibition (SnPP9 10 μM) and NO-independent relaxation.

The effect of heme oxygenase inhibition on NO-independent (presence of L-NAME 3×10^{-5}M) vasorelaxation evoked by acetylcholine (10^{-9}-10^{-6}M) in small porcine pulmonary arteries. The maximum relaxation to acetylcholine was $45.5 \pm 5.8\%$ in controls (o) versus $25.5 \pm 6.4\%$ in the presence of SnPP9 (10 μM) (●), (n=7)($p < 0.05$). Results are expressed as a percent change in phenylephrine contraction and are presented as means $\pm$ SE.
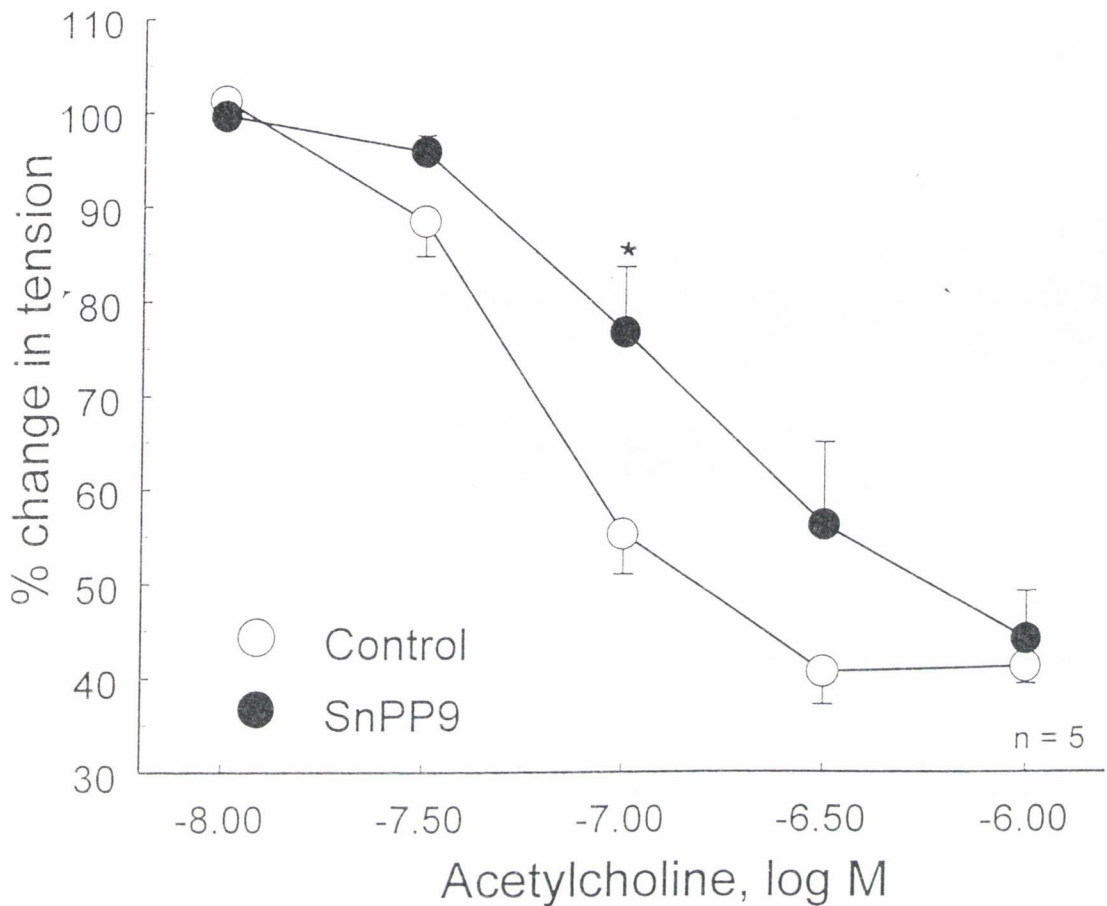
8. Heme oxygenase inhibition (SnPP9 100 μM) and NO-independent relaxation.

The effect of heme oxygenase inhibition on NO-independent (presence of L-NAME 3×10^{-5}M) vasorelaxation evoked by acetylcholine (10^{-9}-10^{-6}M) in small porcine pulmonary arteries. The maximum relaxation to acetylcholine was not different in controls (○), $58.7 \pm 1.8\%$, versus in the presence of SnPP9 (100 μM) (●), $55.9 \pm 5.1\%$(n=5)(p>0.05;NS). However, SnPP9 did produce a rightward shift in the curve. (*) Denotes significantly different from control (p<0.05). Results are expressed as a percent change in phenylephrine contraction and are presented as means $\pm$ SE.
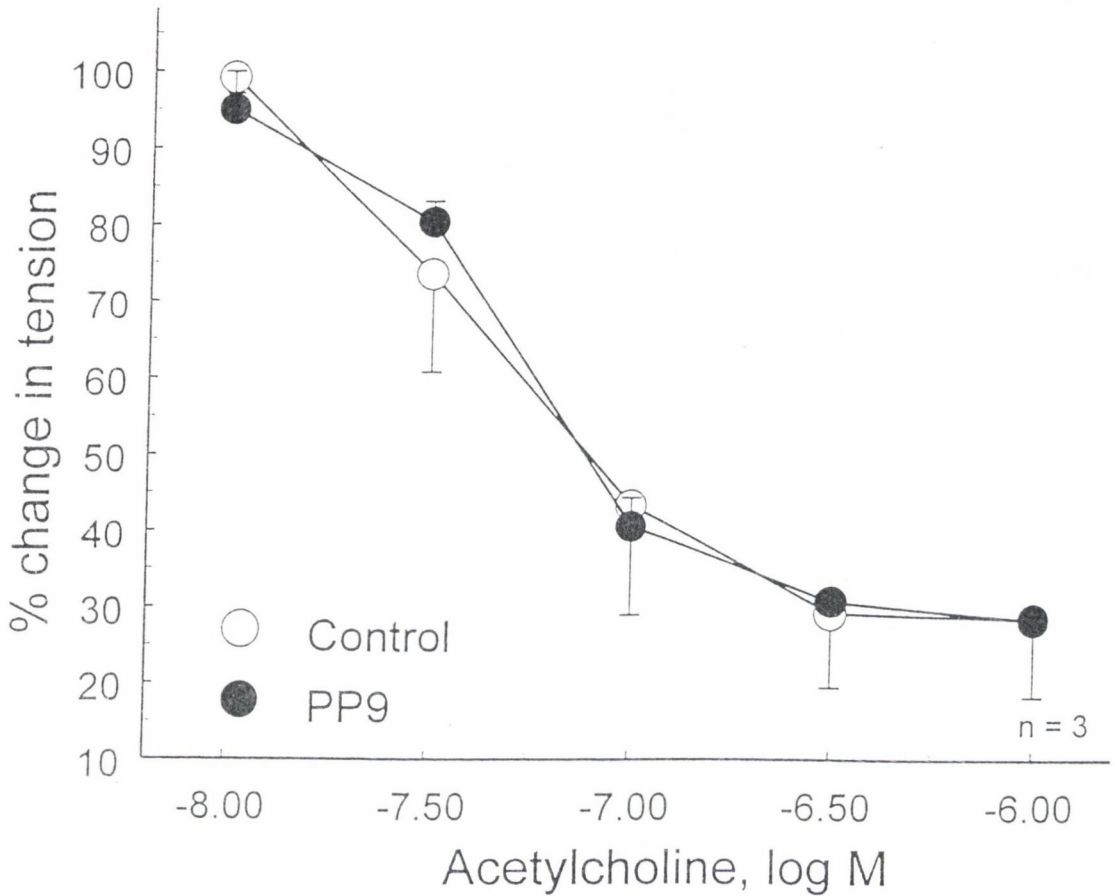
9. Effect of PP9 on NO-independent relaxation.

The effect of the porphyrin, PP9 (10 μM), on NO-independent (presence of L-NAME 3×10^{-5} M) vasorelaxation evoked by acetylcholine (10^{-9}-10^{-6} M) in small porcine pulmonary arteries. The maximum relaxation to acetylcholine was not significantly different in controls (○), $71.5 \pm 10.24\%$, versus in the presence of PP9 (●), $71.7 \pm 1.7\%$ (n=3)(p>0.05;NS). Results are expressed as a percent change phenylephrine contraction and are presented as means $\pm$ SE.
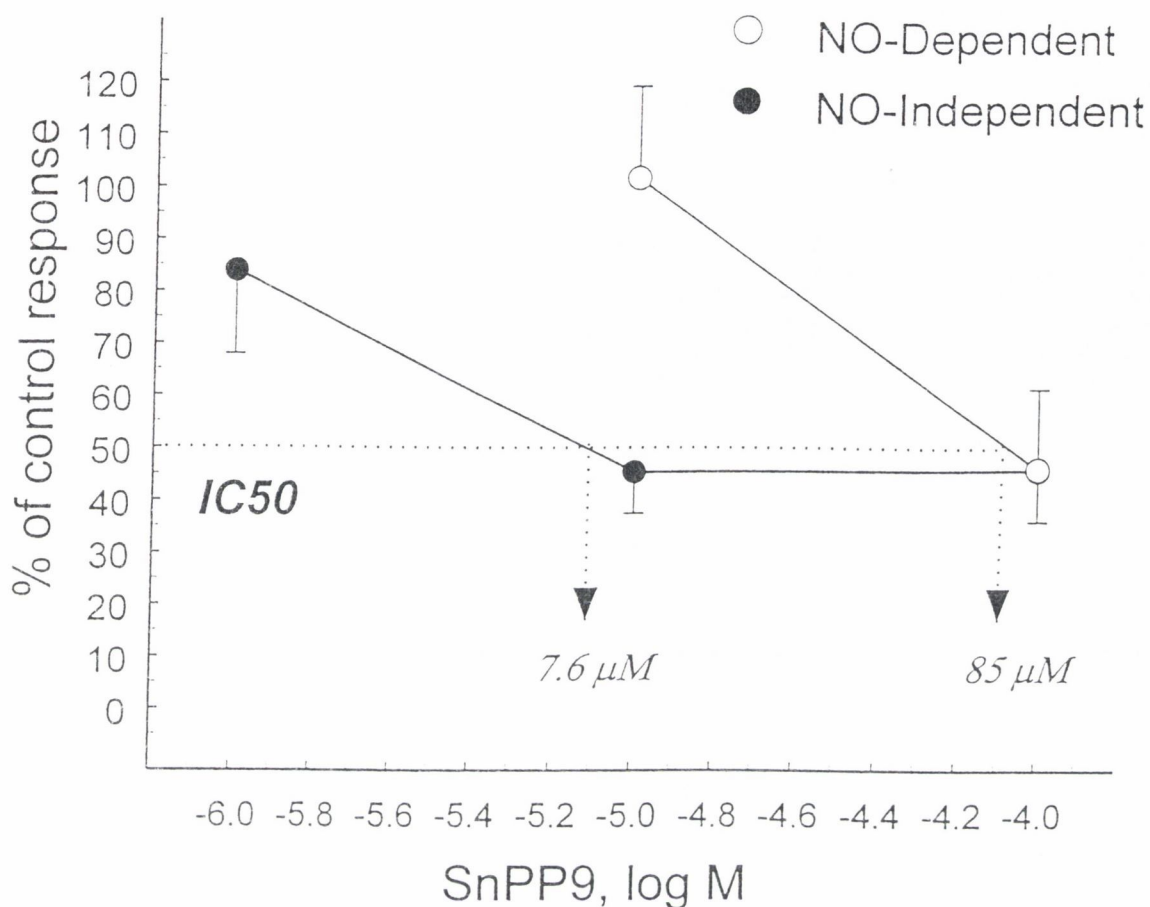
10. Inhibitory effect of SnPP9 on NO-dependent and independent relaxation at the EC_{70} level.

The inhibitory effect of the SnPP9 was determined at the IC_{30} level of response to acetylcholine (the concentration producing 30% relaxation in control arteries and arteries treated with L-NAME), which was interpolated from concentration-response curves. The IC_{50} for SnPP9 calculated for NO-independent and dependent relaxation was $7.6 \pm \mu M$ (n=4) and $85 \pm \mu M$ (n=7) respectively. Results are expressed as means $\pm$ SE.
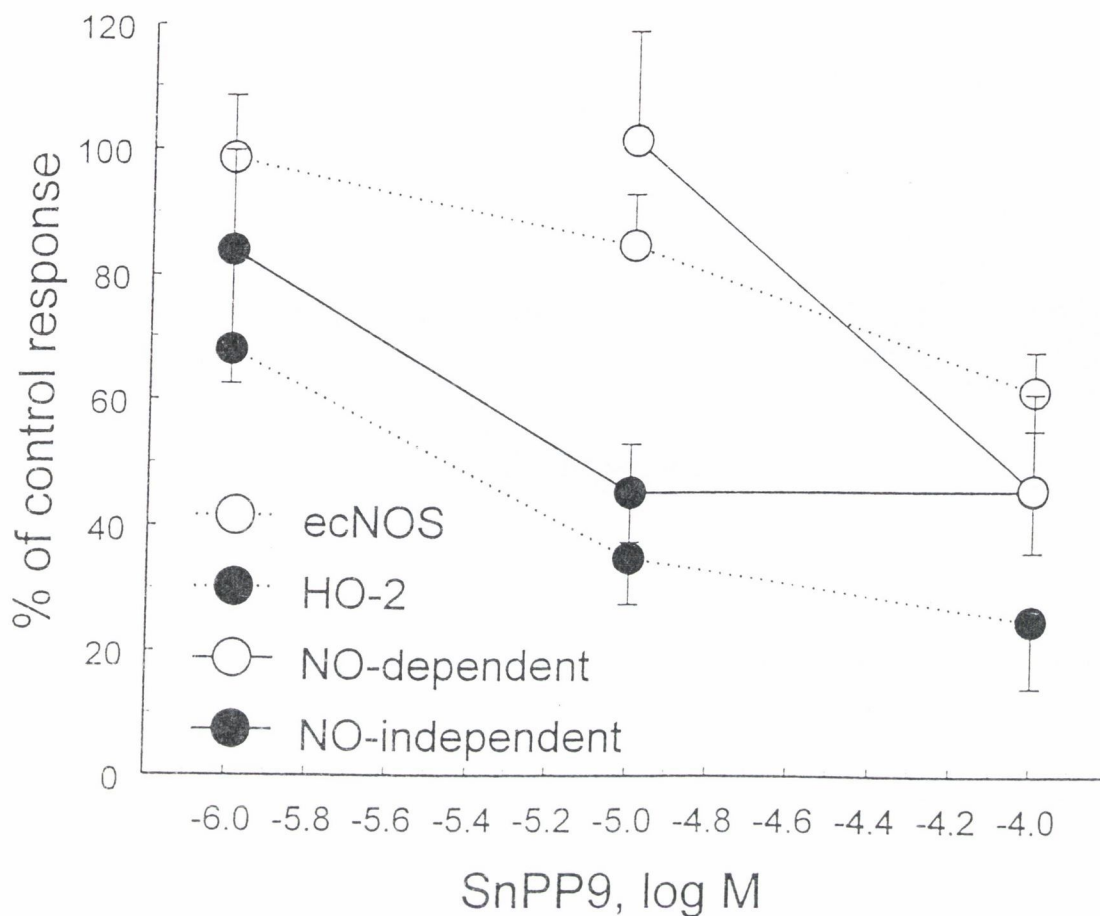
11. Correlation between enzyme assay results and vascular reactivity studies.

A comparison of the concentration-dependent effects of SnPP9 on enzyme assay studies of HO (n=3) and NOS (n=3), and on NO-dependent (n=7) and independent (presence of L-NAME)(n=4) relaxation to acetylcholine in vascular reactivity studies. Results are expressed as means ± SE.
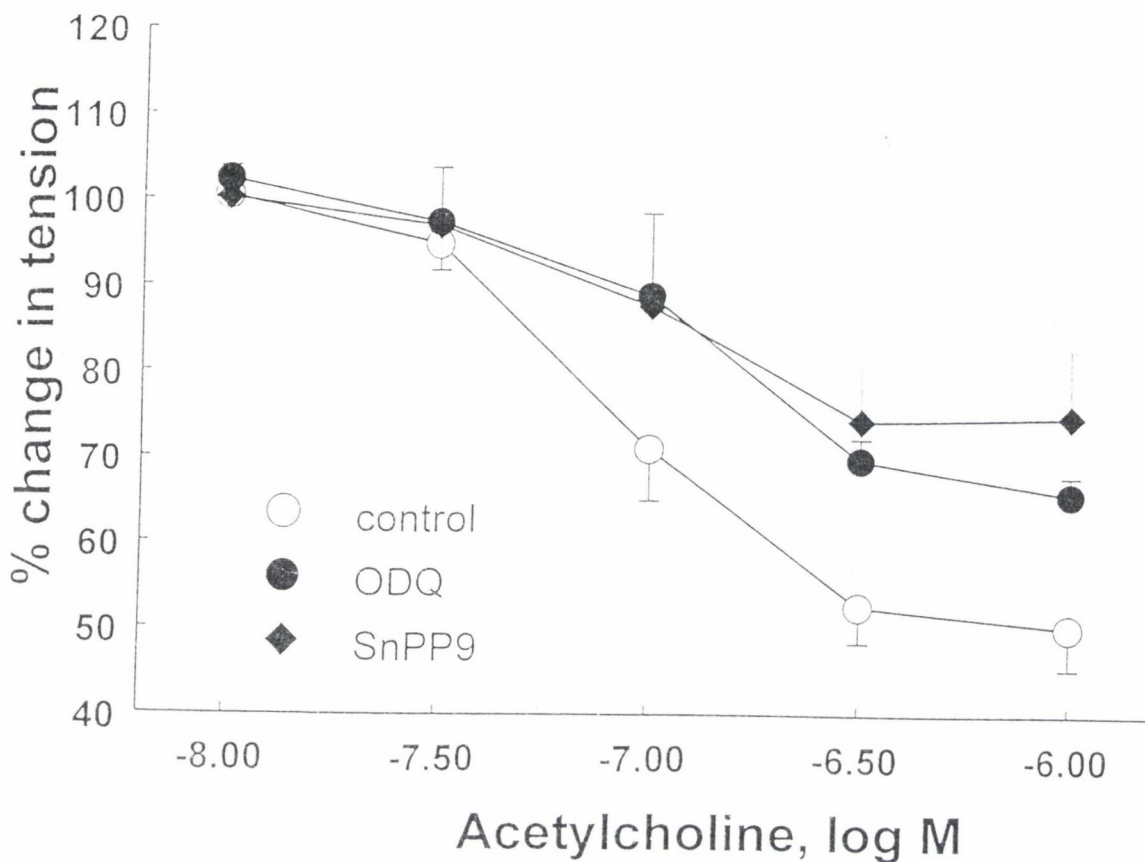
12. Comparison of soluble guanylyl cyclase and heme oxygenase inhibition.

A comparison between the inhibitory effect of SnPP9 (10 μM)(n=7) and the soluble guanylyl cyclase inhibitor, ODQ (10 μM)(n=4) on the vasodilator response to acetylcholine (10^{-9}-10^{-6} M) in small porcine pulmonary arteries pretreated with L-NAME (3×10^{-5}M). Both ODQ and SnPP9 produced similar inhibition of NO-independent relaxation. The control group depicted is a combination of the control groups for the ODQ and SnPP9 experiments (n=11). Results are expressed as a percent change in phenylephrine contraction and are presented as means $\pm$ SE.
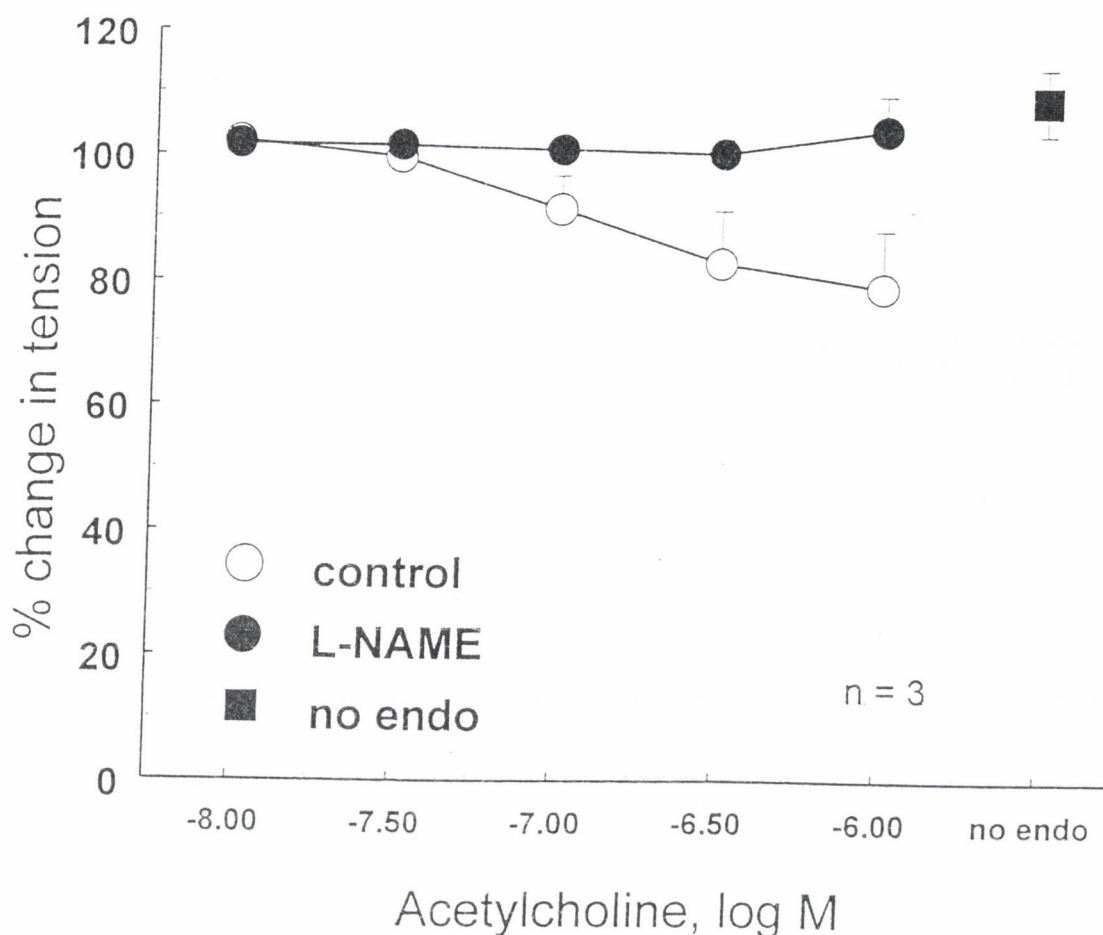
13. NO-dependent and independent responses to acetylcholine in proximal porcine pulmonary artery.

Vasodilator response to acetylcholine (10^{-9}-10^{-6} M) in proximal porcine pulmonary arteries in the presence (●) and absence (○) of L-NAME (3×10^{-5} M) and following endothelial removal (■). The maximum relaxation to acetylcholine in the absence of L-NAME was $20.6 \pm 9.2\%$ (n=3). Endothelial removal abolished relaxation to acetylcholine resulting in contraction, $9.3 \pm 5.3\%$ (n=3)(p<0.05). Endothelium-dependent relaxation was abolished in the presence of L-NAME resulting in contraction, $4.5 \pm 5.5\%$ (n=3)(p<0.05). Results are expressed as a percent change in phenylephrine contraction and are presented as means $\pm$ SE.
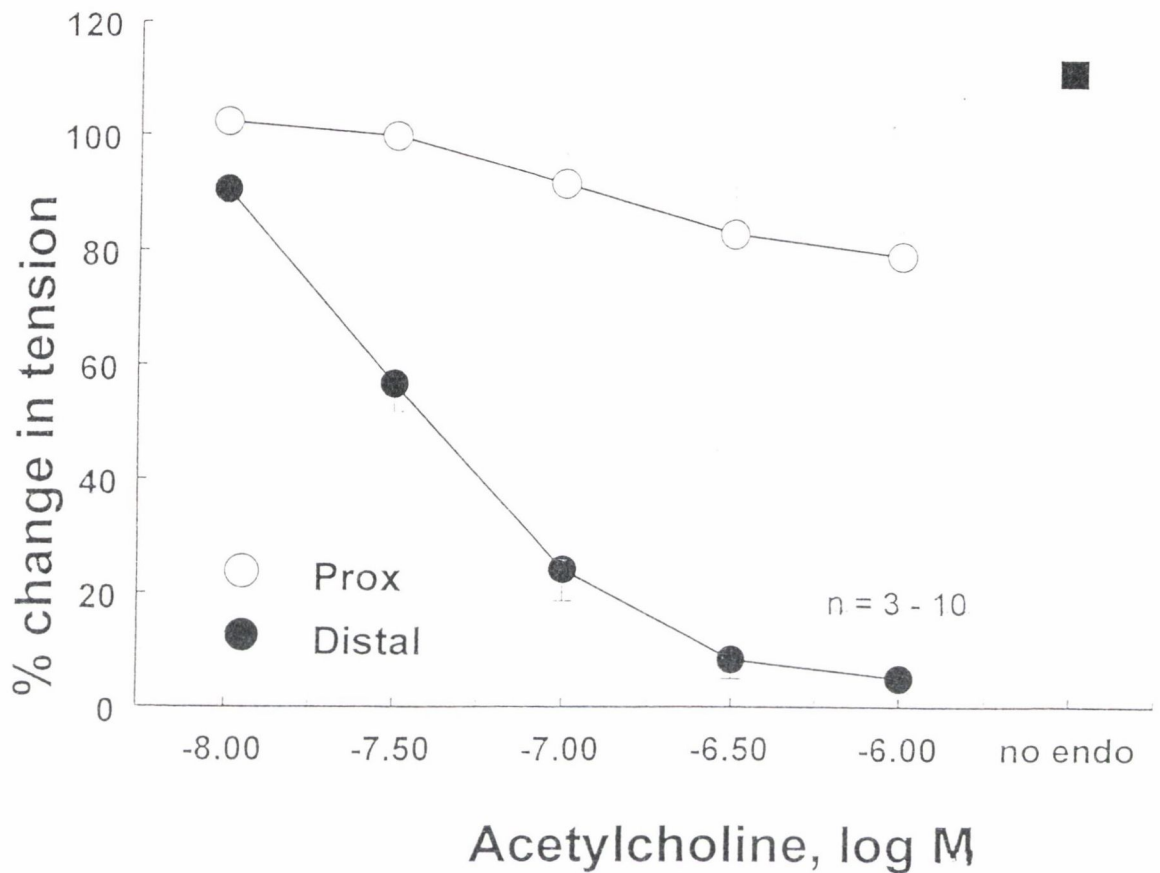
**14. Comparison of NO-dependent relaxation in proximal and distal porcine pulmonary artery segments.**

Comparison of vasodilator response to acetylcholine (10^{-9}-10^{-6}M) in proximal ($\circ$) and distal ($\bullet$) porcine pulmonary arteries in absence of L-NAME. The maximum relaxation to acetylcholine was $20.6 \pm 9.2\%$ (n=3) in proximal rings and $95.1 \pm 2.4\%$ (n=10) in distal rings. Results are expressed as a percent change in phenylephrine contraction and are presented as means $\pm$ SE.
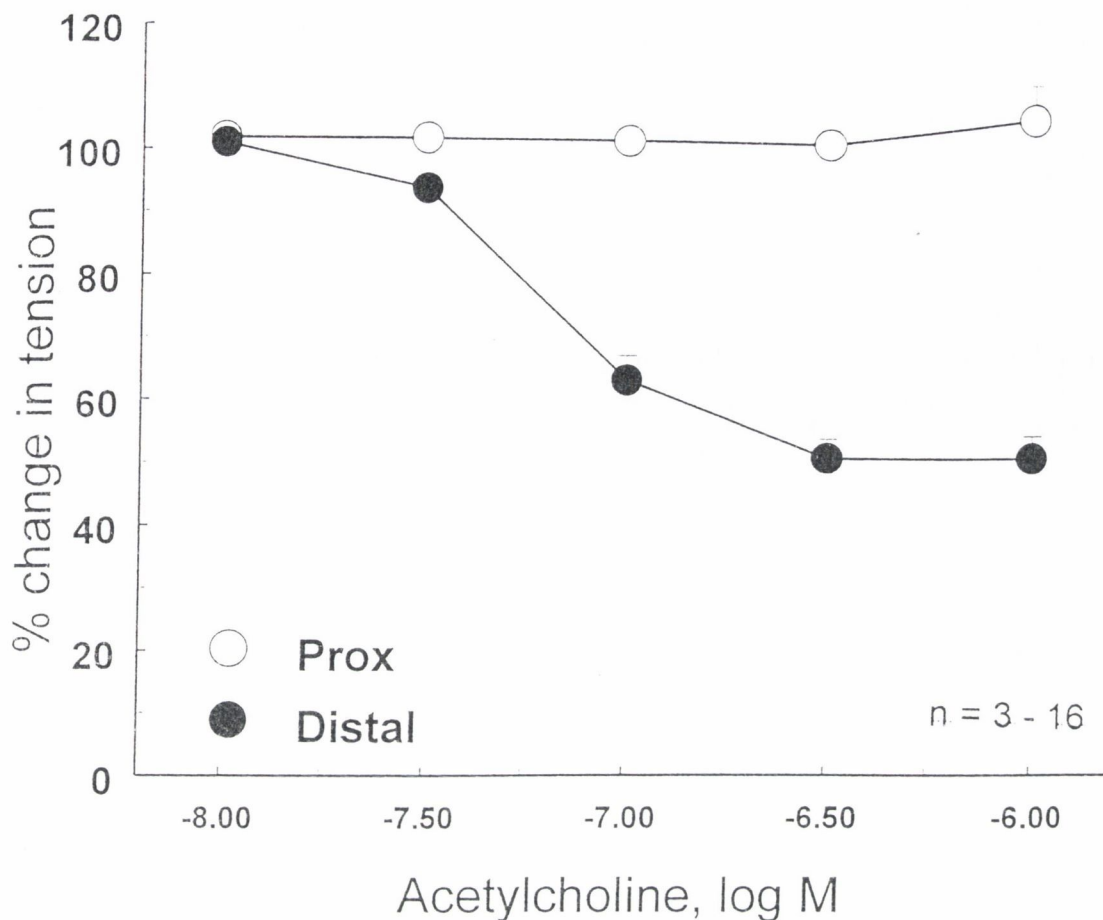
15. Comparison of NO-independent relaxation in proximal and distal porcine pulmonary artery segments.

Comparison of vasodilator response to acetylcholine $(10^{-9}\text{-}10^{-6}\text{M})$ in proximal ($\circ$) and distal ($\bullet$) porcine pulmonary arteries in the presence of L-NAME $(3 \times 10^{-5}\text{M})$. Proximal rings contracted by $4.5 \pm 5.5\%$ (n=3) in response to acetylcholine (10^{-6}M) while the maximum relaxation was $49.4 \pm 3.6\%$ (n=16) in distal rings. Results are expressed as a percent change in phenylephrine contraction and are presented as means $\pm$ SE.
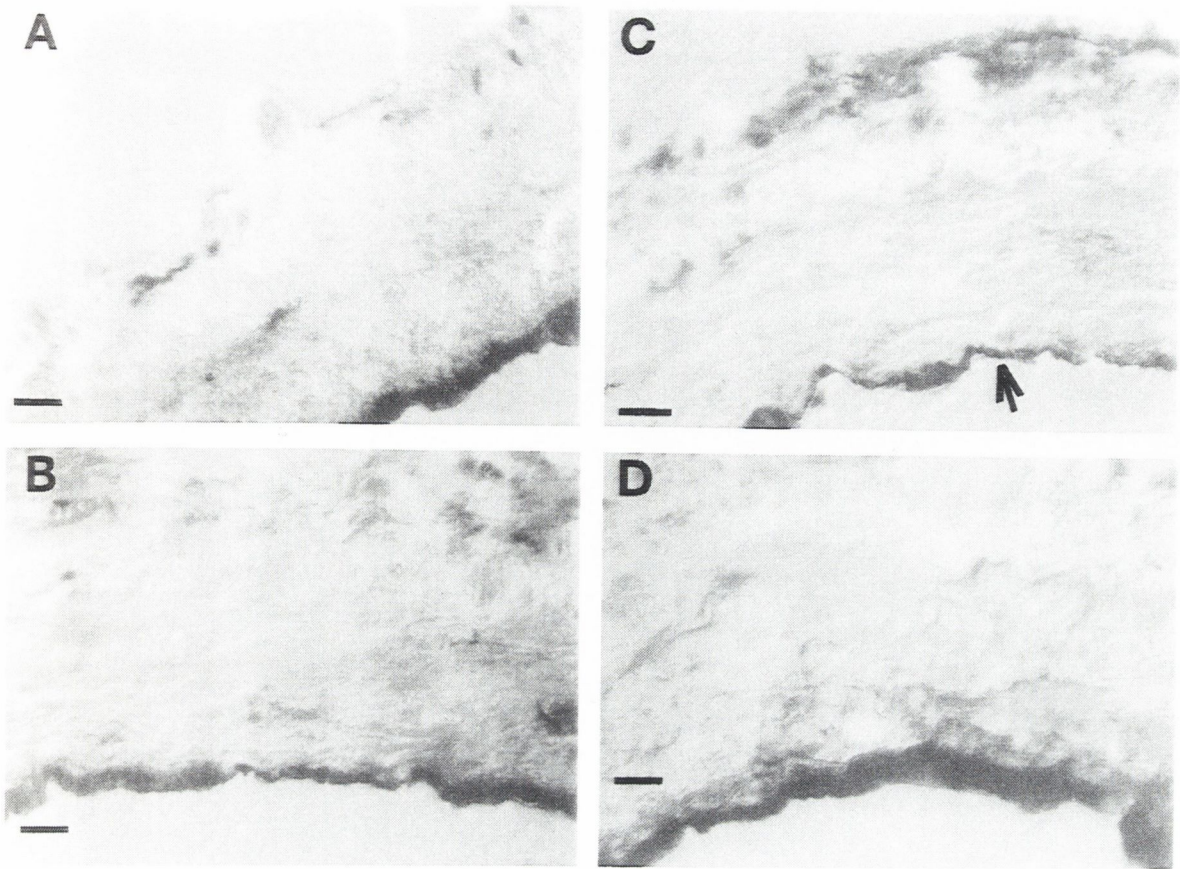
16. Immunohistochemistry of ecNOS and HO-2 in the proximal and distal porcine pulmonary artery.

Immunohistochemistry was performed as described in methods. The arrow points to staining in the vascular endothelium. These figures are representative of results from three separate animals. Localization of HO-2 in the (A) distal and (B) proximal porcine pulmonary artery. ecNOS localization in the distal (C) and proximal (D) porcine pulmonary artery. Preabsorption of HO-2 and ecNOS antiserum with the immunizing peptide abolished immunoreactivity (Bar = 100 μM).
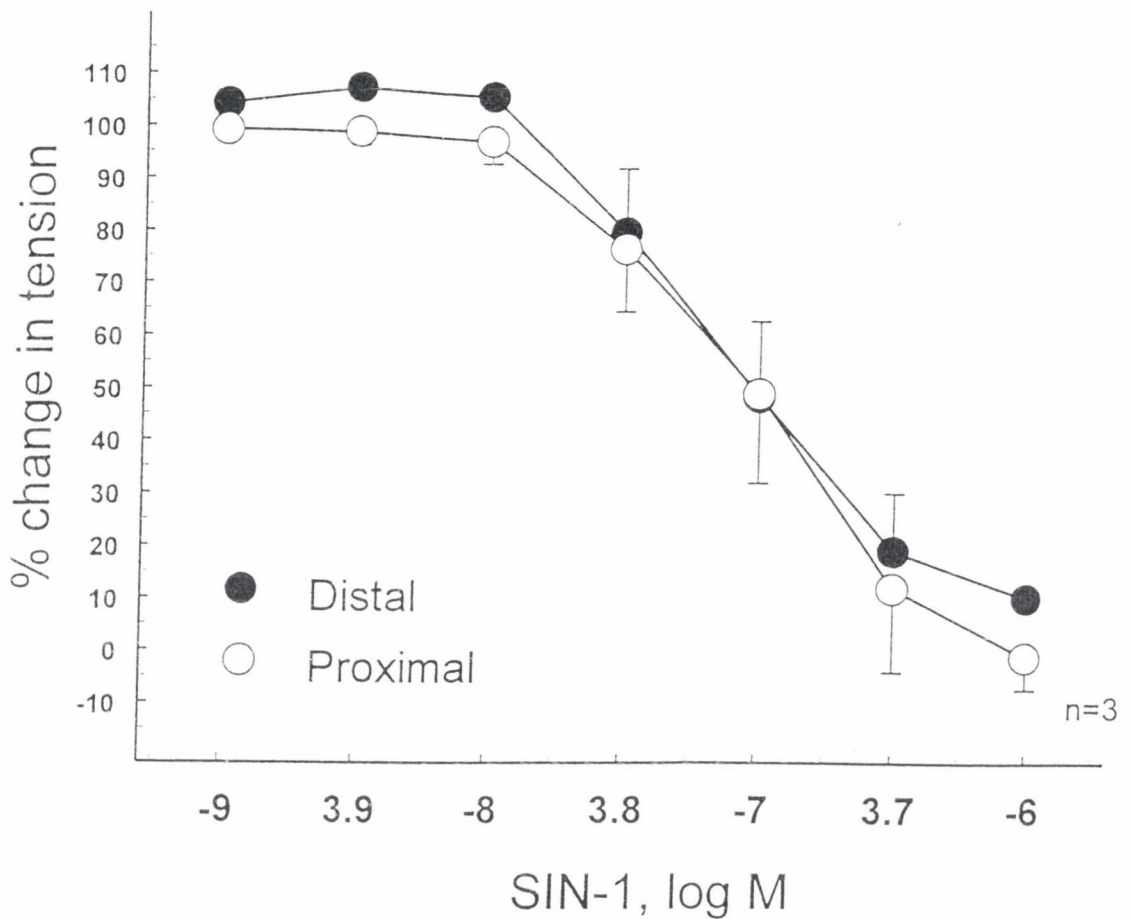
17. Vasodilator response to SIN-1 in proximal and distal porcine pulmonary arteries.

Relaxation to SIN-1 (10^{-9}-10^{-6} M) were assessed in arterial rings contracted with phenylephrine (EC_{50}). The maximum relaxation to SIN-1 was $111.92 \pm 16.0\%$ (n=3) in proximal rings and $110 \pm 0.5\%$ (n=3) in distal rings. The EC_{50} measured 7.01 ± 0.23 for proximal (○) and 7.01 ± 0.2 for distal arteries (●). Results are expressed as a percent change in phenylephrine contraction and are presented as means $\pm$ SE (n=3).

Regional Response to Carbon Monoxide

Proximal Artery

ED 50 ——————

Baseline ————

Distal Artery

ED 50 ——————

Baseline ————

0.25ml   0.5ml   1.0ml

1 min
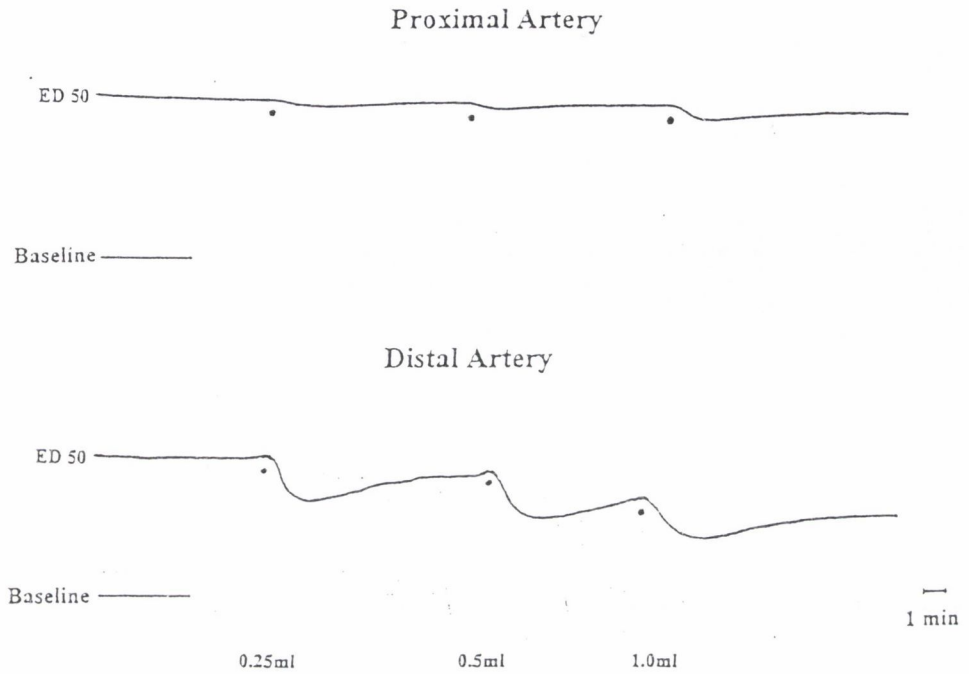
18. Tracing demonstrating the effect of carbon monoxide on tension in proximal and distal porcine pulmonary artery

Representative tracings (n=3) demonstrating the effect of carbon monoxide (25, 50, 100 μM) on tension in proximal and distal porcine pulmonary artery. Rings were contracted with phenylephrine (EC_{50}).
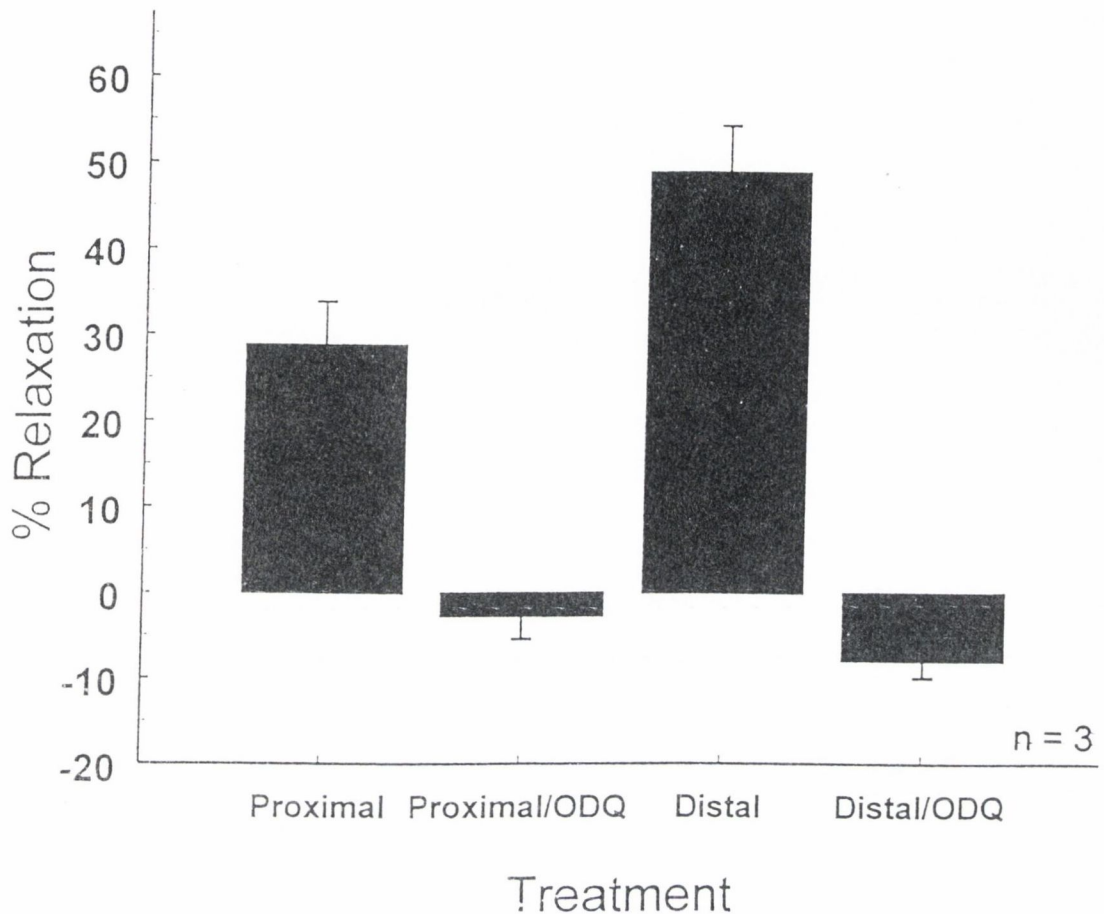
19. Effect of CO on proximal and distal pulmonary arteries.

Effect of CO ($100\mu M$) on proximal and distal pulmonary arteries contracted with phenylephrine (EC_{50}) in the presence of L-NAME ($3\times10^{-5}M$) and following ODQ ($10\mu M$) Distal arterial rings relaxed by $48.8 \pm 5.5\%$, while proximal rings relaxed by $28.8 \pm 5\%$ of the phenylephrine precontraction ($n=3$; $p<0.05$). The response to CO following ODQ ($10\mu M$) produced a contraction of $2.7 \pm 2.7\%$ and $7.7 \pm 1.9\%$ in proximal and distal rings respectively ($n=3$;$p>0.05$;NS). Endothelium-removal did not effect responses to CO. Results are expressed as a percent change in phenylephrine contraction and are presented as means $\pm$ SE.

Control
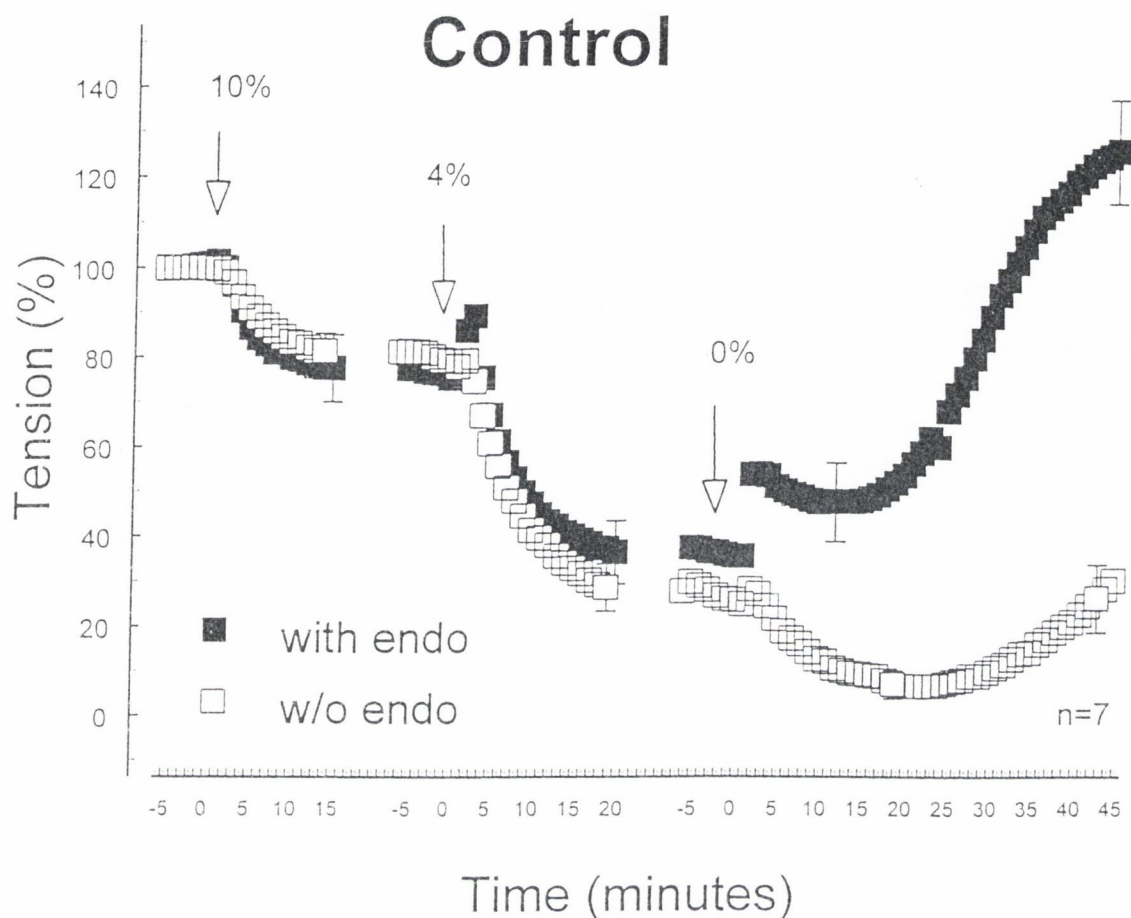
20. The effect of hypoxia on vascular tone in the proximal porcine pulmonary artery

Effects of hypoxia (10% and 4%) and anoxia (0% O_2) on contractile response to phenylephrine in proximal porcine pulmonary arteries with and without endothelium. The protocol was performed without interruption, and contractile tension was determined at one min intervals for the entire time course of each experiment. Results are expressed as a percentage of normoxic response to phenylephrine and are presented as means or means $\pm$ SEM at selected time points (n=7).
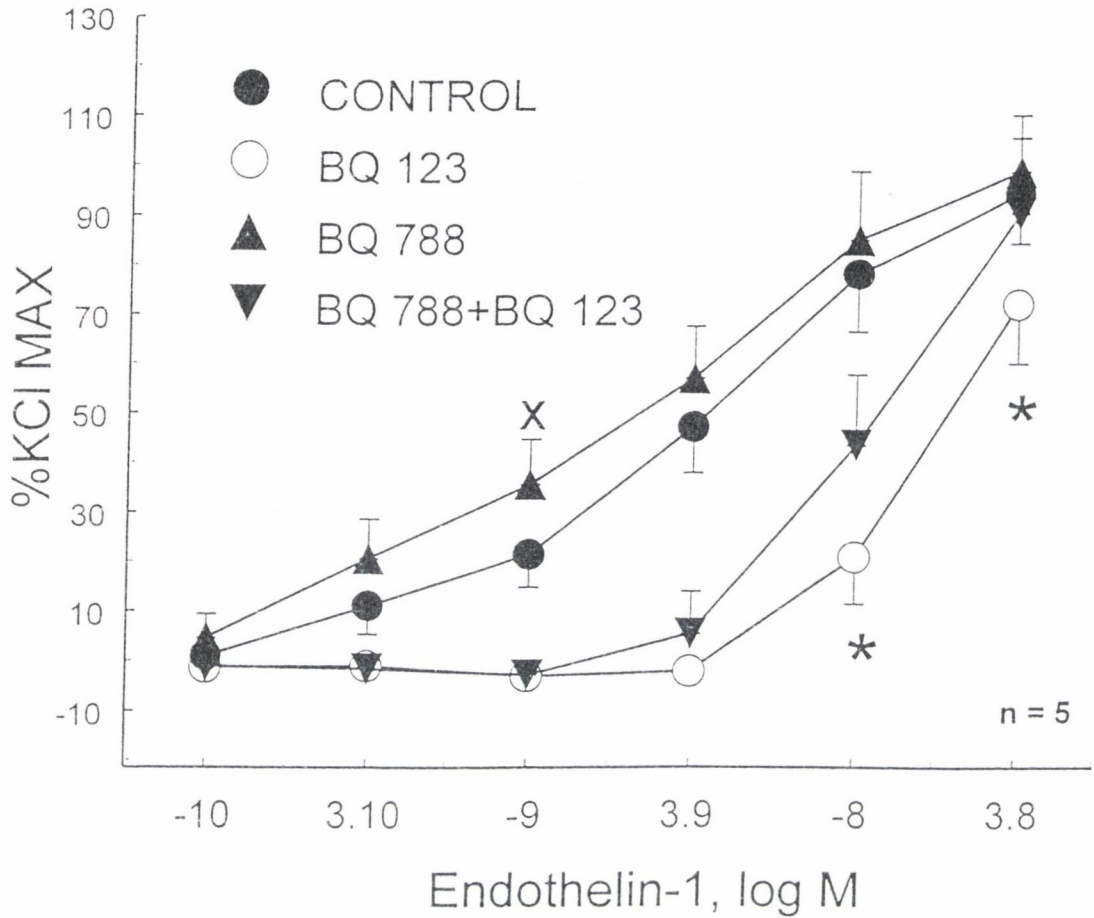
21. Effect of endothelin-1 on endothelium-intact proximal pulmonary arteries.

Effect of the ET_A receptor antagonist, BQ123 (10^{-6}M), and/or the ET_B receptor antagonist, BQ788 (3×10^{-7}M), on contractile response to endothelin-1 (10^{-10} - 3×10^{-8}M) in porcine pulmonary arteries with endothelium (n=5). Results are expressed as a percentage of the maximum contraction to KCl (60mM), obtained prior to administering the receptor antagonists. The symbol (X) denotes significantly different from control ($p < 0.05$) and (*) denotes significantly different from BQ 123 and BQ 788 combined ($p < 0.05$). Data is presented as means $\pm$ SEM (n=5).
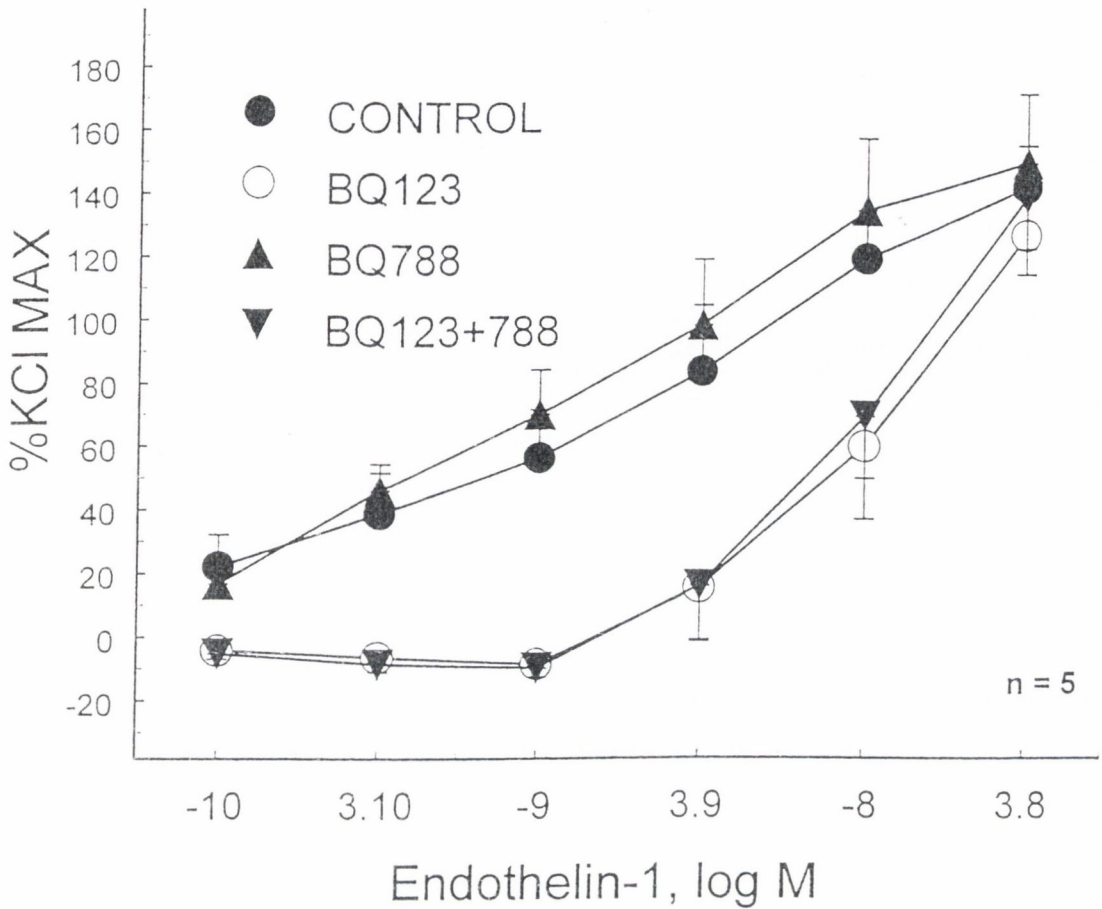
22.   Effect of endothelin-1 on endothelium-denuded proximal pulmonary arteries.

Effect of the ET_A receptor antagonist, BQ123 (10^{-6}M), and/or the ET_B receptor antagonist, BQ788 (3×10^{-7}M), on contractile response to endothelin-1 (10^{-10} - 3×10^{-8}M) in porcine pulmonary arteries without endothelium (n=5). Results are expressed as a percentage of the maximum contraction to KCl (60mM), obtained prior to administering the receptor antagonists. Data is presented as means $\pm$ SEM (n=5).

Endothelium Intact
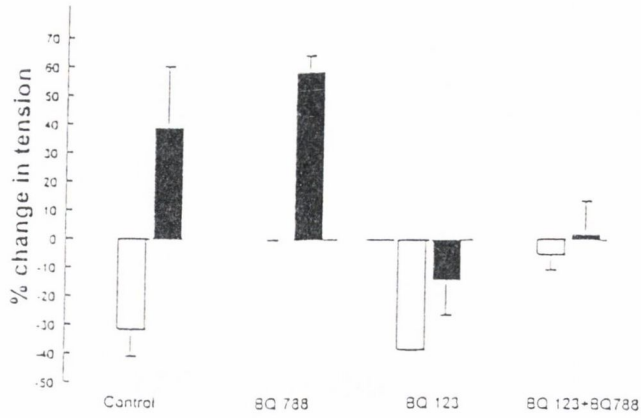
Endothelium Denuded
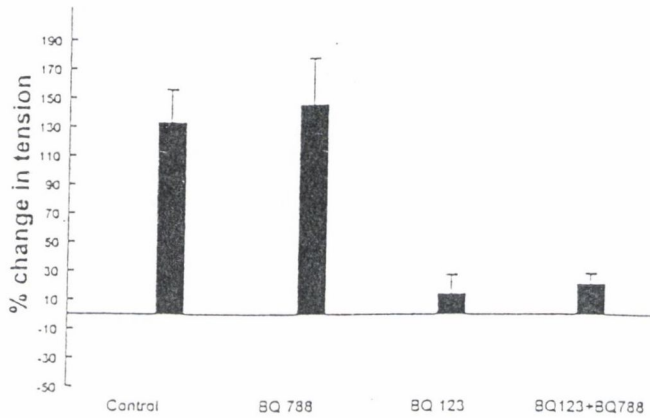
23. Effect of the ET_A and/or the ET_B receptor antagonists on contractile and relaxant responses to endothelin-1 in pulmonary artery rings.

Effect of the ET_A receptor antagonist, BQ123 (10^{-6}M), and/or the ET_B receptor antagonist, BQ788 (3×10^{-7}M), on contractile and relaxant responses to endothelin-1 (3×10^{-9}M) in pulmonary artery rings (with and without endothelium) (n=3). Arterial rings were first precontracted to $EC_{50(KCl)}$ with phenylephrine. Relaxation was determined as the lowest level of tension reached (clear box), and contraction as the tension reached ten minutes following administration of endothelin-1 (3×10^{-9}M) (filled box). Responses to endothelin are expressed as a percentage change in contractile response to phenylephrine and are presented as means $\pm$ SEM (n=3).

BQ 123
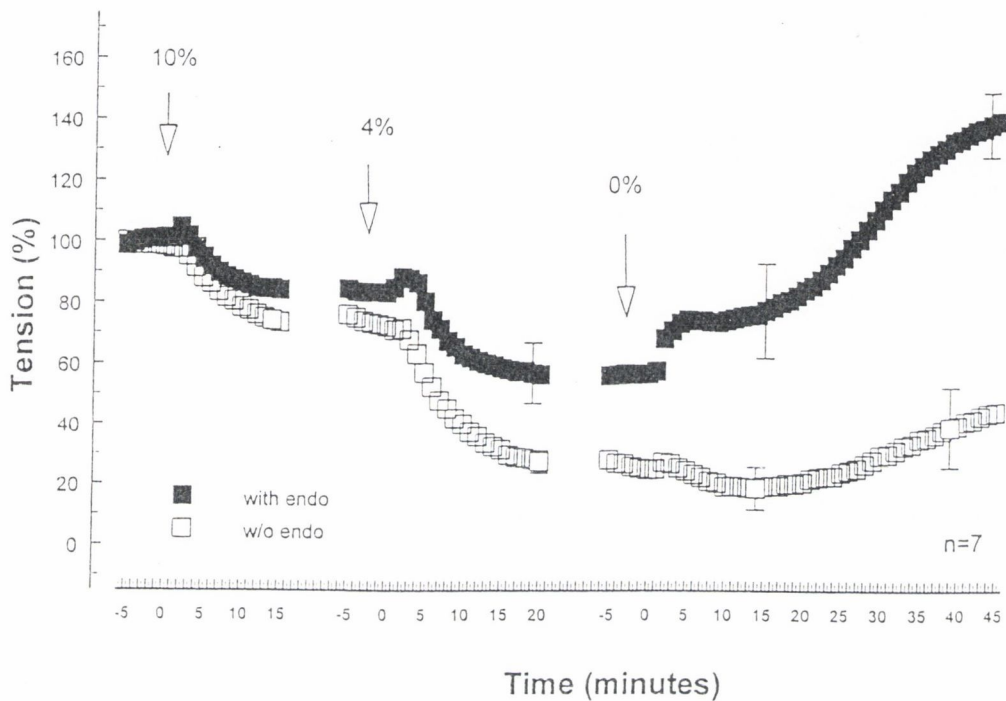
24a. Effects of the ET_A receptor antagonists on hypoxic responses in proximal porcine pulmonary arteries.

Effects of moderate (10% and 4% O_2) and severe hypoxia (0% O_2) on tension of proximal porcine pulmonary arteries (with and without endothelium) in the presence of the ET_A antagonist, BQ 123 $10^{-6}M$. The protocol was performed without interruption, and contractile tension was determined at one min intervals for the entire time course of each experiment. Results are expressed as a percentage of normoxic response to phenylephrine and are presented as means or means $\pm$ SEM at selected time points (n=7).

BQ 788
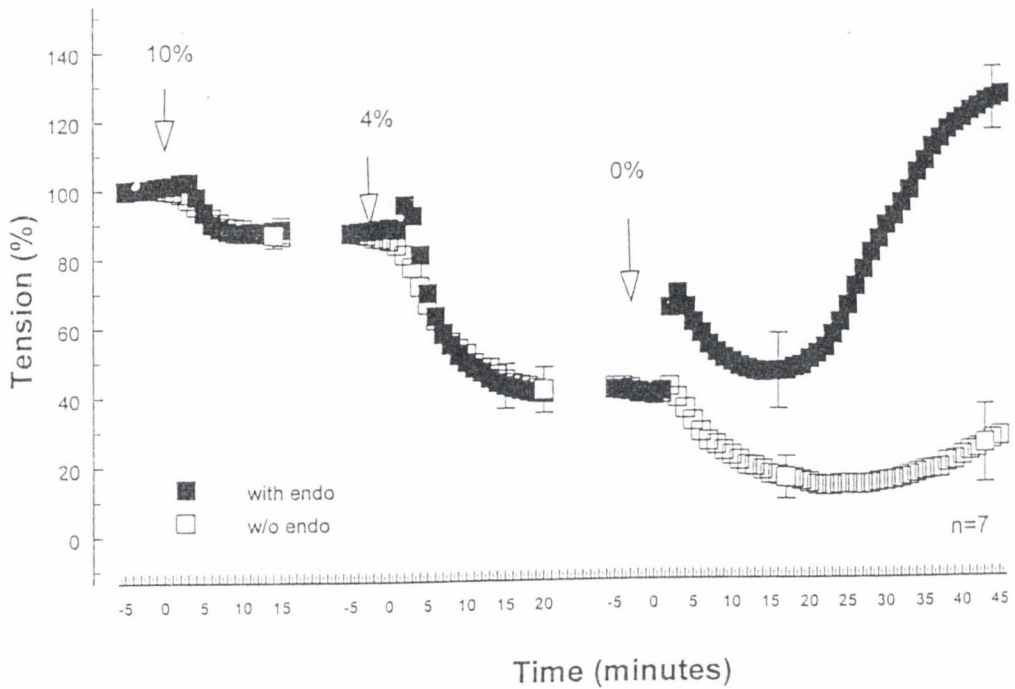
24b. Effects of the ET_B receptor antagonists on hypoxic responses in proximal porcine pulmonary arteries.

Effects of moderate (10% and 4% O_2) and severe hypoxia (0% O_2) on tension of proximal porcine pulmonary arteries (with and without endothelium) in the presence of the ET_B antagonist, BQ 788 $3x10^{-7}M$. The protocol was performed without interruption, and contractile tension was determined at one min intervals for the entire time course of each experiment. Results are expressed as a percentage of normoxic response to phenylephrine and are presented as means or means $\pm$ SEM at selected time points (n=7).

BQ123 and BQ788

24c. Effects of the ET_A and ET_B receptor antagonists on hypoxic responses in proximal porcine pulmonary arteries.

Effects of moderate (10% and 4% O_2) and severe hypoxia (0% O_2) on tension of proximal porcine pulmonary arteries (with and without endothelium) in the presence of the combination of the ET_A antagonist, BQ 123 10^{-6}M and the ET_B antagonist, BQ 788 3×10^{-7}M. The protocol was performed without interruption, and contractile tension was determined at one min intervals for the entire time course of each experiment. Results are expressed as a percentage of normoxic response to phenylephrine and are presented as means or means $\pm$ SEM at selected time points (n=7).

ED 50

ED 50

ED 50

ED 50

Bradykinin $3 \times 10^{-7} \mu M$ 1 min

25. Release of an EDRF from the pulmonary valve leaflet.

The representative tracings above depict four endothelium-denuded pulmonary arterial rings precontracted with phenylephrine to ED_{50}. The response to bradykinin (3×10^{-7}M) was used to confirm the absence of endothelium. The top three rings then had a pulmonary valve leaflet placed into the lumen while the bottom tracing is a control ring without a valve leaflet. In response to bradykinin (3×10^{-7}M) the arterial rings containing the valve leaflets now relaxed indicating the release of an EDRF from the valve leaflet. The control ring contracted. These tracings are representative of experiments with arterial rings from three separate animals (n=3).
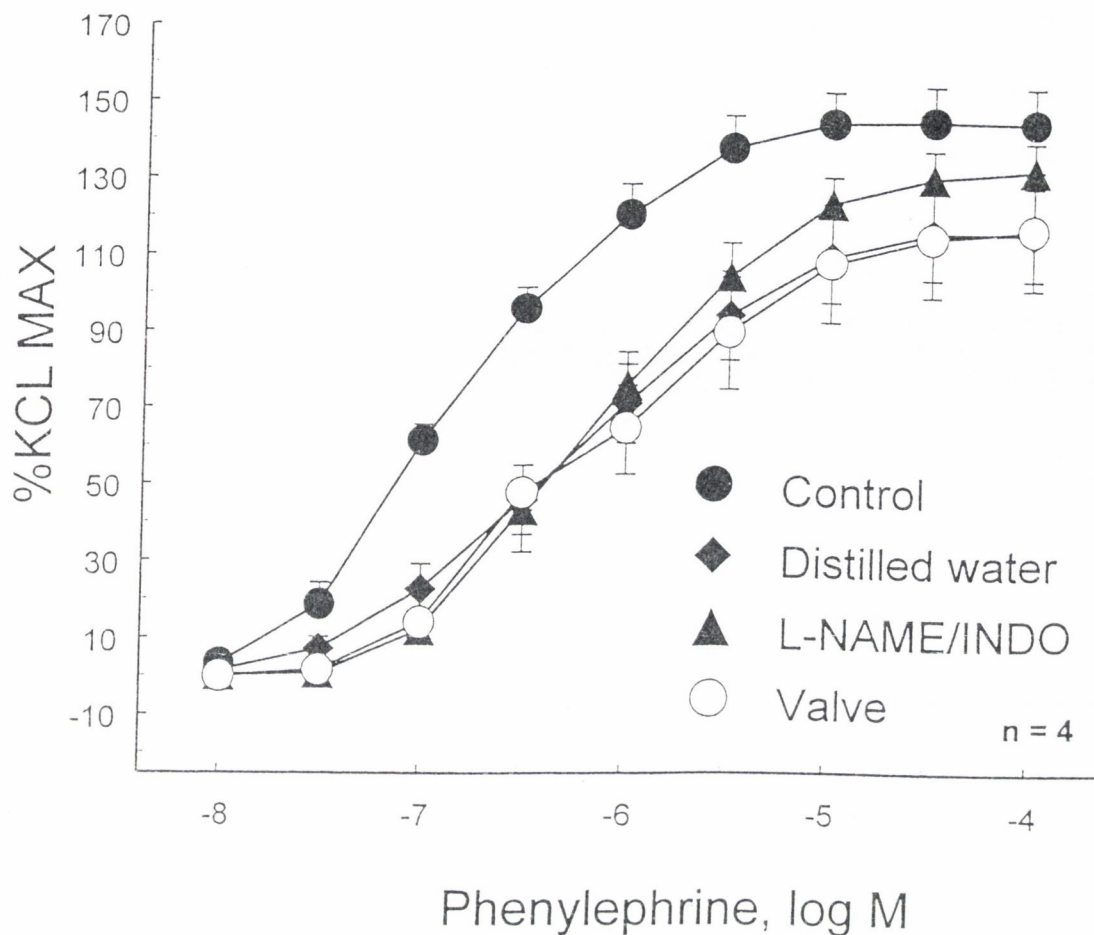
26. Effect of valve leaflet on contractile responses of pulmonary artery rings to phenylephrine (without endothelium).

Prior to placement of the pulmonary valve leaflet, endothelial-denudation was confirmed by lack of response to acetylcholine or bradykinin, and phenylephrine concentration response curves were determined. Some valve leaflets were treated with distilled water (4°C, 4 hours) to damage the endothelial cells, L-NAME and indomethacin, were administered after placement of the valve leaflet, and 30 min before administering phenylephrine. Results are expressed as a percentage of the maximum contraction to KCl (60mM). Data is presented as means $\pm$ SEM (n=4).

FIRST HYPOXIC EXPOSURE   SECOND HYPOXIC EXPOSURE

Time Control 1.

Valve 1.

Valve 2.

Valve 3.

Time Control 2.

Baseline

0% OXYGEN   0% OXYGEN

5 min
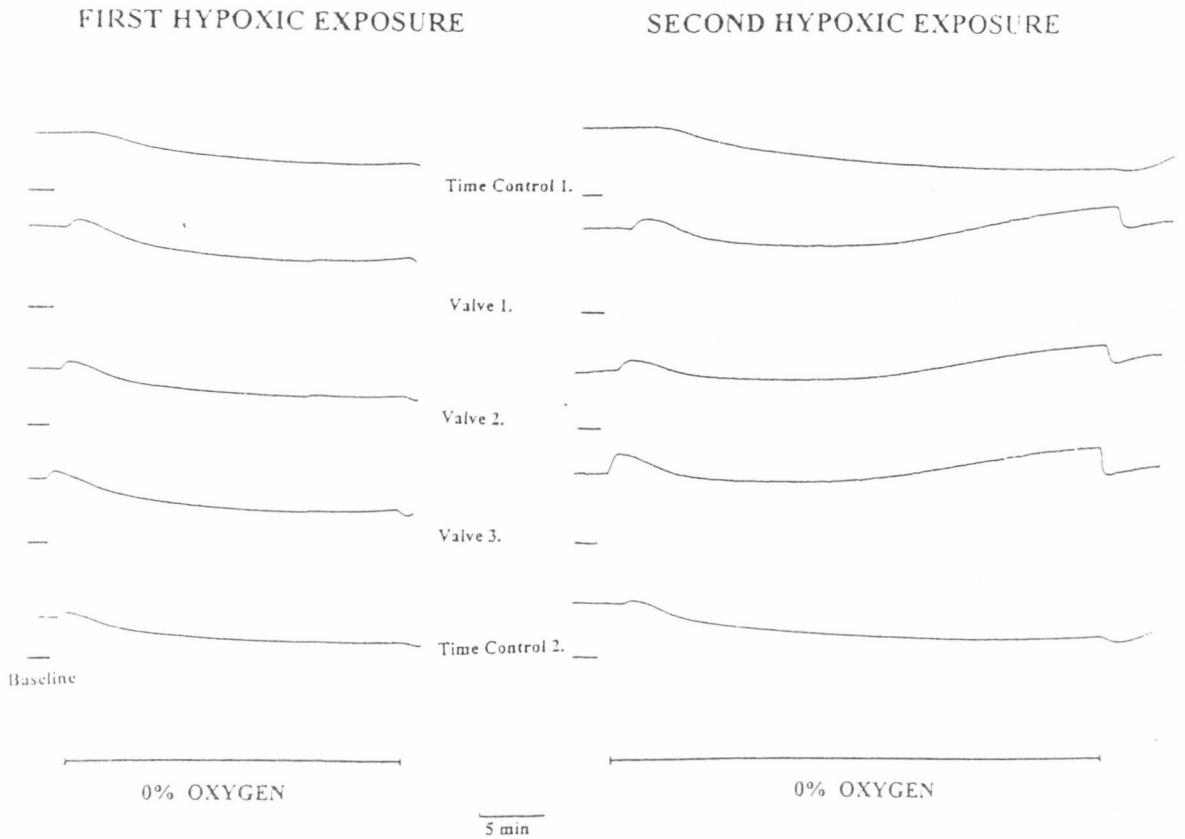
27. Representative tracing demonstrating the transfer of endothelium-derived mediators from a pulmonary valve leaflet to an endothelium-denuded pulmonary arterial ring.

First hypoxic exposure: During contraction to the $EC_{50(KCl)}$ level of tension with phenylephrine, five endothelium-denuded rings relaxed when exposed to an abrupt change in oxygen concentration (16% O_2 to 0% O_2).

Second hypoxic exposure: The rings were again contracted to $EC_{50(KCl)}$ with phenylephrine. In response to severe hypoxia (0% O_2), the two control rings (top and bottom) relaxed, whereas the presence of a pulmonary valve leaflet (Valve 1,2,3) uncovered a late-phase contraction.

These tracings are from experiments on the tissue of one animal on a single occasion. They are representative of results from five separate animals.

Control
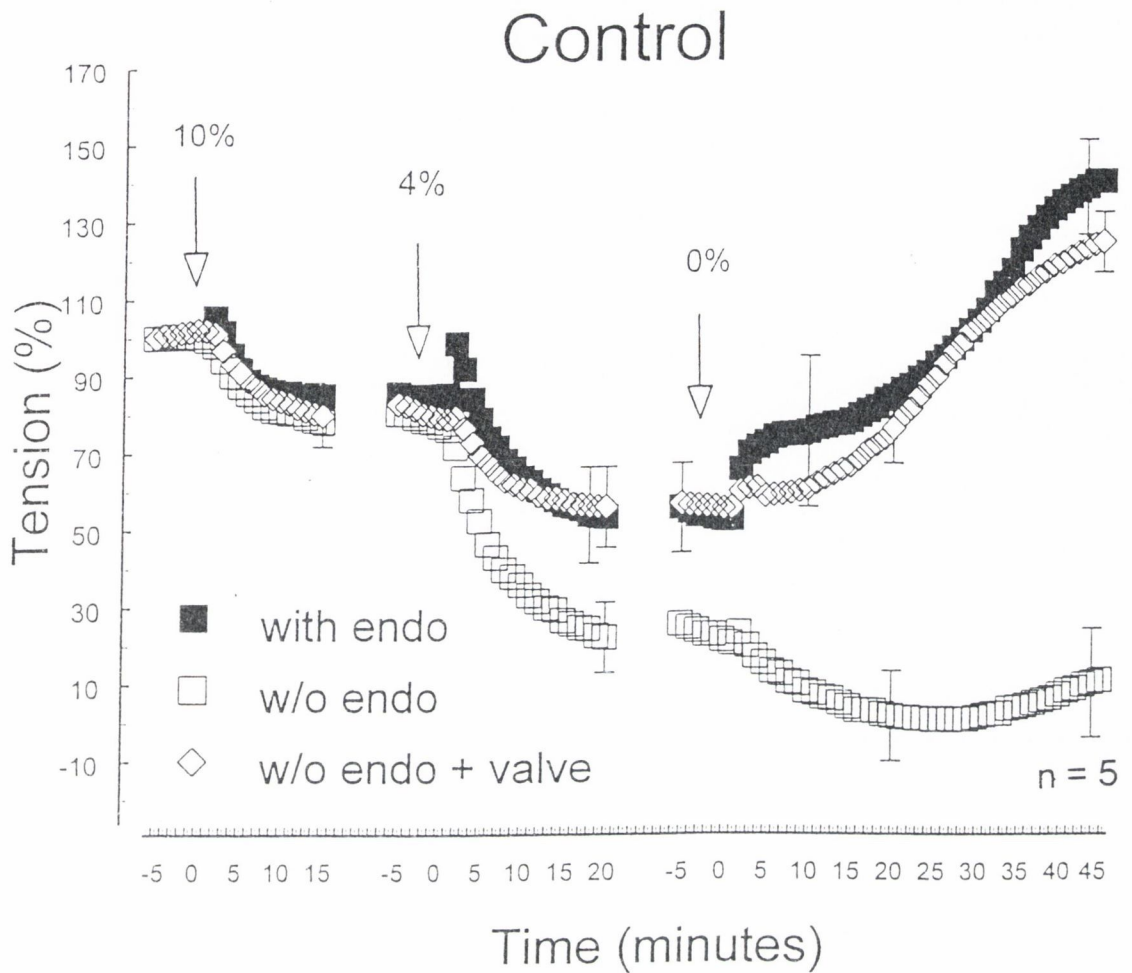
28. Transfer of an hypoxic EDCF from pulmonary valve leaflets to endothelium-denuded arterial rings.

Endothelium-containing rings, endothelium-denuded rings and endothelium-denuded rings containing a pulmonary valve leaflet were studied. Arterial rings were contracted to $EC_{50(KCl)}$ level of tension with phenylephrine prior to the hypoxic exposure. Oxygen tension was then decreased in a stepwise manner (from 16% to 10%, 4%, and 0% O_2), allowing time for tone to stabilize at each level. Results are expressed as a percentage of normoxic response to phenylephrine and are presented as means or means +/-SEM at selected time points. (n=5)

L-NAME/INDO
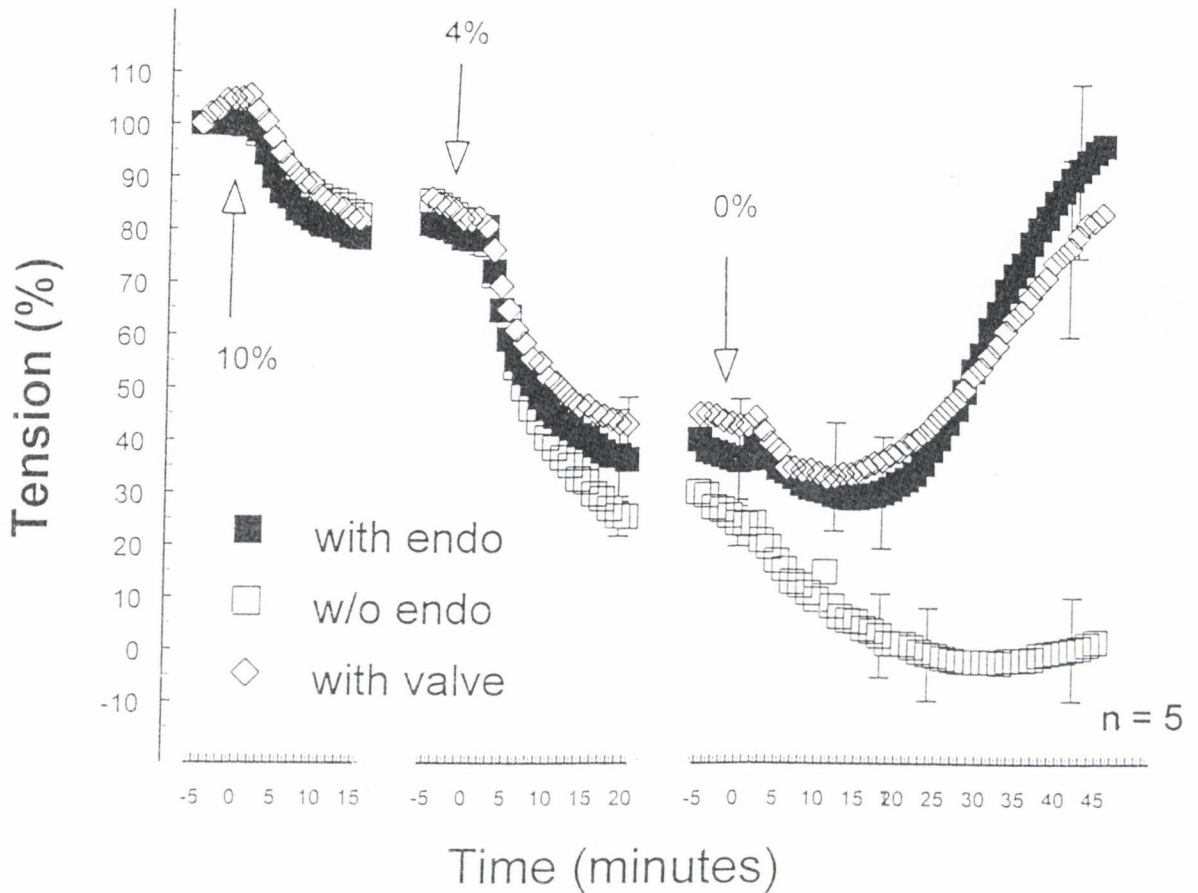
29. Transfer of an hypoxic EDCF from pulmonary valve leaflets to endothelium-denuded arterial rings. Response in the presence of L-NAME and indomethacin.

Endothelium-containing rings, endothelium-denuded rings and endothelium-denuded rings containing a pulmonary valve leaflet were studied. Arterial rings were treated with L-NAME ($3 \times 10^{-5}M$) and indomethacin ($10^{-5}M$), and contracted with phenylephrine prior to hypoxic exposure. Oxygen tension was then decreased in a stepwise manner (from 16% to 10%, 4%, and 0% O_2), allowing time for tone to stabilize at each level. Results are expressed as a percentage of normoxic response to phenylephrine and are presented as means or means +/-SEM at selected time points. (n=5)
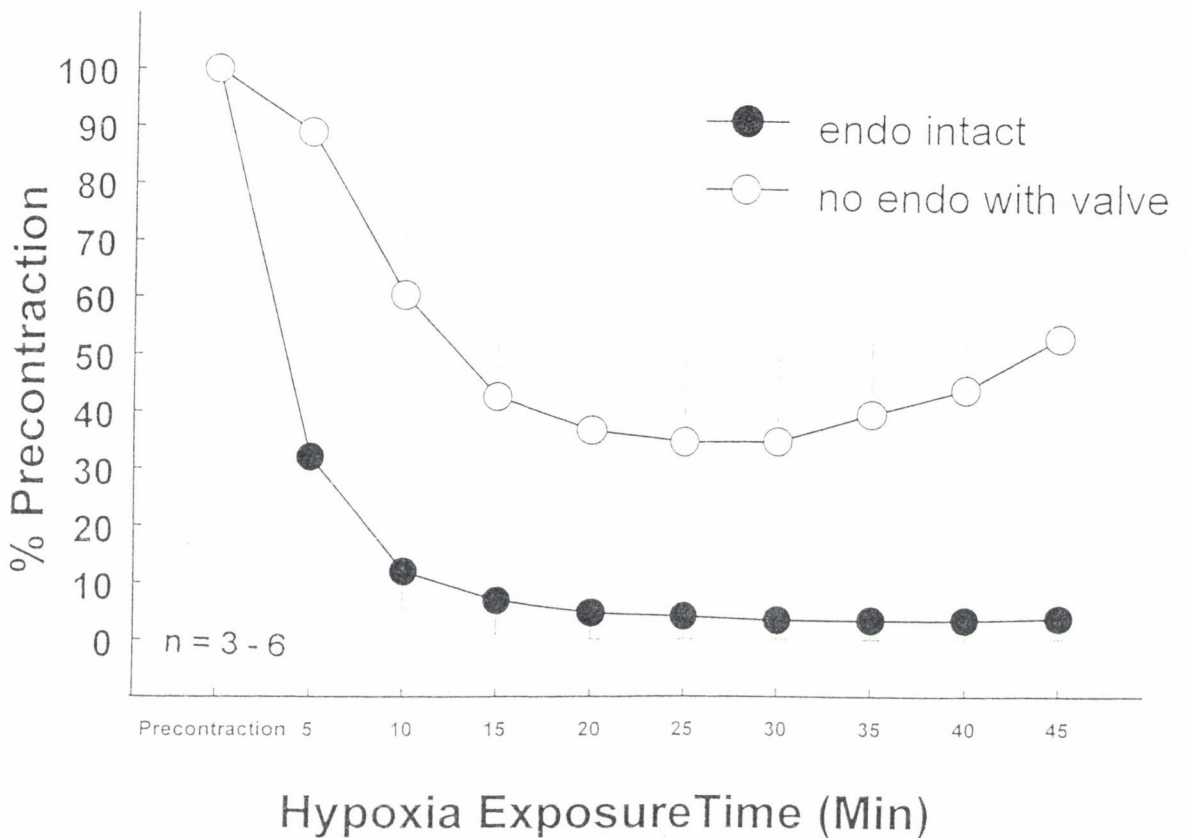
30. Effect of a valve leaflet on the internal iliac artery response to hypoxia.

Effects of severe hypoxia (0% O_2) on tension of the internal iliac artery. Endothelium-containing rings, endothelium-denuded rings and endothelium-denuded rings containing a pulmonary valve leaflet were studied. Arterial rings were contracted to $EC_{50(KCl)}$ level of tension with phenylephrine prior to the hypoxic exposure. L-NAME (3 x 10^{-5} M) and indomethacin (10^{-5} M) were present prior to and during exposure to phenylephrine and hypoxia. Oxygen tension was then decreased abruptly from 16% to 0% O_2 . While endothelium-intact rings relaxed completely 96.3 $\pm$ 3.6%(n=6), rings containing the pulmonary valve leaflet only relaxed by 46.8 $\pm$ 16.5% (n=3; p<0.05). Results are expressed as a percentage of normoxic response to phenylephrine and are presented means +/-SEM.

Transfer Experiments using Dialysis Membrane

Lane 1. A. B.

Lane 2.

Lane 3.

Lane 4.

Baseline

C.

Hypoxia (0% O_2)
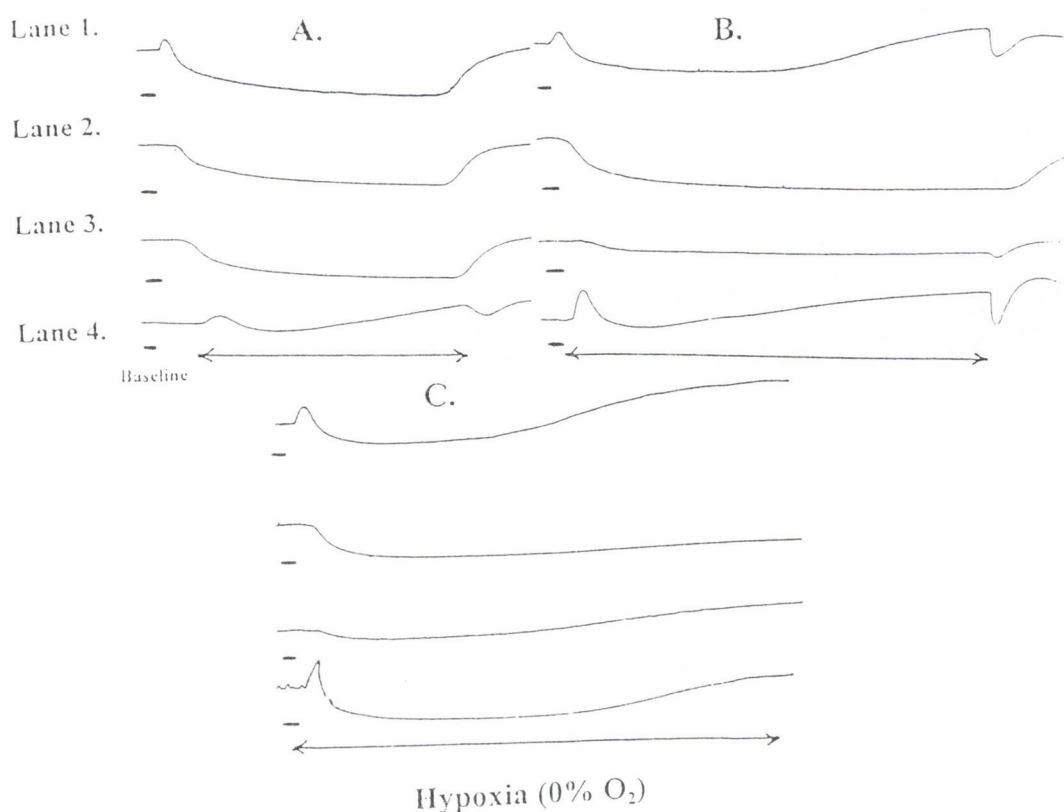
31. Bioassay to determine the molecular weight of the endothelial hypoxic factor.

Arterial rings in lanes 1-4 were contracted to $EC_{50(KCl)}$ level of tension with PE.

(A). In lanes 1-3 the absence of endothelium was confirmed by the lack of an hypoxic contraction (16-0%O_2). The endothelium was intact in lane 4. Oxygen was restored to 16% O_2 after 30 min and PE removed.

(B). In lane 1 a control valve leaflet (y) was placed in the ring lumen, a dialysis bag without a valve was placed in lane 2 and in lane 3 a valve leaflet (x) was placed inside a dialysis bag (MWCO 25K) and placed in the ring lumen. Following recontraction with PE, the rings were again exposed to hypoxia for 45 min. Lane 1 and 4 contracted to hypoxia while 2 and 3 relaxed.

(C). PE was again removed from the chambers. The valve (x) was taken out of the dialysis bag and placed into the lumen of the ring in lane 1. The valve leaflet (y) was placed in a dialysis bag (MWCO 25K) in lane 2 and in lane 3 a control valve (z) was used. Now lanes 1, 3 and 4 contracted to hypoxia while lane 2 relaxed. These tracings are representative of similar results from three separate animals.

Proximal Pulmonary Artery

A.

B.

Lane 1

Lane 2

Lane 3

C.

Baseline

0% Oxygen

1 min
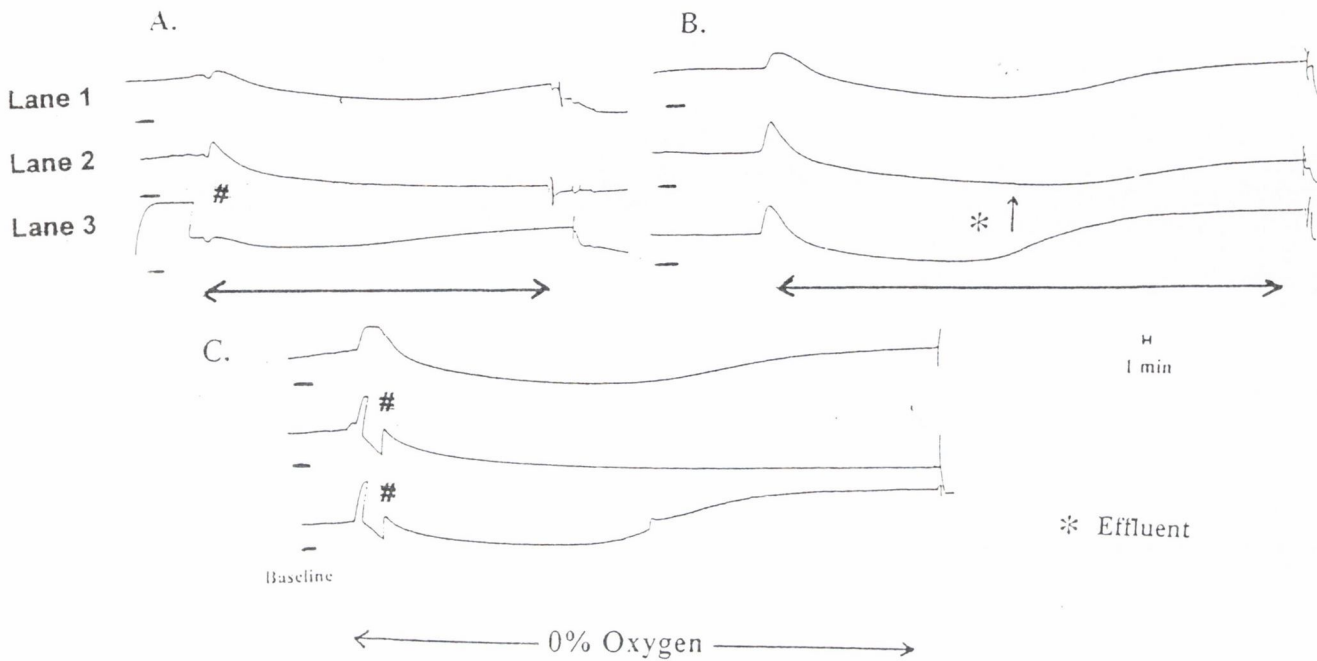
* Effluent

32. Effect of endothelial effluent on hypoxic responses in endothelial denuded proximal pulmonary arterial rings.

Arterial rings in lanes 1-3 were contracted to $EC_{50(KCl)}$ level of tension with PE.

(A). In lane 2 the absence of endothelium was confirmed by the lack of a contractile response on exposure to hypoxia (16-0%O_2). The endothelium was intact in lanes 1 and 3. Oxygen was restored to 16% O_2 after 30 min and PE removed.

(B). Following washout and reoxygenation, the rings were contracted again with PE to ED_{50} and oxygen tension decreased to 0% O_2. At the point of maximum relaxation the effluent (*) was added to the organ chamber resulting in a slowly developing contraction.

(C.) The rings were subsequently washed out and recontracted and exposed to anoxia. The ring in lane 2 did not contract when reexposed to hypoxia in the absence of the effluent. These tracings are representative of results from three separate animals out of a total of six animals studied.

Marks adjustment of the sensitivity of the Grass recorder.
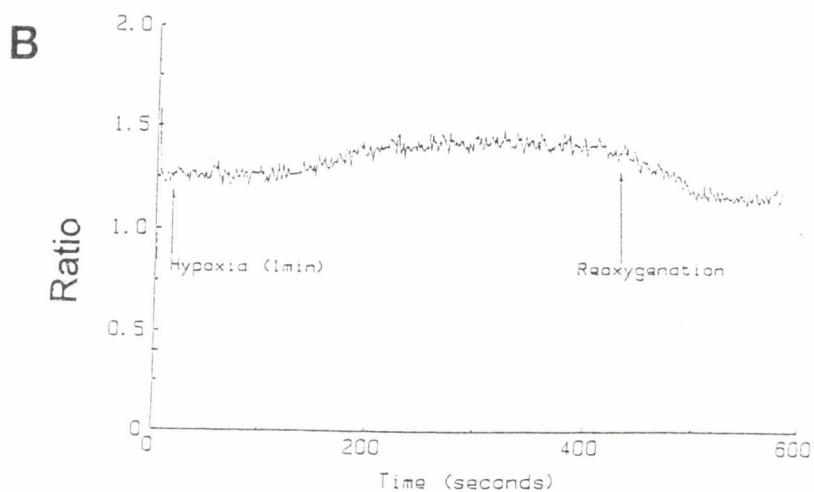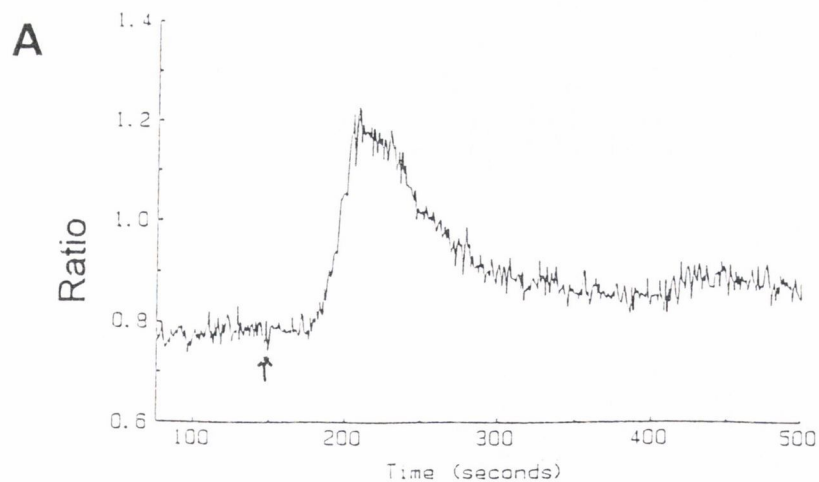
33. Increased intracellular calcium following activation of pulmonary valve leaflet endothelial cells.

(A). Representative tracing (n=3) showing the effect of bradykinin (10^{-6} M)(arrow) on $[Ca^{++}]_i$ of pulmonary valve endothelial cells. Changes in $[Ca^{++}]$ were monitored by measuring FURA-2 fluorescence ratio (R340/380).

(B). Representative tracings (n=2) showing the effect of hypoxia on $[Ca^{++}]_i$ of pulmonary valve endothelial cells. Changes in $[Ca^{++}]$ were monitored by measuring FURA-2 fluorescence ratio (R340/380).
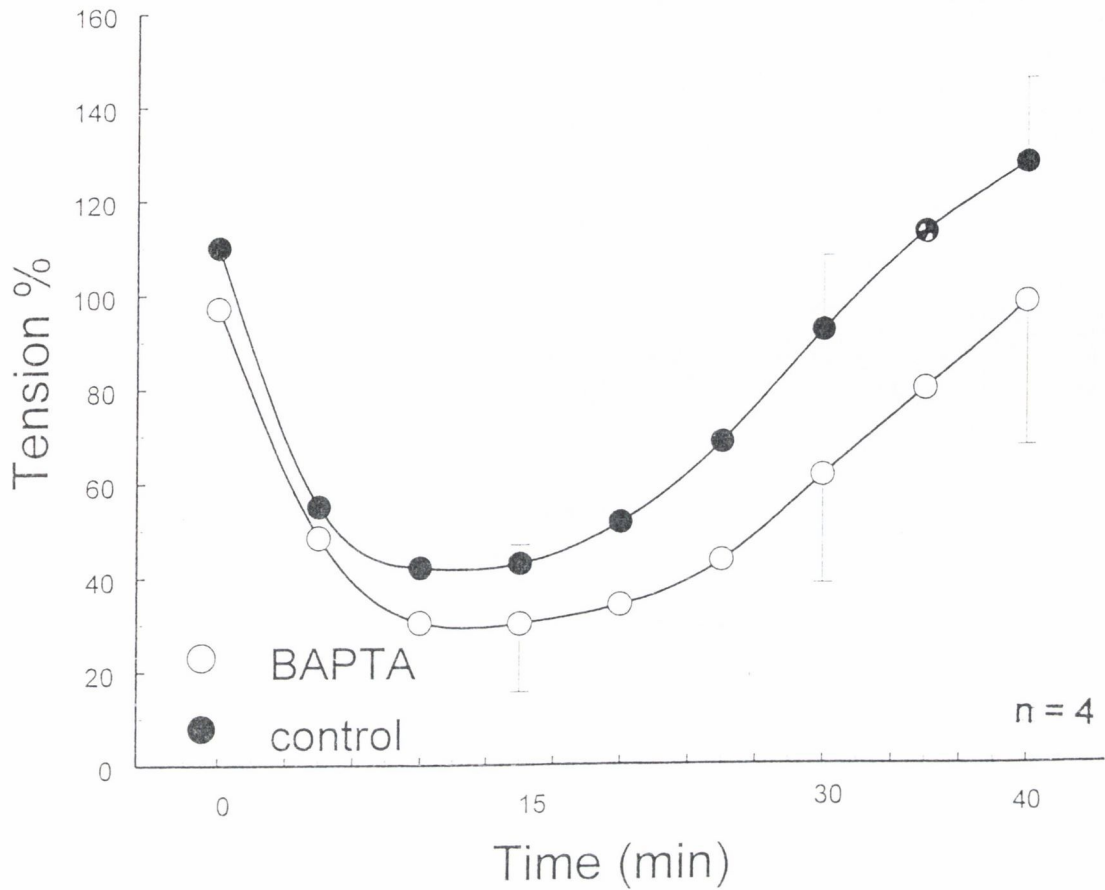
34. Effect of the intracellular calcium chelator, BAPTA, on hypoxic responses.

Effect of BAPTA (75 μM) on response to anoxia (0% O_2) in proximal pulmonary artery contracted with phenylephrine. During normoxia (16% O_2), pulmonary valve leaflets pretreated with BAPTA or control leaflets were placed into the lumen of endothelium-denuded arterial rings. The arterial rings were then contracted to EC_{50} level of tension with phenylephrine. Oxygen tension was abruptly decreased to 0% O_2. There was no significant difference in response between the control and treatment group (n=4; p>0.05;NS). Results are expressed as a percentage of normoxic response to phenylephrine and are presented as means or means ± SEM at selected time points.
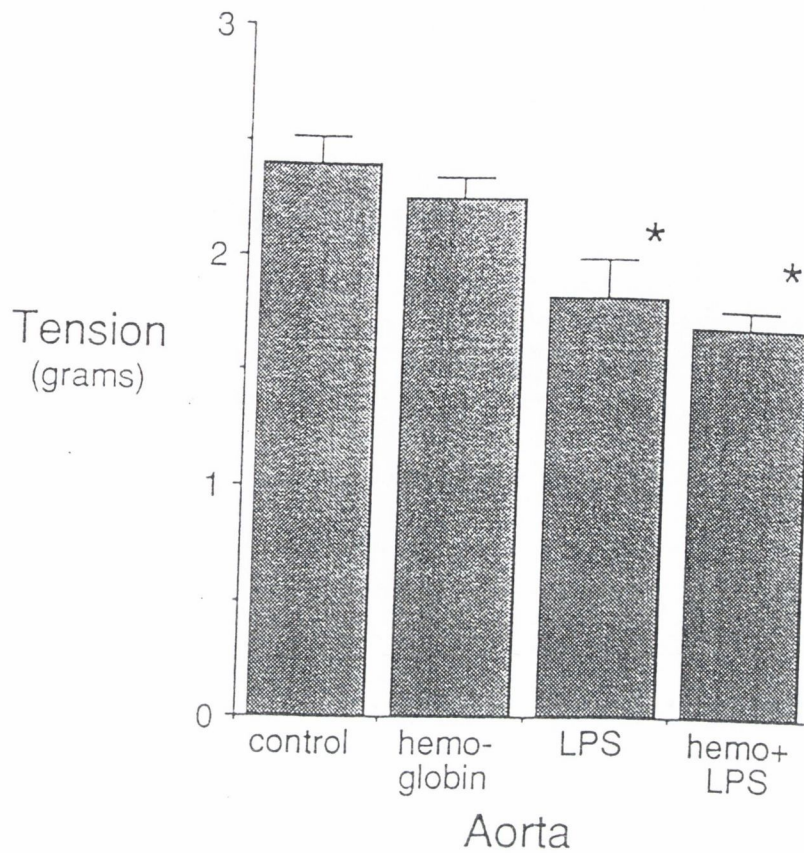
35. The effect of LPS and hemoglobin on KCl evoked contraction in the isolated rat aorta.

LPS inhibited contractions to KCl (60 mM) (n=4; p<0.05). Hemoglobin had no effect on the response to KCl either alone or in combination with LPS. Results are expressed as the maximum tension generated to KCl (60mM). (*) Statistically different from control. Data is presented as means ± SEM (n=4).

Effect of Hemoglobin and LPS on Response to PE in Aorta
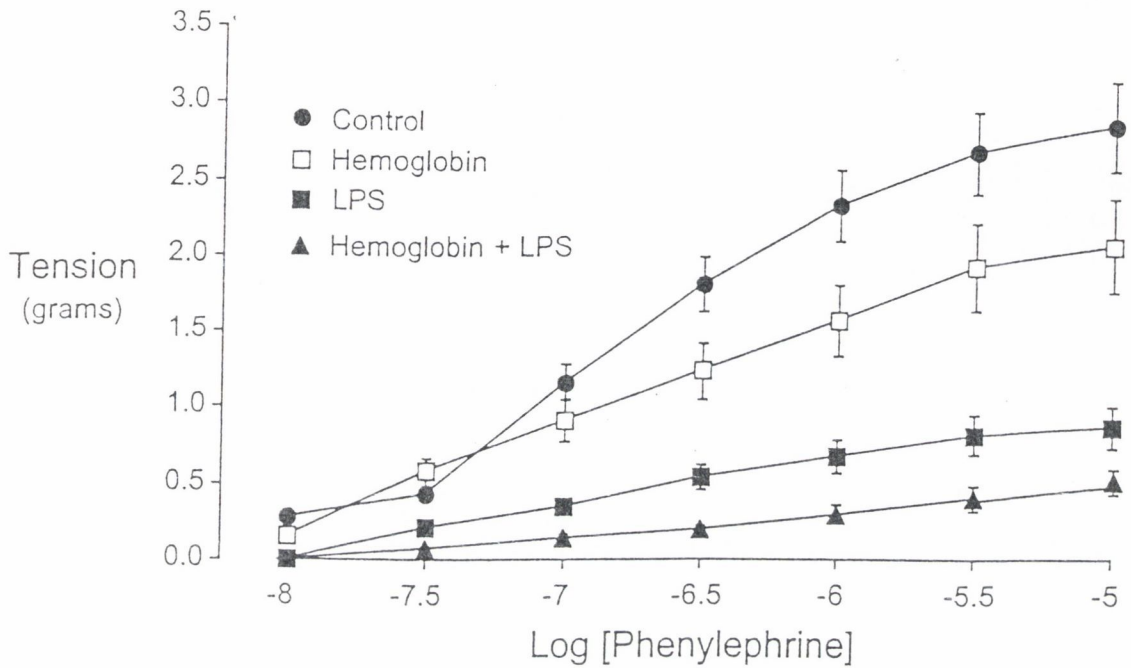
36. The effect of LPS and hemoglobin on phenylephrine evoked contraction in the isolated rat aorta.

The effect of LPS and hemoglobin (HGB) on the concentration response to phenylephrine in the isolated rat aorta (n=4). HGB caused a significant decrease in the PE_{max}. LPS alone and in combination with HGB caused a further decrease in PE_{max}. Results are expressed as the tension generated to PE. Data are presented as means $\pm$ SEM (n=4).

HO-1   **18s rRNA**

Control | LPS | Control | HGB
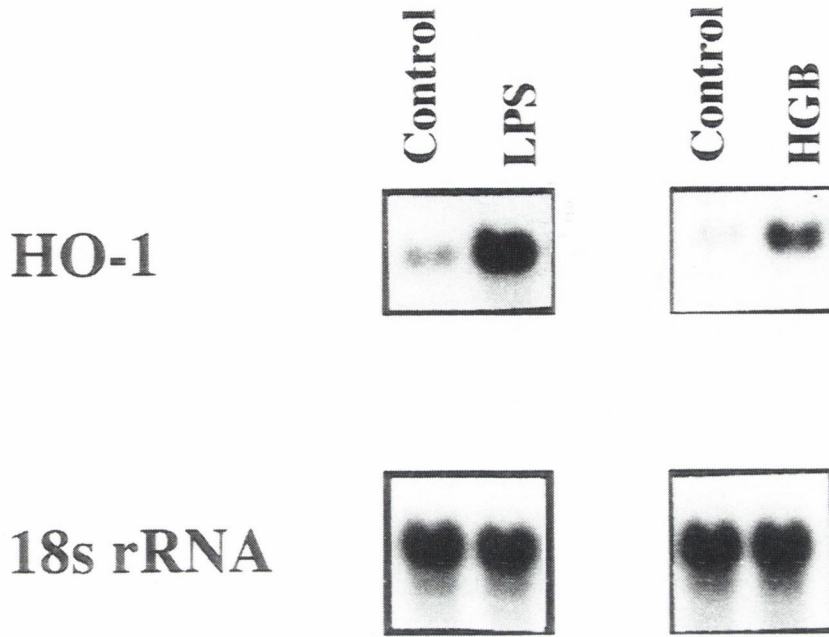
37. Induction of HO-1 in Rat Aorta.

Induction of HO-1 mRNA expression after LPS or HGB treatment. These figures are representative of results from three separate animals. Total RNA was extracted from aorta after LPS (4hours) or HGB (16hours) treatment and analyzed for HO-1 mRNA expression by Northern blot analysis as described in the Methods section. 18S rRNA hybridization is shown as a normalization control. The figure is representative of three separate experiments completed on tissue from three separate animals. Three separate animals were used for LPS experiments and three for hemoglobin experiments.

Effect of SnPP on Response
to PE in Hemoglobin and LPS Treated Aorta
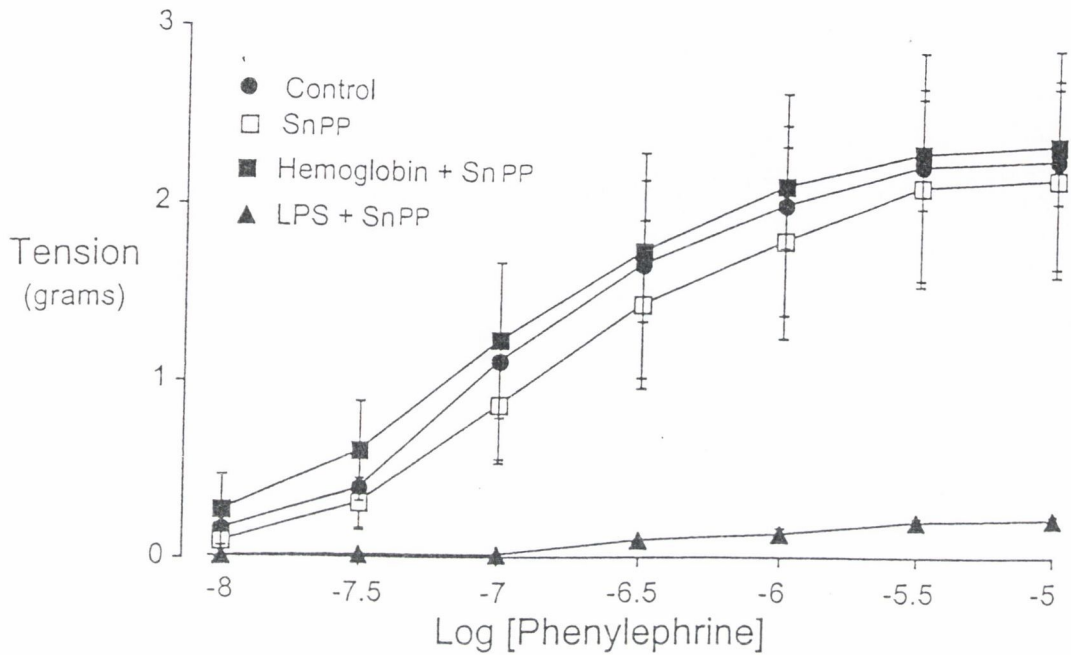
38. The effect of the heme oxygenase inhibitor, SnPP9 (30 μM) on vasodepressor effects of hemoglobin and LPS.

The effect of the heme oxygenase inhibitor, SnPP9 (30 μM) on the concentration response to PE in isolated rat aorta (n=4). There was no difference in the response to PE in control, SnPP9, and SnPP9+HGB vessels. SnPP9+LPS vessels had a marked decrease in PE_{max}. Results are expressed as the tension generated to PE. Data are presented as means $\pm$ SEM (n=4).
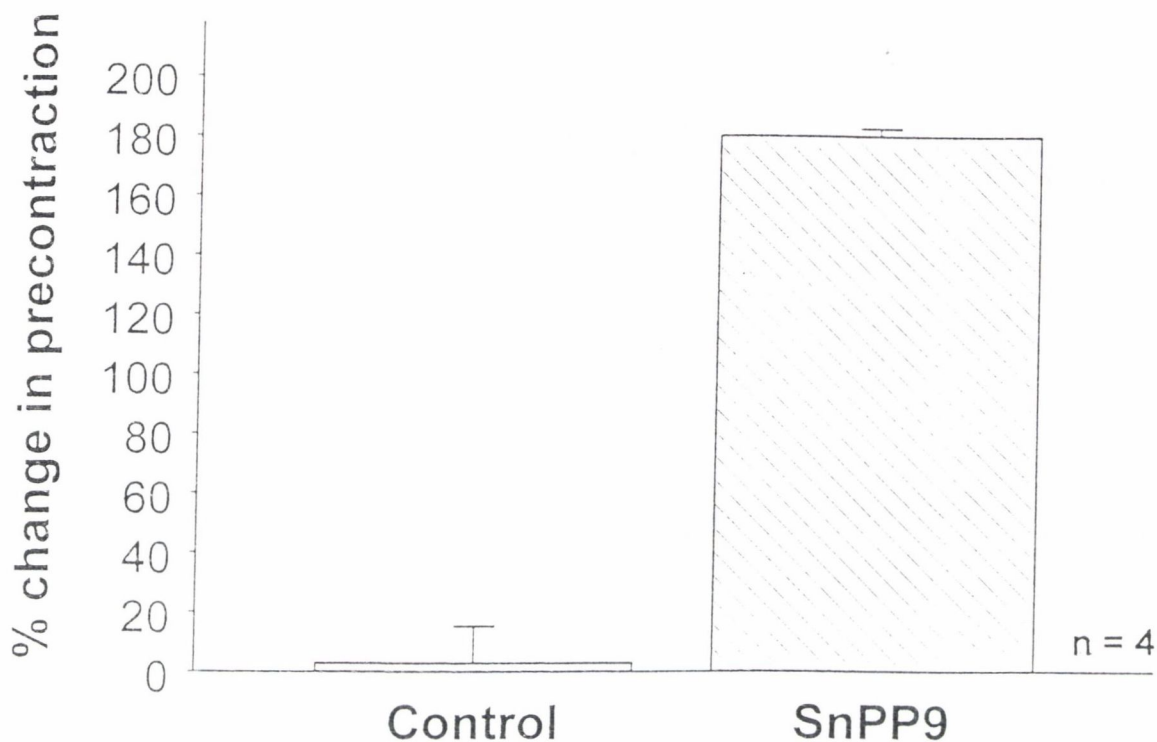
39. The light-dependent effects of SnPP9.

Light dependent effects of SnPP9 were explored in pulmonary artery rings. In the presence of L-NAME (3×10^{-5}M), endothelium-denuded arterial rings were treated with SnPP9 (10 μM). In the presence of light more phenylephrine was added to SnPP9 treated rings to achieve the ED_{30KCl}. When the lights were turned off again the SnPP9 treated arterial rings contracted rapidly (180 ± 12.4% of the precontraction) while there was no change in tone in control rings (2.9 ± 2.6% (n=4)(p>0.05;NS)) Results are expressed as a percent change in the precontraction. Data is presented as means ± SE (n=4).

DISCUSSION

1. INTRODUCTION.

The complex interaction between endothelium and the underlying smooth muscle contributes to the control of vascular tone. Endothelium-dependent relaxing and contracting factors are released in response to various stimuli, including changes in the local cellular environment, and modulate smooth muscle tone. Furthermore, the smooth muscle can also respond to its environment, independent of the endothelium, by relaxing or contracting. The specific responses of both the endothelium and underlying smooth muscle depend also upon the individual vascular bed. Pulmonary vascular endothelium and smooth muscle cells exhibit responses to stimuli such as hypoxia that are quite unique from cells derived from the systemic circulation. In this thesis we explore how the local modulation of pulmonary vascular function through endothelial:smooth muscle interaction contributes to the control of pulmonary vascular tone and contributes to the uniqueness of the pulmonary circulation.

We describe the expression of heme oxygenase-2 in porcine pulmonary vascular endothelium and propose a role for its product, carbon monoxide, as a novel endothelium-dependent relaxing factor. We also demonstrate the presence of a novel endothelium-dependent contractile factor released in response to hypoxia. By developing a unique new mechanism to tease apart the respective contributions of the endothelium and smooth muscle to vascular tone we explore the mechanisms responsible for hypoxic pulmonary vasoconstriction. Using an endothelium-rich valve leaflet to restore endothelial activity to an endothelium-denuded arterial ring we determined that the pulmonary vascular endothelium contributes to the pulmonary artery hypoxic response. We also show that induction of the enzyme heme oxygenase-1 in vascular smooth muscle results in decreased responsiveness to PE that we

speculate is as a result of endogenous CO production by the enzyme. Furthermore, we confirm that the vasodepressor effects of LPS in a rat model of sepsis are not mediated by enhanced CO production following the induction of HO-1.

In this thesis therefore, we demonstrate how a number of novel signaling mediators and mechanisms may contribute to the complexity of endothelial:smooth muscle interaction and indeed may also contribute to the uniqueness of the pulmonary circulation.

2. ENDOTHELIUM-DEPENDENT RELAXATION. A ROLE FOR HO AND CO.

2.1. The emerging role of heme oxygenase and CO. HO-2 expression in the pulmonary circulation.

It was not until the role of NO unfolded, as a vasoactive mediator released by endothelial cells and a neurotransmitter in both the central and peripheral nervous system (Bredt and Snyder 1994a), that the possibility CO might also function as a signaling molecule was explored. It was subsequently determined that constitutive HO-2 was present in central and peripheral nerves as well as in autonomic ganglion, thereby suggesting a role for CO as a neurotransmitter (Stevens and Wang 1993; Verma et al. 1993) (Bredt et al. 1990; Bredt and Snyder 1989; Bredt and Snyder 1994a; Dinerman et al. 1993; Grozdanovic and Gossrau 1996; Marks 1994; Snyder and Bredt 1991). HO-2 was first demonstrated in the vasculature in 1994 in aortic endothelial cells (Ewing et al. 1994). Our group were the first to demonstrate HO-2 expression in the lung. We demonstrated expression of HO-2 in pulmonary artery endothelium and the peripheral nerves of the adventitial layer of pulmonary blood vessels (Ny et al. 1996; Ny et al. 1995; Vollerthun et al. 1995; Zakhary et al. 1996).

Staining for HO-2 was also observed in mesenteric, and basilar artery endothelial cells. While we saw only faint staining for HO-1 and no evidence for the HO-2 in the smooth muscle (Zakhary et al. 1996), both enzymes have been reported in cultured rat smooth muscle (Christodoulides et al. 1995) and CO production has been described from vessel homogenates (Cook et al. 1995; Grundemar et al. 1995). The presence of HO-2 in endothelial cells raises questions about its role in vascular reactivity, and the possibility that CO might function as a novel endothelium-derived relaxing factor similar to NO and indeed that it may contribute to the low tone observed in the pulmonary circulation.

2.2. The selectivity of heme oxygenase inhibitors.

Defining the role of CO in endothelium-dependent relaxation requires selective HO inhibitors. We evaluated the porphyrin inhibitors in both enzyme assay and vascular reactivity studies. Protoporphyrins substituted with various metals (Sn, Zn, Cu) have been widely used as heme oxygenase inhibitors (Maines 1988; Posselt et al. 1986; Vreman et al. 1991). However, there has been considerable controversy regarding their selectivity because protoporphyrins can also inhibit other heme-dependent enzymes important in endothelium-dependent relaxation (Meffert et al. 1994; Zakhary et al. 1996). To determine the specificity of the protoporphyrins, SnPP9 and ZnPP9, their inhibitory effect on the enzymes ecNOS, soluble guanylyl cyclase (sGC) and HO-2 were evaluated and compared with the non-inhibitory protoporphyrin, PP9. Our enzyme assay results demonstrate that SnPP9 is a useful selective inhibitor which is 10 times more potent at inhibiting HO-2 as it is at inhibiting ecNOS or sGC. Furthermore, the enzyme assays suggest that PP9 may be a valuable negative control; 100 μM did not inhibit HO, ecNOS or sGC. Indeed, at 100 μM we

observed a 25% augmentation of sGC activity which had been previously observed (Ignarro et al. 1984). We determined that ZnPP9 was a less useful inhibitor than SnPP9 because it precipitated in krebs solution. ZnPP9 is also 20 times more protein bound than SnPP9 (Greenbaum and Kappas 1991). Curiously, an inhibitory effect of ZnPP9 on ANP (atrial naturetic peptide) and VIP (vasoactive intestinal peptide) mediated relaxation has been reported which may have been through non-specific binding with these peptides, although this possibility was not evaluated in the report (Ny et al. 1995). Given the selectivity of SnPP9 for HO-2 we chose to use it to explore the role of HO and CO in pulmonary arterial endothelium-dependent relaxation.

2.3. NO-dependent and independent relaxation in small porcine pulmonary arteries.

Acetylcholine activates porcine pulmonary artery endothelial cells evoking the release of endothelium-derived relaxing factors that result in smooth muscle relaxation. By inhibiting NOS with L-NAME, relaxation mediated by EDRF's other than NO are uncovered (Zygmunt et al. 1995). L-NAME, at concentrations equal to or lower than used in the these studies, abolishes agonist-evoked endothelium-dependent relaxation in a number of blood vessels including rat aorta, rabbit aorta and proximal pulmonary arteries (Kovitz et al. 1993; Rees et al. 1990a). In other blood vessels, a significant relaxation to endothelium-dependent stimuli remains in the presence of the antagonist (e.g. (Cowan et al. 1993)). However, even in these blood vessels, L-NAME abolishes the increase in cyclic GMP evoked by the agonists, suggesting that the NO-dependent activity is still effectively inhibited (Cowan et al. 1993; Najibi et al. 1994). The L-NAME-resistant relaxant response, which is not associated with cyclic GMP elevation, is associated with hyperpolarization of the smooth muscle and is abolished by inhibition

of potassium channels (Cowan et al. 1993; Nagao and Vanhoutte 1992b; Najibi et al. 1994). Indeed, inhibition of the NOS is generally not associated with inhibition of the hyperpolarization (Nagao and Vanhoutte 1992b)(although there are exceptions when NO does contribute to hyperpolarization, e.g. (Flavahan and Vanhoutte 1995)), and inhibition of the hyperpolarization can be accomplished without affecting the L-NAME sensitive response. Therefore, it has been assumed that the L-NAME resistant relaxation and hyperpolarization is mediated by an NO-independent mediator, endothelium-derived hyperpolarizing factor (EDHF). The concept of EDHF was recently challenged by Cohen et al (Cohen et al. 1997) who proposed that L-NAME does not completely inhibit NOS in the rabbit carotid artery, and that the L-NAME-sensitive and L-NAME resistant components of endothelium-dependent relaxation in rabbit carotid arteries are mediated by NO. This was prompted by their observation that high concentrations of L-NAME or other NOS inhibitors did not completely inhibit NO production in this blood vessel. However, the authors (Cohen et al. 1997) failed to address the fact that mechanisms underlying the relaxation to the L-NAME-sensitive and the L-NAME-resistant responses are distinct. In the rabbit carotid artery, L-NAME abolishes the NO-stimulated cyclic GMP accumulation (Nagao and Vanhoutte 1992b; Najibi et al. 1994). Furthermore, although inhibition of the L-NAME resistant response by charybdotoxin did not reduce the control endothelium-dependent relaxation, it enabled L-NAME to abolish the response (Najibi et al. 1994). This data suggests the L-NAME-sensitive, cyclic GMP-mediated relaxation is entirely distinct from the L-NAME-resistant, cyclic GMP-independent hyperpolarizing response. Although this dichotomy was not addressed by Cohen et al (Cohen et al. 1997), the data suggests that L-NAME resistance does not merely reflect incomplete blockade of NOS. Indeed, the data indicates that L-NAME does abolish NO-cyclic

GMP signaling and the activity of NOS. The source and bioactivity of the L-NAME-resistant production of NO is unknown. One possibility is that the activity reflects release of NO from a preformed pool of the mediator, which would make it resistant to NOS inhibition. However, because the activity is associated with ion channel modulation rather than cyclic GMP signaling, the "NO activity" would have to reside in a form that is chemically distinct from the L-NAME-sensitive "NO activity", such as an altered redox form of NO or a NO-containing molecule (Flavahan and Vanhoutte 1995). The latter possibility would be consistent with stabilization or storage of NO (Flavahan and Vanhoutte 1995). However, an equally likely hypothesis is that the L-NAME-resistant "NO activity" observed by Cohen et al (Cohen et al. 1997) does not represent biologically-active NO. The nature of L-NAME-resistant, endothelium-dependent relaxation must therefore remain uncharacterized and may represent a NO-independent mediator, such as EDHF or CO (Flavahan and Vanhoutte 1995) , or result from non-conventional signaling by NO. Our results demonstrate complete relaxation of small pulmonary blood vessels with acetylcholine. However the presence of L-NAME uncovered significant residual relaxation. Moreover, the cyclooxygenase inhibitor indomethacin, did not effect this residual relaxation implying that it is not accounted for by prostacyclin. Whether this residual relaxation was mediated by CO produced by endothelial HO-2 was therefore evaluated.

2.4. Heme oxygenase inhibitors and endothelium-dependent relaxation.

In the absence of L-NAME, the porphyrin, SnPP9 (10 µM) had no effect on acetylcholine-induced relaxation. This data suggests that low concentrations of SnPP9 do not depress endothelial cell function in a non-specific fashion. At higher concentrations, SnPP9 (100 µM) did decrease this NO-dependent response with an

IC_{50} similar to that exerted on the NOS enzyme in the assay studies. These results would therefore suggest that at high doses SnPP9 inhibits relaxation by its inhibitory effect on NOS.

On the other hand, the acetylcholine-induced relaxation occurring in the presence of L-NAME and mediated by NO-independent mechanisms was highly sensitive to inhibition by SnPP9. Indeed the IC_{50} for this inhibitory effect (7.6 µM) was also similar to the IC_{50} for inhibition of HO-2 in the enzyme assay studies (7.5 µM). This suggests that HO-2, which we demonstrated to be present in endothelial cells, may contribute to NO-independent relaxation. Presumably this results from HO-2 mediated production of CO. CO causes relaxation in a similar manner to NO by stimulating guanylyl cyclase and increasing cGMP (Furchgott and Jothianandan 1991). A role for CO in NO-independent relaxation was further supported by the similar inhibitory effects of ODQ and SnPP9 on L-NAME-resistant, endothelium-dependent relaxation to acetylcholine (Figure 11). Curiously, while SnPP9 100µM did inhibit NO-independent relaxation at lower concentrations of acetylcholine, it did not inhibit maximal relaxation. While we do not know the mechanism responsible for this observation, we speculated that higher concentrations of porphyrins may stimulate guanylyl cyclase thereby counteracting their inhibitory effect on HO. While this has not been described with SnPP9 it can be observed with PP9 (Ignarro et al. 1984).

CO is less than one thousandth as potent as NO as an activator of guanylyl cyclase (Furchgott and Jothianandan 1991). Indeed, because CO and NO both bind the heme moiety of sGC they may interact to modulate enzyme sensitivity. Moreover, CO may indeed have a lower efficacy in activating sGC than NO thereby acting as a "partial agonist". If so, then depending on their relative concentrations, CO could act either to facilitate or to inhibit NO-mediated activation of sGC. To better understand

the mechanism of action of HO in endothelium-dependent relaxation we measured cGMP levels following endothelial activation with acetylcholine in the presence of L-NAME.

2.5. cGMP levels and NO-independent responses.

Changes in heme oxygenase activity can be assessed by analyzing CO mediated changes in cyclic GMP accumulation (Morita et al. 1995; Verma et al. 1993). Our studies demonstrate that in the presence of L-NAME to inhibit NO production, cyclic GMP levels were significantly reduced by removing the endothelium (reduced by 42.9% p<0.05). This suggests that endothelial HO-2 continuously produces CO under baseline conditions. SnPP9 also significantly reduced the endothelium-intact cGMP levels (reduced by 23.9% p<0.05). These results are in keeping with our observations from both our enzyme assay and vascular reactivity studies that SnPP9 at 30 μM should decrease HO activity in intact endothelial cells by approximately 50% (i.e. 42% to 21%) rather than completely abolish HO activity. Furthermore, as expected, SnPP9 had no effect in endothelium-denuded rings (6.5% NS). The absence of an effect of SnPP9 on smooth muscle cyclic GMP levels confirms the selectivity of the inhibitor, and is consistent with the lack of significant expression of HO-1 or HO-2 in porcine pulmonary artery smooth muscle cells under normal physiological conditions. However, in the presence of L-NAME to inhibit NO production, endothelial activation by acetylcholine did not evoke a further increase in cyclic GMP accumulation. While cGMP increased by 38.2% it was not a statistically significant increase. Therefore, while we did not demonstrate statistical significance, there was considerable variability in cGMP levels folowing acetylcholine, and therefore it does raise a question about biological significance. While nitric oxide produces a change in

cGMP by 30 seconds in the order of 1,000% (Furchgott and Jothianandan 1991), one might consider that an increase in the order of 38.2% in cGMP levels following agonist stimulation is not large. However, as we shall discuss in a moment there is evidence to suggest that the same rules that apply to NO and cGMP activation may not be in effect with CO (Schematic 16).

Nevertheless, at first glance these results would suggest that stimulation of the endothelium is not associated with activation of the enzyme HO-2. Although ecNOS is a calcium-dependent enzyme that is activated by, and releases NO in response to, increased intracellular calcium (Busse et al. 1993; Luckhoff et al. 1988), no calmodulin-dependent signaling has been identified in association with HO-2. Therefore, despite a number of similarities between ecNOS and HO-2, a comparable mechanism of agonist-induced activation for CO release has not been described. We therefore speculate that continuous basal activity of the enzyme and continuous CO production amplified endothelium-dependent relaxation to acetylcholine via alternative mechanisms.

These cGMP experiments are open to the criticism, that while there is no statistically significant difference between the control group and the rings treated with acetylcholine, there may be biological significance. Smaller sample sizes in these experiments, and indeed in a number of other experiments in this thesis, are open to the criticism that the minimum number of animals were used to demonstrate a statistically significant effect or to accept the null hypothesis. By "accepting" (that is fail to reject) the null hypothesis when it is indeed false: we commit a type II error. The limitations of small sample sizes are acknowledged. Low numbers in some experiments however may remain convincing where the mean values are similar and the variance relatively small (Figure 4, Heme oxygenase inhibition (SnPP9 10 μM) and NO-dependent

relaxation).

2.5.1. CO, cGMP and NO-independent relaxation.

Endothelium-derived dilator mediators act and interact at the level of the smooth muscle to cause relaxation (Flavahan and Vanhoutte 1995). Although NO, prostacyclin and other endothelium-derived dilators can each cause smooth muscle relaxation independently, they can also amplify the smooth muscle dilator response to the other mediators (Gambone et al. 1997; Shimokawa et al. 1988). For example, prostacyclin can stimulate endothelial cells to release NO, but it also can act in a synergistic manner with NO to cause relaxation (Gambone et al. 1997; Shimokawa et al. 1988). This synergistic interaction appears to be mediated, at least in part, by cyclic GMP-mediated inhibition of cyclic AMP phosphodiesterase (PDE III) (Eckly and Lugnier 1994; Lugnier and Komas 1993; Maurice et al. 1991). Furthermore, interaction between cGMP, cAMP and EDHF at K_{ATP} channels has also been proposed (Gambone et al. 1997).

The continuous basal activity of HO-2 and endothelial production of CO may not have been sufficient to produce relaxation of the blood vessel. Indeed, inhibition of HO activity by SnPP9 did not increase the contractile response to phenylephrine. Furthermore, SnPP9 only affected arterial contractility during endothelium-dependent activation by acetylcholine. Therefore, the background activity of HO-2 and CO may have served to amplify the activity of an endothelium-derived dilator produced in response to acetylcholine. This amplification appears to be cGMP-dependent as it was blocked not only by SnPP9 but also by ODQ. This observation is important because CO has been demonstrated to produce relaxation via cGMP-dependent and independent mechanisms. Activation of K^+_{Ca} channels directly by CO has been

described but would not explain our observations (Coceani et al. 1996; Wang et al. 1997a; Wang et al. 1997b).

Therefore, there are a number of possible mechanisms by which HO-2 and CO might influence vascular reactivity without evoking a further increase in basal cGMP levels. Basal cGMP production by CO may act through the cGMP-dependent phosphodiesterases (PDE III). These phosphodiesterases reduce cAMP levels but are inhibited by cGMP and presumably by CO (Eckly and Lugnier 1994; Lugnier and Komas 1993; Torphy et al. 1993). Reduction in cGMP levels by SnPP9 could therefore result in increased activity of this enzyme and therefore decreased cAMP levels and a reduction in relaxation. Furthermore, ATP-sensitive K^+-channels are regulated by cAMP-dependent kinase. By phosphorylating the K^+-channel the channel is stabilized and activated. Indeed cAMP-dependent kinase appears to be co-localized to the plasma membrane with the K^+_{ATP} channel. Therefore, basal cGMP from endothelial HO-2 production of CO may increase cAMP activity through inhibition of phophodiesterase III activity. This would result in enhanced activation of the K^+_{ATP} channel. The endothelium-derived hyperpolarizing factor(s) (EDHF) can also cause relaxation by activating K_{Ca} or K_{ATP}-channels and may indeed be the alternative mediator responsible for a significant portion of our NO-independent relaxation (Nagao and Vanhoutte 1992a; Standen et al. 1989; Vanhoutte 1993). It has previously been demonstrated that EDHFs can interact synergistically with other endothelium-derived mediators such as NO and PGI_2 to cause endothelium-dependent relaxation (Gambone et al. 1997). Therefore, basal cGMP generation by CO might synergize with cAMP and EDHF, produced in response to acetylcholine to produce relaxation. Neither the cGMP nor cAMP contribute to relaxation alone, but in the presence of EDHF they might enhance relaxation. In hindsight it would have been instructive to

have explored the role of glibenclamide, a K^+_{ATP} channel inhibitor, in NO-independent relaxation in the presence and absence of HO inhibitors.

2.6. Heterogeneity in endothelium-dependent relaxation along the pulmonary artery.

The description of heterogeneity in response to endothelial activators along the pulmonary artery is not new (Kovitz et al. 1993; Zellers et al. 1991). The potential mechanisms responsible for these differences are multiple. Variations in the number and type of endothelial receptors, coupling with different G-proteins, and each mediator having different sensitivities to changes in intracellular calcium, will result in the release of a heterogeneous pattern of endothelium-derived relaxing and contracting factors (Flavahan and Vanhoutte 1995). Moreover, the degree of contraction or relaxation will similarly also depend on smooth muscle receptor and signal transduction characteristics. Our results demonstrate differences in the response to acetylcholine between proximal and distal segments of the pulmonary artery in keeping with previous work on the subject in swine (Kovitz et al. 1993; Zellers and Vanhoutte 1989). While the proximal pulmonary artery relaxes to around 20% of the PE precontaction, distal rings relax almost completely. This is opposite to the observation in the rat pulmonary artery where a brisk relaxation has been observed proximally and a small blunted relaxation in more distal rings (Leach et al. 1992). These differences represent interspecies variability in endothelium-dependent relaxation.

Pretreatment with the NOS inhibitor L-NAME abolished relaxation in proximal arterial rings and resulted in a contraction, similar to that seen following endothelial removal, consistent with uncovering of the smooth muscle muscarinic receptor mediated response. These results indicate that the proximal porcine pulmonary artery

response to acetylcholine is mediated by release of NO, without any significant NO-independent response. By contrast, the distal pulmonary artery demonstrates a significant NO-independent response. Previous authors have described a decreasing influence of NO in more distal vessels, particularly in the microcirculation (Xue et al. 1994) and have demonstrated increased activity of EDHF (Bauersachs et al. 1994). However, while we demonstrate increased NO-independent activity in the distal pulmonary artery, the NO-dependent component was also greater more distally (i.e. NO-dependent relaxation in distal arterial rings was > 50% of the PE precontraction while it was only 20% proximally). On the other hand the expression of endothelial NOS appears to decrease more distally on immunohistochemical staining in keeping with a previous report in young pigs (Hislop et al. 1995). This contradiction, increased NO-dependent relaxation in distal vessels despite decreased NOS expression, is not explained by an increase in the sensitivity of distal smooth muscle cells to NO as the relaxation evoked by the NO-donor SIN-1 (Schror et al. 1989) was identical in both proximal and distal arterial segments. The most likely explanation, therefore, for this difference in NO activity along the porcine pulmonary artery appears related to enhanced signaling in distal compared with proximal endothelial cells in response to agonist.

In contrast to ecNOS we did not observe a difference in expression of HO-2 between the proximal and distal pulmonary artery, but we were unable to demonstrate any HO-dependent component in proximal arteries. Furthermore, while the NO-donor SIN-1 had equal activity along the pulmonary artery, we observed that the response to exogenous CO was different between the proximal and distal vessels. The reason for this difference between exogenous NO and CO was puzzling. Potential explanations for this differential regulation of responses to NO and CO include possible differences

in oxidant stress along the pulmonary artery which could preferentially affect the response to NO and result in diminished relative activity of NO proximally (Gryglewski et al. 1986; Rubanyi and Vanhoutte 1986). Furthermore, the difference in response to NO and CO along the pulmonary artery may occur as a result of additional actions of CO or NO that would alter dilator potency. However, any additional action of the mediators would have to be explained in the light of the finding that responses to CO and NO were inhibited by ODQ, an inhibitor of soluble guanylyl cyclase. Although Furchgott demonstrated that CO is less than one thousandth as potent as NO as an activator of guanylyl cyclase he did not emphasize in his report that the levels of cyclic GMP generated to produce similar degrees of relaxation were significantly smaller when CO was used compared to NO (Furchgott and Jothianandan 1991). By graphing (not done in his paper) the cGMP responses measured following concentrations of NO and CO that produced similar degrees of relaxation in the rabbit aorta this startling revelation is even more apparent (Schematic 16).

This evidence suggests that CO may have effects on the activity or response to cyclic GMP, perhaps by augmenting the action of cGMP dependent kinases, that could be altered between proximal and more distal sections of the pulmonary circulation (Ignarro et al. 1987b). In support of this theory it has recently been demonstrated in the lamb ductus arteriosus that CO produces relaxation without a significant change in cGMP levels despite partial inhibition by antagonists of soluble guanylyl cyclase (Coceani et al. 1996).

Further alternative mechanisms of action for CO have been described. The hyperpolarizing effects of CO can be either cGMP-dependent or independent acting directly through a K_{Ca} channel (Utz and Ullrich 1991; Wang et al. 1997b). Using a patch clamp technique, in freshly dispersed human jejunal circular smooth muscle cells,

cGMP Levels following NO and CO

Similar Effects on Tone
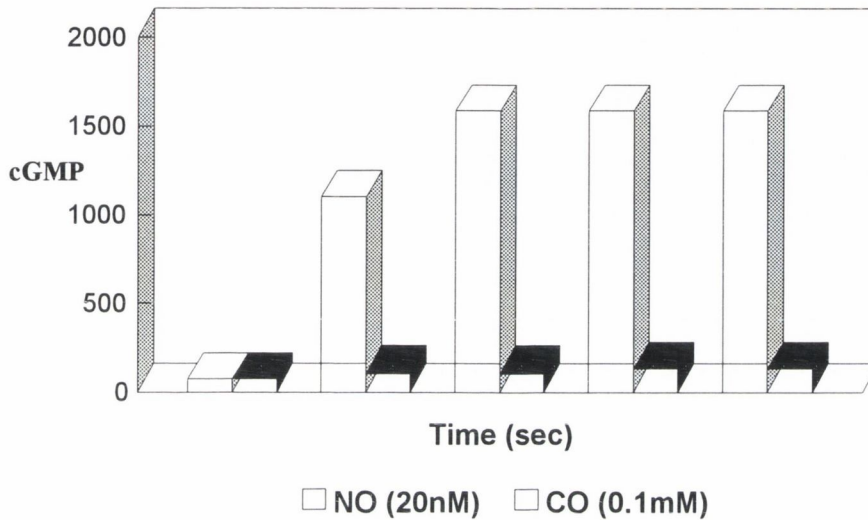
Schematic 16. The effect of exogenous CO and NO on smooth muscle cGMP levels when producing similar degrees of relaxation graphed from data extracted from Furchgott's paper in 1991. cGMP measured as pmol/g protein.

it was determined that exogenous CO can hyperpolarize smooth muscle by stimulating an outwardly directed potassium current (I_K) and leak current (Farrugia et al. 1993). CO thereby hyperpolarized the resting membrane potential (E_M), a finding similar to that described with NO in colonic smooth muscle. Similarly CO has been shown to activate I_K in corneal epithelial cells (Rich et al. 1994).

Therefore, it appears that HO-2 and CO may indeed contribute to the heterogeneity in the porcine pulmonary artery. Despite observing staining for HO-2 in proximal arteries the effect of HO-2 on vascular reactivity is observed more distally where exogenous CO is also more active. By demonstrating increased activity of CO in

distal rings we propose that HO-2, and presumably CO, contribute to the enhanced NO-independent relaxation seen more distally.

While the question of whether L-arginine analogues completely inhibit NOS is currently somewhat controversial, a significant number of observations in this thesis serve to support our conclusions that L-NAME at 3×10^{-5}M (30μM) completely inhibited nitric oxide synthase activity. L-NAME at 3×10^{-5}M completely inhibited endothelium-dependent relaxation to acetylcholine and ET-1 in the proximal porcine pulmonary artery while there was residual relaxation to acetylcholine in distal arterial rings. In the cGMP experiments, the presence of L-NAME completely inhibited any increase in cGMP in response to acetylcholine. If ecNOS in the distal porcine pulmonary artery were not completely inhibited by L-NAME one would expect a substantial increase in cGMP following acetylcholine given that NO is 1,000 times more potent at stimulating sGC than CO. While some authors have used the measured concentration of NO in response to acetylcholine to show that NO release persists despite higher concentrations of L-NAME than used in this thesis, the same authors did not measure cGMP or use hemoglobin or sGC antagonists to confirm that the residual relaxation is indeed mediated by NO (Cohen et al. 1997).

In summary, we demonstrate that endothelial HO-2 may contribute to endothelium-dependent relaxation and perhaps to regional differences in endothelial function in the pulmonary circulation. We propose that CO functions as a novel signaling molecule with a mechanism of action distinct from NO. We suggest that basal release of CO from endothelial HO-2 increases the dilator response to other endothelium-derived dilator mediators, presumably EDHF. This effect is observed in more distal arteries where NO-independent mediators are active and where the dilator activity of CO is enhanced.

3. ENDOTHELIUM-DEPENDENT HYPOXIC CONTRACTION. A NOVEL DIFFUSIBLE FACTOR, DISTINCT FROM ENDOTHELIN.

3.1 Endothelium-dependent hypoxic vasoconstriction is not mediated by endothelin.

The hypoxic response of pulmonary arteries have been studied in many laboratories. With a few exceptions (Holden and McCall 1984; Madden et al. 1985; Ogata et al. 1992; Yuan et al. 1990), it has been necessary to pretreat the vessels with vasoconstrictors in order to obtain responses of sufficient magnitude and reproducibility to study (Archer et al. 1989; Bennie et al. 1991; Hoshino et al. 1988; Jin et al. 1992; Johns et al. 1989; Rodman et al. 1990). Concern that such pretreatment may not be physiologic has been tempered by the realization that *in vivo* vessels are bathed continuously by vasoactive substances and the observation that vasoconstrictors could prevent the loss of hypoxic responsiveness otherwise seen in isolated lungs perfused with physiological salt solutions (Berkov 1974; McMurtry 1984). Precontraction could allow the artery to achieve some threshold state required for the expression of direct hypoxic stimulation as change in vasomotor tone and/or provide a process for indirect hypoxic modulation. These effects could occur in vascular smooth muscle, endothelium or both.

Our *in vitro* model of hypoxic vasoconstriction, which appears to be mediated by an EDCF, is a useful model to evaluate the role of endothelin in hypoxic pulmonary constriction. To define the endothelin receptor phenotype we first explored the response to endothelin in the proximal pulmonary artery. Our results demonstrate that in the proximal porcine pulmonary artery endothelin-1 caused contraction that was inhibited by BQ 123 (Ihara et al. 1992), with a K_B (100 nM) consistent with inhibition

of ET_A receptors (Bax and Saxena 1994). Endothelin-1 also caused relaxation that was abolished by endothelial removal, and by BQ 788 a selective ET_B receptor antagonist (Ishikawa et al. 1994). Therefore, in the proximal porcine pulmonary artery endothelin-1 mediates contraction via the ET_A receptor on the smooth muscle, and relaxation via endothelial ET_B receptors (Sudjarwo et al. 1993). Despite their potency at inhibiting contractile responses to endothelin-1, the endothelin receptor antagonists did not inhibit the endothelium-dependent, late phase hypoxic contraction in the proximal pulmonary artery thereby implying that endothelin does not play a role in mediating this response. However, one possibility is that hypoxia augmented the activity of a distinct endothelin receptor on vascular smooth muscle that was not inhibited by these antagonists (e.g. atypical B receptors, ET_C). Indeed, BQ 123 caused a non-parallel shift in the endothelin-1 concentration-response curve being more potent at low compared to high levels of tension. For example, in endothelium-denuded rings BQ 123 caused a significantly greater shift in the curve at the EC_{30KCl} level compared to the EC_{100KCl} level of tension (log shifts in the concentration-response curve of 1.3 ± 0.2 (20-fold shift) and 0.55 ± 0.14 (3.5-fold shift), respectively, n=5, p<0.05). [EC_{30KCl} and EC_{100KCl} levels are equivalent to approximately 20% and 70% of the maximal response to endothelin-1]. This pattern was not influenced by blockade of ET_B receptors (BQ 788) and may therefore reflect activation, by endothelin-1, of a non-ET_A, non-ET_B receptor of low activity (e.g. ET_C) (Flavahan et al. 1984). However, endothelin-1 ($3 \times 10^{-9}M$), when given to endothelium-denuded rings during anoxia (0% O_2), caused contraction that was still inhibited by the ET_A receptor antagonist BQ 123 ($10^{-6}M$). These results indicate that hypoxia does not uncover a novel ET receptor and that endothelin-1 does not mediate the late phase endothelium-dependent contraction to hypoxia

To further characterize the nature of the hypoxic mediator, transfer experiments were performed using pulmonary valve leaflets as a source of endothelial cells. The cardiac valve leaflet is a thin fibrous flap lined on each side by a confluent layer of endothelium that was confirmed by the endothelium-specific fluorescent dye, Di-L acetylated LDL. Bradykinin, an endothelial activator, evoked a characteristic biphasic increase in intracellular calcium in valve leaflets loaded with FURA-2AM, a similar response to that seen in endothelial cells isolated from the proximal pulmonary artery. We devised a novel method to evaluate endothelial:smooth muscle interaction *in vitro*. By placing a valve leaflet into the lumen of a previously denuded arterial ring endothelial responses; release of EDRF and an hypoxic EDCF, were restored. Although endothelium-denuded pulmonary artery rings normally relax in response to hypoxia, the presence of a valve leaflet in endothelium-denuded rings restored the endothelium-dependent contraction to severe hypoxia (0% O_2). The contraction persisted following L-NAME and indomethacin, confirming that the contraction was not mediated by inhibition of endothelial dilator mediators, but rather indicates the release of a diffusible contractile factor by the endothelium. Placing the leaflet into an endothelium-denuded ring caused a rightward shift in the phenylephrine concentration-effect curve, that was unaffected by L-NAME and indomethacin, or by pretreatment of the valve with distilled water to damage the endothelium. This suggests that the leaflet does not release significant amounts of dilator mediators under basal conditions, but that it does exert a slight mechanical effect on arterial contractility. Indeed, although the presence of the valve in endothelium-denuded arteries restored the late endothelium-dependent contractile response, it did not restore the initial, transient endothelium-dependent contraction to moderate hypoxia (Figure 28) (Kovitz et al. 1993). This transient response is mediated by hypoxic inhibition of the basal activity of

endothelium-derived NO (Kovitz et al. 1993). Therefore this suggests that either the valve leaflet produces less NO than intact endothelium, or that the preparation does not favor optimum transfer of NO to the smooth muscle.

The role of endothelium in mediating or modulating hypoxic pulmonary vasoconstriction *in vivo* has not been clearly defined. Hypoxia has been demonstrated in the absence of endothelium to directly produce contraction in cultured pulmonary artery smooth cells (Murray et al. 1990). Hypoxia may initially increase intracellular calcium through the release of stores in the sarcoplasmic reticulum (Salvaterra and Goldman 1993), or by directly inhibiting the delayed rectifier K-channels in smooth muscle cells, leading to depolarization, thereby opening the voltage-operated calcium channels (VOCC) allowing the influx of extracellular calcium (Post et al. 1992; Yuan et al. 1993). The calcium influx stimulates further sustained increases in intracellular calcium via calcium-induced calcium release (CICR) from internal stores resulting in smooth muscle contraction (Cornfield et al. 1994; Post et al. 1992; Yuan et al. 1993). However, in freshly-dispersed smooth muscle cells, the contraction is rather small and raises the question whether it completely explains the phenomena of hypoxiic pulmonary vasoconstriction (HPV). The endothelium, by releasing a contractile factor, may therefore act to amplify this response (Robertson et al. 1995).

Such an endothelium-derived contractile factor could be released in response to hypoxia (Kourembanas et al. 1991; Kovitz et al. 1993), or it could be released continuously but be active only under hypoxic conditions. In our results the late-phase, endothelium-dependent contraction to hypoxia was observed in pulmonary, but not in systemic arteries. Isolated porcine iliac arteries exposed to anoxia for 45 minutes relaxed by $97 \pm 3\%$ (n=6) of the phenylephrine precontraction. This is in keeping with previous findings in isolated rat systemic arteries (Leach et al. 1994). Current opinion

favors a difference in the arterial smooth muscle response to hypoxia to explain how the pulmonary vascular bed constricts, while systemic arteries relax, in response to hypoxia. Differences in K^+ channels in pulmonary and systemic smooth muscle cells have been demonstrated that appear to support this heterogeneity (Yuan et al. 1990). An alternative possibility is for differences to emerge due to heterogeneity in systemic and pulmonary endothelial responses to hypoxia. Our results demonstrate that while both endothelium-denuded and intact systemic iliac arteries relax in response to hypoxia, the placement of a pulmonary valve leaflet as a source of the hypoxic contractile factor alters the systemic response. The pulmonary valve either evoked hypoxic contraction or inhibited complete hypoxic relaxation (Figure 30). These results suggest that the endothelium, in part, determines the nature of the vascular response to hypoxia.

3.2 Characterization of a novel hypoxic factor.

The slowly developing endothelium-dependent response to hypoxia in the proximal pulmonary artery is similar to that seen following endothelin. However, one feature that is distinctly different from endothelin is the rapid restoration of tone upon reoxygenation. Our results evaluating the effect of endothelin antagonists suggest that endothelin is indeed not the mediator of this hypoxic contraction. We sought to determine the molecular weight of the endothelium-dependent hypoxic 'factor' by placing the valve leaflet in sealed dialysis membrane before placing them in the arterial lumen during organ chamber experiments. While our results are preliminary, two points are worthy of note. Firstly, the 'factor' appeared to be retained by the 25K membrane or to be sufficiently labile that it could not traverse the membrane. Secondly, the valve experiments are open to the criticism that the observed valve-

dependent contraction to hypoxia might be due to mechanisms distinct from the endothelium, such as the result of stearic distortion of the arterial ring and the increased PE requirement to achieve precontraction (PE_{50KCl}). However, while in hindsight it would have been helpful to have repeated the hypoxia experiments with valve leaflets treated with distilled water to explore such non-specific interference with the endothelium-independent relaxation, the dialysis membrane with or without the valve leaflet did not restore hypoxic contraction despite producing significant stearic distortion.

Isolation of the hypoxic factor was also attempted using pulmonary valve leaflets. The factor resisted freezing to $-70^{\circ}C$ and produced contraction under both normoxic and hypoxic conditions. Demonstrating that the factor was not present during normoxia suggests that it is produced in response to hypoxia rather than being always present but active only under hypoxic conditions. The hypoxic effluent had no effect on baseline tone which is also consistent with the observation that in isolated porcine arterial rings hypoxia does not produce contraction without PE precontaction. Finally, the dialysis membrane cut-off suggests that the 'factor' has a MW of >25,000 or that it is sufficiently labile that it cannot cross the membrane. Unfortunately, our isolation experiments are preliminary and incomplete. An endothelium-dependent 'factor' released during hypoxia from isolated rat lungs has previously been presented, however, the proposed factor was considerably smaller than ours (<3000) but equally difficult to define (Robertson et al. 1994). This alternative hypoxic 'factor' from the rat lung appeared to be a peptide that also demonstrated activity during normoxic and hypoxic conditions, but did not have activity in systemic vessels.

3.3 Signal transduction mechanisms in hypoxia.

While increased intracellular calcium plays a central role in the smooth muscle response to hypoxia, the effect of hypoxia on endothelial cell calcium is largely unknown. Our preliminary evidence from FURA-2AM loaded pulmonary valve endothelial cells suggests a small increase in calcium may occur in response to hypoxia, however, we did not observe an inhibitory effect of chelating intracellular calcium using BAPTA-AM on the hypoxic vasoconstriction. However, the implications from these are at best tentative because effective chelation of intracellular calcium was not demonstrated. These results suggest that while hypoxia may indeed increase endothelial intracellular calcium, that release of the endothelium-dependent hypoxic factor may not be calcium dependent.

3.4 Exploring a possible unifying role for HO and CO in the pulmonary circulation.

Hypoxia has been demonstrated to increase HO-1 expression and activity and result in increased CO production with the accumulation of smooth muscle cGMP (Kourembanas and Bernfield 1994; Morita et al. 1995). When co-cultured with endothelial cells, smooth muscle HO generated CO was demonstrated to decrease the expression of the endothelial hypoxia inducible genes, endothelin and vascular endothelial growth factor (VEGF) (Morita and Kourembanas 1995). This was inhibited by SnPP9 and addition of hemoglobin to the culture media (Morita and Kourembanas 1995). So hypoxia, via HO-1 induction and CO production, may modulate the expression of certain genes.

Current evidence points to a heme protein as oxygen sensor in vascular tissue. The sensor responds to a reduction in oxygen tension by activating a number of

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transcription factors (Wang and Semenza 1996). However, CO is an effective oxygen mimic and therefore, if at a high enough concentration, might "fool" the oxygen sensor. Morita et al demonstrated that the increase in HO-1 following exposure to hypoxia decreased within twenty four hours. However, the presence of SnPP9 resulted in sustained elevation in HO mRNA (Morita et al. 1995). Therefore, it would appear that product inhibition controls HO expression; i.e. CO turns off transcription of HO when levels of HO are high. A fall in HO, and therefore CO production, would allow the oxygen sensitive heme sensor to activate again unless oxygen tension had returned to normal in the mean time.

These studies focused on smooth muscle HO-1 induction in response to hypoxia which begs the question what role might constitutive HO-2 in pulmonary vascular endothelium play in modulating endothelial responses to hypoxia. Porcine pulmonary and systemic microvessels (100 - 150 μM) demonstrate a unique difference in the response to hypoxia (Liu and Flavahan 1997). Endothelium-intact coronary microvessels dilate in response to hypoxia, while endothelial-denuded coronary microvessels contract. The current evidence suggests that endothelial-cells from microvessels release a relaxing factor distinct from either NO or prostacyclin that activates coronary smooth muscle K_{ATP}- channels (Liu and Flavahan 1997). In contrast, pulmonary microvessels with or without endothelium contract in response to hypoxia. This would suggest that in the microvascular bed the response to hypoxia is not different between coronary or pulmonary vascular smooth muscle, but that the hypoxic sensor in the pulmonary endothelial cell is somehow turned off. This could support our hypothesis that the background production of CO by pulmonary constitutive HO-2 in endothelium could turn off the hypoxic sensor. Moreover, the coronary endothelium had less pronounced expression of HO-2 (Zakhary et al. 1996).

Testing this hypothesis in microvessels was problematic however because the protophyrins used to inhibit HO are photolabile and light microscopy is required measure microvascular responses to hypoxia. We were unable to demonstrate any effect of HO inhibition with SnPP9 on the hypoxic response in the proximal pulmonary artery.

Therefore, in the porcine proximal pulmonary artery hypoxia produces contraction via an endothelium-derived contracting factor distinct from endothelin. The factor, which is released during hypoxia, has effects on pulmonary as well as systemic arterial rings. The nature of this mediator and the mechanisms responsible for its release and activity remain to be determined.

4. EFFECT OF HO-1 INDUCTION ON VASOREACTIVITY IN RAT AORTA.

We sought to determine the effect of increased endogenous CO production on vascular reactivity in a rat model of HO-1 induction. Injection of hemoglobin, results in induction of HO-1 in multiple organs including, lung, liver, heart, spleen, adrenal gland and kidney (Otterbein et al. 1995). We confirmed that hemoglobin injection also resulted in HO-1 induction in rat aortic smooth muscle (Figure 37). We therefore had a model to induce HO-1 *in vivo* in order to evaluate the effect on vascular responses *in vitro*. By placing aortic rings with increased expression of smooth muscle HO-1 in organ chambers the effect of HO-1 on vascular reactivity could be determined.

This model also allowed us to simultaneously explore the role of HO-1 induction on the vascular effects of sepsis with LPS. Septic shock induced by endotoxin or its active component, lipopolysacharide (LPS), produces hypotension and decreased responsiveness to a wide variety of vasoconstrictors (Guc et al. 1990;

Wakabayashi et al. 1989). Antioxidants appear to be protective and have been shown to inhibit the decrease in vascular tone following endotoxin (Loegering et al. 1995) implying that free radicals play a role in mediating the detrimental effects of LPS. Furthermore, protein synthesis inhibitors also inhibit the depression in contractility, suggesting that new protein synthesis is involved (Beasley et al. 1990; McKenna 1990). Indeed, oxidant stress results in the induction of a number of enzymes (Camhi et al. 1995b), some of which may contribute to the development of septic shock, including iNOS (Scott et al. 1996), cyclooxygenase (Yamanaka et al. 1993) and heme oxygenase-1 (HO-1)(Choi and Alam 1996).

Heme oxygenase-1 (HO-1) has been demonstrated to be highly induced in response to oxidative stress (Applegate et al. 1991; Keyse et al. 1990; Keyse and Tyrrell 1989) and in particular to LPS (Camhi et al. 1995a). It has been suggested that increased levels of HO-1, induced by LPS, may be protective in septic shock (Otterbein et al. 1995). Otterbein et al showed that rats treated with LPS (40mg/kg) die within 10 hours (Otterbein et al. 1995). Pretreatment with an inhibitor of HO, SnPP9, increased the susceptibility of rats to LPS resulting in 100% mortality at 3 hours. However, when hemoglobin, a potent inducer of HO-1, was given 16 hours prior to administration of LPS, mortality dropped to 0%. If SnPP9 was added prior to hemoglobin only 20% of the rats survived (Otterbein et al. 1995). These results suggest that induced heme oxygenase may protect against the detrimental effects of endotoxin. How HO might produce this beneficial effect is not known, although attention has focused on the anti-oxidant properties of bilirubin (Otterbein et al. 1995; Stocker et al. 1987) and on the induction of ferritin by the release of free iron from heme breakdown (Balla et al. 1992; White and Munro 1988). Transition metals such as iron are important in free radical reactions and can amplify oxidant-mediated injury

(Balla et al. 1990). Therefore, by evoking increased expression of ferritin the amount of transition metals available for destructive oxidative reactions would be reduced.

However, HO also produces carbon monoxide (CO) in the breakdown of each heme ring (Maines 1988). Increased concentrations of smooth muscle CO might be expected to stimulate soluble guanylyl cyclase and produce significant vasodepression. Indeed, the vasodepressor effects of LPS might be in part a result of the induction of HO-1 and increased CO production. We therefore evaluated the effect of hemoglobin and LPS, both potent inducers of HO-1, on vascular reactivity to PE and sought to determine if CO produced by induced HO-1 might also influence vascular tone. We also sought to determine whether the protective effect of HO-1 induction by hemoglobin treatment demonstrated *in vivo* was also present *in vitro*, in the response of isolated vascular rings to PE.

Our results demonstrate that pretreatment of rats with hemoglobin attenuated the response to phenylephrine and that the HO inhibitor, SnPP9, reversed the effect of hemoglobin suggesting that HO-1 induction was responsible, perhaps through production of CO. The effect of SnPP9 was specific in this study with no effect noted on control rings. However, the decreased responsiveness to PE following LPS was not influenced by HO inhibition. Indeed, arterial rings were less responsive to PE in the presence of both LPS and SnPP9 together, paralleling the increased mortality noted in rats that were treated with both LPS and SnPP9 (Otterbein et al. 1995). Therefore, our results confirm that HO induction is not the mechanism responsible for the vasodepressor effects of LPS (Wu et al. 1994). While treatment with hemoglobin was protective *in vivo* against LPS (Otterbein et al. 1995), the hemoglobin did not protect arterial rings from the observed reduction in responsiveness following LPS to PE *in vitro* and indeed further depressed vascular responsiveness, over either agent alone.

So while pretreatment of rats with hemoglobin by Otterbein et al. was shown to correlate with induction of HO-1 and was associated with enhanced survival when the animals were subsequently exposed to LPS (Otterbein et al. 1995), hemoglobin did not potentiate or restore vascular responses to PE *in vitro* following LPS. However, our results do support the induction of HO-1 following hemoglobin and indeed suggest enhanced production of physiologically significant CO following hemoglobin treatment. It is possible that circulating hemoglobin could bind increased NO or CO *in vivo* attenuating depressed responses to PE and maintaining tone, an effect not seen *in vitro*. While LPS also increases HO-1, the more substantial effect of LPS on vascular tone is not related to HO-1 induction, and overwhelms any protective effect of increased HO-1 on the response to LPS in our experimental model.

In summary, pretreatment with hemoglobin, a potent inducer of the enzyme HO-1, results in a depressed vasoconstrictor response to PE. This effect is reversed by the HO inhibitor SnPP9, suggesting increased production of CO is responsible for the vasodepression. This may provide a useful model to further explore the mechanism of action of HO and CO in the vasculature. *In vivo* treatment with LPS, also an inducer of HO-1, depressed vasoconstrictor responses to PE, however, this effect was independent of the induction of HO-1.

5. PHOTORELAXATION. ANOTHER ROLE FOR CO?.

We have demonstrated the presence of HO-2 in the porcine pulmonary artery and suggested a possible role for its product, carbon monoxide, in endothelium-dependent relaxation. Furthermore, we demonstrated that induction of HO-1 results in depressed responsiveness to PE in rat aorta, again presumably, as a result of CO. We also explored the phenomena of photorelaxation and explored a hypothesis that CO

might also play a role there. Photorelaxation, vascular relaxation on exposure to light, is a well described phenomena in isolated vessel rings (Furchgott and Jothianandan 1991; Jacob and Tallarida 1977; Trivedi et al. 1978). Furchgott, who over thirty years ago reported that UV light causes relaxation of vascular smooth muscle (Furchgott et al. 1961), went on to demonstrate that that the relaxation was independent of endothelium, and was mediated through a cGMP-dependent mechanism (Furchgott 1991; Furchgott and Jothianandan 1991; Matsunaga and Furchgott 1989) that could be enhanced by the cGMP phosphodiesterase inhibitor M&M 22948 and inhibited by hemoglobin and/or metylene blue (Furchgott et al. 1984). These results would support the presence of a soluble guanylyl cyclase dependent mediator not unlike NO. However, it has been suggested that the hemoglobin used in these experiments may absorb the UV light passing through the chamber media and therefore the inhibition of relaxation may have been artifactual (Furchgott 1991). Similarly, the use of metylene blue has been criticized because it may under certain conditions act as a photosensitizing agent generating reactive oxygen species such as singlet oxygen (Chen and Gillis 1993), thereby enhancing photorelaxation (Ignarro et al. 1984).

Early observations that photorelaxation was potentiated by superoxide dismutase and by inorganic nitrite ($NaNO_2$) (which releases NO in response to light), led a number of authors to conclude that NO was indeed the mediator involved in photorelaxation, although the source of the endogenous NO was not known because NOS antagonists did not inhibit the response (Kubaszewski et al. 1994). The rapid relaxation in response to light and the development of tolerance following repeated exposure led some investigators to the suggest that NO was released from a depletable intracellular store (Kubaszewski et al. 1994; Venturini et al. 1993) and that thiols, glutathione, cystine or albumin, could bind NO and act as an NO sink and decompose

photochemically to release NO (Ignarro and Gruetter 1980; Williams 1985). Alternatively, nitrite which is a product of NO oxidation can be photolytically decomposed with the regeneration of NO (Matsunaga and Furchgott 1989).

An alternative explanation has been that UV light photochemically reduces oxygen yielding H_2O_2 which then activates sGC and increases cGMP (Ignarro et al. 1984). UV light promotes increased H_2O_2 metabolism via catalase with an increase in O_2-consumption, independent of mitochondrial respiration, an effect that is inhibited by hypoxia and cyanide (Wolin et al. 1991). However, carbon monoxide may indeed make a more plausible mediator of photorelaxation than NO or H_2O_2. CO is bound more avidly than oxygen or NO to hemoglobin. However carboxyhemoglobin is 125 times more sensitive to the dissociation effects of light, resulting in rapid dissociation of CO. This was first described by Haldane in 1896 (Haldane and Smith 1896), (Hartridge and Roughton 1923)(Gibson et al 1957). Endogenous CO may therefore be generated and rapidly bound to intracellular hemeproteins, but rapidly released in response to light thereby producing the rapid on and off effect on vascular tone observed. The produced CO would rapidly bind to cellular heme proteins under dark conditions, but be rapidly dissociated in the presence of light to produce cGMP mediated relaxation.

Our results have demonstrated that SnPP9 in the presence of light markedly enhances photorelaxation. However porphyrins are known to increase the release of oxygen radicals in response to light, an effect that should produce vasoconstriction rather than relaxation and SOD should inhibit the effect. However, we saw no effect of SOD in our set-up nor has SOD consistently affected photorelaxation in other studies (Venturini et al. 1993). The photorelaxation in SnPP9 treated arterial rings was inhibited by ODQ and methylene blue, did not require endothelium, and exhibited

tolerance following repeat exposure, suggesting that the traditional pathway for photorelaxation was involved. While the relaxation in response to light was slowly developing initially, once maximal relaxation had occurred the effects on tone of turning lights on and off were rapid. This would seem to suggest the slow production of a substance by the combination of light and SnPP9, but once the substance was produced it was relatively stable. It is worthy of note also that the light used in our experiments was overhead fluorescent tubes rather than wavelength specific UV lamps.

The porphyrins have been known to have a number of light dependent effects. The metal porphyrins are light sensitive photosensitizers, therefore all experiments and drug preparation must be performed in the dark. Moreover, PP9 has been used clinically as a method for the photodynamic destruction of tumors (Land 1984). It is reported that light activates porphyrins to release singlet oxygen species ($^{\cdot}O_2$) that mediate the tumorcidal effects. Light dependent effects of ZnPP9 have recently been described in the rat isolated hepatic artery (Zygmunt et al. 1994) where it was reported that ZnPP9 inhibited NO-independent relaxation in the presence of light, but had no effect in the dark. Following maximum relaxation to acetycholine in the dark, light produced contraction in rings treated with ZnPP9. However, these results are at odds with ours. The reason for the difference is not clear, although the authors do use an alternative approach to drug preparation by using DMSO and pluronic acid rather than the more traditional method of dissolving the SnPP9 at alkaline pH.

So while the porphyrins may produce light dependent effects through the production of radicals, the photogeneration of carbon monoxide by porphyrins has also been studied (Vreman et al. 1990). When SnPP9 (5 μM) is exposed to cool white light from fluorescent tubes CO is generated (Vreman et al. 1990). CO production increases with increasing SnPP9 concentration. Comparison of SnPP9 with other

metalloproteins found that SnPP9 was the most photoreactive and ZnPP9 the least. While SnPP9 by itself in phosphate buffer increased CO production, the effect was relatively small when compared with the effect of SnPP9 and light in the presence of other biological compounds such as NADPH, human albumen and plasma. The production of CO was also related to the oxygen concentration of the head space gas. Higher oxygen concentration presumably increases the likelihood of reactive oxygen species generation. The combination of SnPP9 and NADPH produced CO in response to light, but by 15 min there appeared to be complete photodegradation of NADPH as measured spectrophotometrically. Heme is not a photosensitizing metalloporphyrin and did not generate CO in association with NADPH. We hypothesized therefore that the porphyrins act in the presence of light to produce CO from destruction by radicals of NADPH. The CO produced by the effect of light and SnPP9 could therefore produce the observed photorelaxation that is soluble guanylyl cyclase dependent.

Whether CO accounts for photorelaxation in the absence of porhyrins is tantalizing, but remains speculative. The presence of a photoactivatable store may have important effects *in vivo*. Phototherapy, using overhead fluorescent light, has been demonstrated to prevent closure of the ductus arteriosus in neonates in hospital (Rosenfeld et al. 1986). Infants who require phototherapy for the hyperbilirubinemia associated with prematurity may also be treated simultaneously with metalloporphyrins such as SnPP9 which are approved to reduce the conversion of heme to biliverdin and ultimately bilirubin. The combination of porphyrins and light on vascular tone seen *in vitro* in our experiments may be potentially hazardous *in vivo* to the neonate.

6. GENERAL CONCLUSION

In this thesis we explore the complex interaction between the endothelium and smooth muscle in the control of vascular tone. We describe two novel endothelium derived factors and a new method to explore the relative contribution of endothelium and smooth muscle to vascular responses. Developing the valve leaflet method to produce an 'endothelium transplant' we have perhaps opened the door to a number of new experimental approaches in vascular biology. By treating the valve leaflets separate from the vascular smooth muscle, with antagonists or indeed manipulating gene function, the endothelium can be 'restored' to the arterial ring to measure the effect of these manipulations on arterial function. Furthermore, our *in vivo* induction in the rat of aortic HO-1 with demonstrable effects on *in vitro* vascular function is another novel approach to exploring mechanisms in vascular biology.

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