A study of equine pulmonary blood vessel function *in vitro*

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Abstract

This study characterised the responses of isolated equine pulmonary artery vessels to hypoxia and a range of agonists, and compared responses of isolated bovine pulmonary vessels. *In vivo* studies have demonstrated an inability of the equine pulmonary vascular system to ensure that blood is directed to well-ventilated regions of the lung in lateral or dorsal recumbency. This phenomenon is accentuated when accompanied by general anaesthesia: the resulting hypoxaemia contributes to the morbidity and mortality associated with this procedure. Proper function is further hampered by the lack of a strong hypoxic vasocontrictor (HPV) response redirecting blood away from poorly ventilated lung regions. Both the HPV response and vasoactive agent-induced vasoconstriction have direct medial smooth muscle and endothelial cell components which were examined in this study. A simple and reproducible method of isolating and culturing vascular endothelial cells was developed and used to measure the release of vasoactive substances during normoxia and hypoxia. The rate of release of endothelin from cultured equine pulmonary artery endothelial cells was similar to that from bovine pulmonary artery and equine aorta cells; 4 h hypoxia had no effect on the rates of release from any of these cell types. Hypoxia stimulated prostacyclin release from bovine but not equine pulmonary endothelial cells. Isolated equine pulmonary artery had similar sensitivities to phenylephrine and 5-hydroxytryptamine, yet contracted with approximately 50% of the force when compared to bovine vessels. The contractile response to endothelin appeared to be mediated via the ET_A receptor in equine pulmonary artery. The response of both equine and bovine pulmonary vessels to hypoxia was similar, although bovine vessels contracted more strongly than equine vessels in both absolute and relative terms. In conclusion, equine and bovine pulmonary vessels responded to vasoactive agents and hypoxia, but equine vessels of equivalent diameter contract less strongly. These differences do not appear to be caused by altered endothelial cell function. Instead they may reflect the presence of less smooth muscle within the medial layer and an intrinsically weaker response of equine pulmonary smooth muscle to hypoxia.

Dedicated to the memory of Margaret Pamela Schiemer

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Declaration

I, Karen Elaine MacEachern, do hereby declare that the work carried out in this thesis is original, was carried out by myself or with due acknowledgement, and has not been presented for the award of a degree at any other University.

Publications

Papers

MacEachern, K.E., Smith, G.L. and Nolan, A.M. (1997) Methods for the isolation, culture and characterisation of equine pulmonary artery endothelial cells. *Research in Veterinary Science* (In Press).

Morris, B.J., Simpson, C.S., Mundell, S., MacEachern, K.E., Johnston, H.M. and Nolan, A.M. (1997) Dynamic changes in NADPH-diaphorase staining reflect activity of nitric oxide synthase: evidence for a dopaminergic regulation of striatal nitric oxide release. *Neuropharmacology* (In Press).

Abstracts

MacEachern, K.E., Smith, G.L. and Nolan, A.M. (1995) Investigation of the hypoxic pulmonary vasoconstrictor response in horse tissue. *British Equine Veterinary Congress*, Harrogate.

MacEachern, K.E., Smith, G.L. and Nolan, A.M. (1996) Characterisation of equine vascular endothelial cells *in vitro*. *Association of Veterinary Teachers and Research Workers Meeting*, Scarborough.

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Abbreviations

5-HT	5-hydroxytryptamine
AA	arachidonic acid
AC	adenylate cyclase
ACh	acetylcholine
Bk	bradykinin
BNF	buffered neutral formalin
Ca^{2+}	calcium ions
CaM	calmodulin
cAMP	3',5' cyclic adenosine monophosphate
cGMP	3',5' cyclic guanosine monophosphate
cNOS	constitutive nitric oxide synthase
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
EC_{50}	50% effective concentration
EDHF	endothelium-derived hyperpolarising factor
EDRF	endothelium-derived relaxing factor
ELISA	enzyme-linked immunosorbent assay
ET	endothelin
g	grams
GC	guanylate cyclase
G protein	GTP-binding protein
g tension/wet wt	grams tension/gram wet weight
h	hour(s)
HPV	hypoxic pulmonary vasoconstriction
i.d.	internal diameter
iNOS	inducible nitric oxide synthase
$Ins(1,3,4,5)P_4$	inositol (1,3,4,5) tetra-kis-phosphate
$Ins(1,4,5)P_3$	inositol (1,4,5) trisphosphate
IP_3	inositol triphosphate
L-NAME	N^{ω}-nitro-L-arginine methyl ester

min	minute(s)
MLCK	myosin light chain kinase
mm Hg	millimeters of mercury
mRNA	messenger RNA
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
o.d.	outer diameter
P(A - a) O_2	alveolar arterial oxygen tension difference
P_AO_2	alveolar partial pressure of oxygen
P_aO_2	arterial partial pressure of oxygen
PB	phosphate buffer
PBS	phosphate buffered saline
PE	phenylephrine
pen/strep	penicillin/streptomycin
$PGF_{1\alpha}$	prostaglandin $F_{1\alpha}$ (stable metabolite of PGI_2)
PGI_2	prostacyclin
PIP_2	phosphatidylinositol (4,5)-biphosphate
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PL-A	phospholipase A
PL-C	phospholipase C
PLB	phospholamban
PO_2	partial pressure of oxygen
RIA	radioimmunoassay
s.e.m.	standard error of the mean
SD	standard deviation
sec	second(s)
SFM	serum free medium
SR	sarcoplasmic reticulum
TxA_2	thromboxane (as released from cells, unstable)
TxB_2	thromboxane (stable metabolite)
V/Q	ventilation / perfusion ratio

Chapter 1: General Introduction

1.1 Pulmonary function in the horse

The experimental work described in this thesis consists primarily of *in vitro* measurements from equine pulmonary blood vessels and endothelial cells. Abnormal or inefficient pulmonary function is thought to be the basis for a number of clinical conditions. As summarised in this first section, the physiology of equine lung is not simply a scaled-up version of human lung function; it has several features unique to the horse which are reflected in the behaviour of pulmonary tissue *in vitro*.

1.1.1 Anatomy of the equine lung

The morphology of the equine lung differs significantly from other species, even when compared to mammals of similar size.

1.1.1.1 Gross anatomy

The equine lungs form elongated structures within the thoracic cavity surrounded by a thick pleural membrane. Unlike most mammalian species, including man, equine lungs do not have an obvious lobar structure apart from the separation between left and right lobes. Furthermore, the separation of lobes into lobules is incomplete, giving the equine lung an amorphous appearance, unlike the distinct lobes and lobules of bovine lung.

1.1.1.2 Pulmonary airway organisation

The basic structure of airways common to all mammals consists of a trachea, which divides into right and left main bronchi. These airways divide again to form lobar and then segmental bronchi. This process continues down to the terminal bronchioles and in some species these terminal bronchioles differentiate to form respiratory bronchioles. In the horse, the main bronchus divides within the lung into a small cranial section and a larger caudal section. As described above, the bronchi divide into bronchioles, which divide further to form terminal bronchioles which

end in alveoli. This contrasts with dog, cat and bovine lung which have well developed respiratory bronchioles, prior to their termination in alveoli (Tyler et al 1971). These differences in structure of the terminal bronchial tree may reflect variations in control of ventilation between these species and may underlie the differential response of the respiratory system to various disease processes, exercise and anaesthesia. While the detailed organisation of the equine bronchial tree is unclear, other mammals with incomplete separation of lobules have well developed collateral ventilatory pathways (Tyler et al 1971). Collateral ventilation is the movement of air between adjacent regions of lung through pathways other than the tracheo-bronchial tree, i.e. accessory pathways such as communicating respiratory bronchioles and alveolar ducts. The role of this section of the pulmonary airway in the horse is unknown, it may have a role in prevention of atelectasis (alveolar collapse) in horses suffering from airway obstruction (Robinson 1982; Lekeux 1993), a phenomenon that may be occur in lateral or dorsal recumbency (section 1.1.4.3).

1.1.1.3 Pulmonary vascular organisation

The pulmonary artery divides and accompanies the bronchi into the left and right lobes of the equine lung. These arteries are mainly elastic. They branch and eventually form resistance arteries adjacent to bronchioles; at this level the artery wall is predominantly smooth muscle. The terminal branches of the arteries, the pulmonary arterioles, consist of an elastic lamina and a single endothelial cell layer and lead to the pulmonary capillary network. The pulmonary veins conduct blood from the capillaries to the left atrium of the heart. The bronchial circulation serves airways, large blood vessels and the thick pleura of the equine lung. The bronchial artery also supplies some interalveolar septa in the horse. This type of systemic vessel has not been seen at the alveolar level in any other species other than man (Tyler et al 1971).

1.1.2 Detailed structure of pulmonary blood vessels

The diameter of the main pulmonary artery in an adult thoroughbred is approximately 5 cm. The typical artery is composed of three layers: (i) tunica intima, consisting of a single layer of endothelial cells (50 μm long, 15 μm wide and 1 μm thick), a subendothelial layer of delicate fibroelastic connective tissue and the internal elastic membrane. (ii) Tunica media consists mainly of smooth muscle cells (50 - 100 μm long, 5 - 8 μm diameter), arranged in a circular fashion. Interspersed between the smooth muscle cells are varying amounts of elastic and collagenous fibres. (iii) The outer coat, the tunica adventitia is composed of longitudinally arranged smooth muscle cells and connective tissue. Pulmonary arteries are thinner walled than equivalent vessels in the systemic circulation. This is associated with a lower blood pressure in the pulmonary circulation (Leeson et al 1985). The wall of the main pulmonary artery consists mainly of elastic fibres with few smooth muscle cells. As this vessel branches to form muscular arteries (0.3 - 10 mm i.d.), the composition of the wall changes to favour smooth muscle with relatively fewer elastic fibres (Rhodin 1980).

Resistance arteries (and arterioles) have a diameter of 0.1 - 0.3 mm; the tunica intima consists an endothelium and internal elastic membrane. No subendothelial tissue is recognisable. The tunica media is composed of 1 to 5 distinct layers of muscle cells, among which are some scattered elastic fibrils. The number of layers of muscle decrease as the calibre of the vessel decreases. Arterioles are able to control the distribution of blood to different capillary beds by vasodilation and vasoconstriction in localised regions. They are the prime controllers of systemic and pulmonary blood pressure. In the adult pig and cow, the medial layer of these small pulmonary arteries is significantly thicker than those of horse, sheep and dog which have only a thin smooth muscle layer (Robinson 1982). The amount of smooth muscle in the medial layer determines the ability of the vessels to constrict to stimuli including alveolar hypoxia and other neural and humoral stimuli (Robinson 1992).

1.1.3 Ventilation of the equine lung

Ventilation is the movement of gas in and out of the alveoli. A resting horse of about 480 kg body weight might typically have a respiratory frequency (f) of 12·min^{-1} and a tidal volume (V_T) of 5 l, giving rise to a minute ventilation (V_E) of 60 l·min^{-1} (Robinson 1991). The tidal volume consists of a physiological dead space and a portion participating in gas exchange (alveolar ventilation). The physiological dead space is made up of the conducting airways (anatomical dead space) and a volume associated with poorly perfused alveoli, where gas exchange can not occur optimally (functional dead space). In the horse, the percentage of the tidal volume occupied by physiological dead space is approximately 60% (Robinson 1991), compared to approximately 30% in man (West 1990). This high value of physiological dead space in the horse can increase further at higher ventilation frequencies.

1.1.3.1 Influence of gravity and posture on pulmonary ventilation

The influence of gravity on human lung function was described by West (1964). This work indicated that the main determinant of ventilation in the lung was the degree of distension. West (1964) showed that the weight of the lungs suspended within the thoracic cavity caused the intrapleural pressure to be more subatmospheric in the upper (dorsal) region of the lung than the in lower parts (ventral), suggesting that the upper lung is more distended and less compliant than the dependent (ventral) regions. During inhalation, air will enter more compliant (ventral) regions of the lung preferentially, giving rise to a vertical gradient of ventilation (West 1977a). Similar vertical gradients of ventilation have been observed in the standing horse (Amis et al 1984), that were matched by a gradient of perfusion as shown in Figure 1.1 and further discussed in section 1.1.4.

In addition to gravity, posture and general anaesthesia markedly influence the ventilation of the lungs. The recumbent horse can adopt 3 basic positions, (i) sternal recumbency, where the horse rests on the sternum and abdomen, (ii) lateral recumbency; i.e. lying on left or right side and (iii) dorsal recumbency, i.e. the horse

lies on its back, a posture not uncommon during surgery. Posture alone is a difficult factor to study since horses do not normally spend significant time in dorsal or lateral recumbency. While a number of studies have investigated changes in alveolar gas composition in conscious recumbent horses (Hall 1984; Rugh et al 1984), little work has directly addressed the pattern of ventilation of equine lungs in the absence of anaesthesia. McDonell & Hall (1974) reported a reduced functional residual capacity of anaesthetised laterally recumbent horses, in particular there was a greater reduction in the capacity of the lower lung. Dorsal recumbency was shown to reduce the ventilation of equine lungs even more than lateral recumbency (Nyman et al 1987). The cause of the reduced ventilation is thought to be due to compression of the lower lung by the abdominal viscera causing collapse of the alveoli. The horse is thought to be particularly prone to this phenomenon due the prominent dome shape of the diaphragm (Taylor 1984).

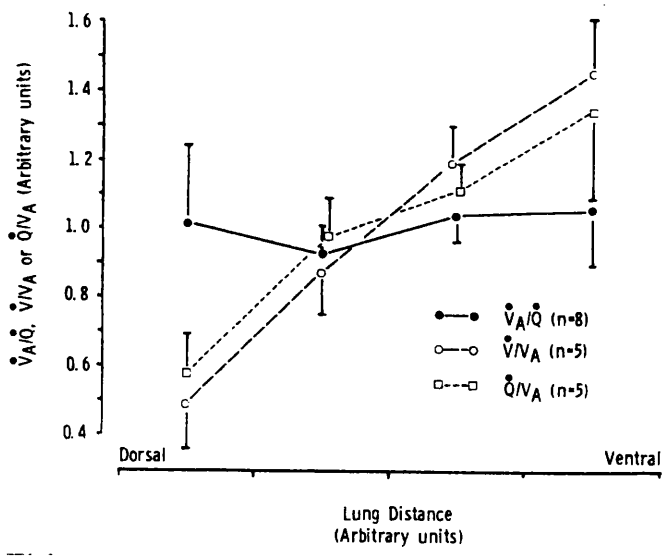


Figure 1.1: Vertical distribution (mean $\pm$ 1 SD) of ventilation perfusion ratio (V_A/Q), pulmonary ventilation (V/V_A) and pulmonary perfusion (Q/V_A) in the lungs of healthy conscious standing horses at the level of the ninth rib. Adapted from Amis et al (1984).

1.1.3.2 Equine ventilatory pattern

Horses, donkeys and mules are obligate nasal breathers and exhibit a biphasic inspiratory and expiratory ventilatory pattern which is unique to equidae. Koterba

et al (1988) suggested that the cause of the biphasic pattern is an active and passive component to both inspiration and expiration. Unlike most mammals, the horse uses both the intercostal muscles and diaphragm for breathing at rest. Inspiration is caused by relaxation of intercostal muscles followed by a delay of 1 - 2 seconds, then by contraction of the diaphragm. Conversely, expiration is caused by relaxation of the diaphragm followed after 1 - 2 seconds by contraction of the intercostal muscles. This unique ventilatory technique probably reflects an adaptation to the unusually high resistance to flow presented by nasal breathing and a long trachea (Lavoie et al 1995).

1.1.3.3 Pulmonary ventilation during exercise

Exercise is an activity which highlights the differences in pulmonary function between human and equine. Studies have revealed consistent changes in the respiratory frequency and tidal volume associated with varying levels of exercise as illustrated in Table 1.1 (Bayly et al 1987; Koterba et al 1988; Wagner et al 1989).

	n	Velocity ($m \cdot min^{-1}$)	Step frequency (min^{-1})	Respiratory frequency (min^{-1})	Tidal volume (l)	Minute volume ($l \cdot min^{-1}$)
Standing	23	0	0	35 ± 7	4.4 ± 1.5	157 ± 63
Walk	30	98 ± 8	60 ± 4	71 ± 7	6.0 ± 1.0	421 ± 68
Slow trot	30	222 ± 25	87 ± 6	82 ± 8	8.1 ± 1.5	653 ± 101
Fast trot	28	288 ± 28	92 ± 4	85 ± 8	9.1 ± 1.6	766 ± 114
Canter	26	356 ± 36	109 ± 4	107 ± 4	8.9 ± 1.5	950 ± 148
Gallop	27	532 ± 70	120 ± 8	118 ± 8	11.3 ± 1.4	1337 ± 165

Table 1.1: Gait and respiratory parameters at different exercise levels in the horse (adapted from Hornicke et al 1983).

While maximally exercising humans can increase their minute ventilation by 15 times the value at rest, horses can only manage a factor of 10 times their resting value during a fast gallop. Table 1.1 also illustrates the very tight coupling between step frequency and respiration frequency. Detailed studies by Attenburrow (1983)

suggest that there is an interaction between the muscles of locomotion and the mechanics of respiration, such that during the canter and gallop respiration is mechanically assisted by limb movements. Despite this mechanical assistance, significant alveolar hypoxia and hypercapnia develops during exercise in the horse (Bayly et al 1987; Erickson et al 1994; Wagner 1995), resulting in significant hypoxaemia. The main cause for this phenomenon is inadequate ventilation (Wagner et al 1989) and contrasts to the situation in humans, where only the mildest alveolar hypoxia occurs during extreme exercise. The basis for the restricted increase in ventilation frequency in horses may be the requirement for respiration and gait to be synchronised during exercise (Attenburrow 1983).

1.1.4 Pulmonary perfusion in the equine lung

The equine pulmonary vascular system receives the output from the right side of the heart, which is approximately 30 - 40 $l \cdot min^{-1}$ in the standing thoroughbred (Bonagura & Muir 1991). Horses have a mean pulmonary artery pressure of approximately 30 - 40 mm Hg (Robinson 1991), higher than most other mammals including humans (approximately 15 mm Hg, Detweiler 1993). It should be noted that, unlike the systemic circulation, pulmonary artery pressure depends on both the pulmonary arteriolar resistance and pulmonary capillary resistance. It is possible that the unusually high pulmonary pressure in the horse at rest may be a consequence of a chronically active hypoxic pulmonary vasoconstrictor (HPV) response. However, Pelletier & Leith (1993) studying standing and exercising horses were able to monitor pulmonary pressures while controlling inspired gases. Their results showed that raising the PO_2 of the inspired gases above normal levels did not affect the mean pulmonary pressures suggesting that the high pulmonary pressures in the standing and exercising horse were not caused by an active HPV response. As yet, there is no satisfactory explanation of the high pulmonary vascular resistance in the standing horse.

1.1.4.1 Hypoxic pulmonary vasoconstriction

Von Euler & Liljestrand (1946) first proposed the mechanism of hypoxic pulmonary vasoconstriction whereby pulmonary capillary blood flow is adjusted to alveolar ventilation. A decrease in oxygen tension induces vasodilation in most systemic arteries but leads to vasoconstriction in the pulmonary arterial bed (Staub 1985). The strength of the HPV response appears to vary across the mammalian species. In domestic animals, the HPV response has been found to be most vigorous in cattle and pigs, less vigorous in horses and minimal in dogs and sheep (Bisgard et al 1975; Elliott et al 1991). Since von Euler & Liljestrand (1946), a considerable amount of work has been done to determine the mechanisms underlying HPV and the segments of the pulmonary vascular tree responsive to hypoxia. Early work investigated whether the HPV response was a result of systemic hypoxaemia, detected by the carotid and aortic chemoreceptors, and mediated via a reflex increase in sympathetic tone to the pulmonary blood vessels. While this reflex appears to be highly developed in the foetal circulation of most mammals, it is not thought to make a significant contribution to the HPV response in adults (reviewed by Fishman 1976). Instead, HPV responses are observed in denervated lung, isolated lung segments and in isolated pulmonary vessels (reviewed by Staub 1985) suggesting that the HPV response is a local reaction to reduced PO_2. The cellular basis for the local HPV response is not understood; the range of possible mechanisms and the experimental evidence is discussed in detail in section 1.4.

1.1.4.2 Sites of hypoxic pulmonary vasoconstriction

Work on isolated perfused lung preparation has shown that the hypoxic stimulus determining the HPV response is a function of alveolar PO_2 and that the sites of vasoconstriction are located within the small precapillary arterioles (100 - 300μm diameter, Marshall & Marshall 1983; Voelkel 1986). However, recent work has shown that an HPV response is seen in both small (1 - 3 mm) and large (3 - 10 mm) pulmonary arteries (Harder et al 1985; Rodman et al 1989; Kovitz et al 1993) and raises the question as to the role of the large pulmonary arteries in the HPV response. While these larger vessels will be influenced directly by alveolar

hypoxia, the bronchial circulation supplies the vasa vasorum to the walls of all but the smallest arteries. Thus the PO_2 within the wall of large vessels will be influenced by both alveolar and arterial PO_2. However, it is difficult to assess the contribution that the HPV response in large vessels alone can make to the total pulmonary vascular resistance. In an elegant set of experiments, Marshall et al (1991) perfused hypoxic blood through the bronchial artery of a sheep while ventilating the lungs with air, thus maintaining alveolar, arterial, and mixed venous PO_2. With this system, they applied a hypoxic stimulus to all but the smallest arteries and measured the resulting change in vascular resistance. Their results showed that hypoxic vasoconstriction of the large pulmonary arteries alone can make a significant contribution to the increased vascular resistance seen in hypoxia. One shortcoming of the study was the lack of comparison of the increased total pulmonary vascular resistance due to alveolar hypoxia with that which was simply due to hypoxic vasoconstriction of the large pulmonary arteries. Thus, while the major site for the hypoxic vasoconstrictor response appears to be the small precapillary arterioles, vasoconstriction of the larger arteries (greater than approximately 1 mm diameter) may also play a role in the increased pulmonary resistance.

1.1.4.3 Influence of gravity and posture on pulmonary blood flow

As with ventilation, gravity determines the distribution of blood flow throughout the lung. The concept of separating the ventilation and perfusion of the lung into different zones arose from studies on human lungs (West 1977b). As illustrated in Figure 1.2, in upper regions of the lungs, the pulmonary arterial pressure may be less than alveolar pressure, and under these conditions, flow through the pulmonary capillaries is not possible (Zone I).

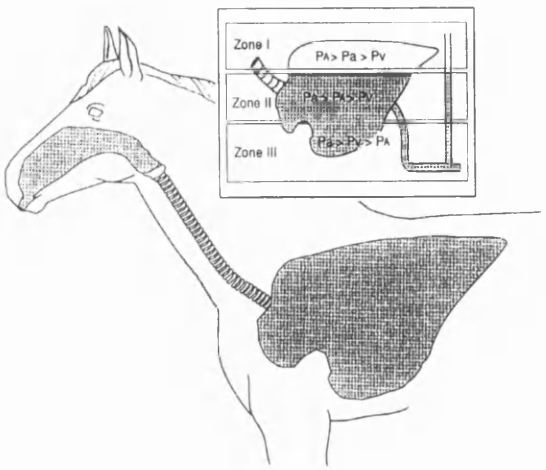


Figure 1.2: The zones of pulmonary perfusion in the horse (pulmonary arterial pressure (Pa), pulmonary venous pressure (P_V) and alveolar pressure (P_A)). Adapted from Robinson (1991).

Further down the lung, pulmonary arterial pressure is greater than pulmonary alveolar and venous pressure (Zone II), under these conditions the capillary is open for part of its length until the point where alveolar pressure exceeds capillary pressure, thus blood flow in zone II will occur only during systole. In the lower part of the lung, (Zone III) pulmonary arterial and venous pressure both exceed alveolar pressure and capillary beds are perfused throughout their lengths. Finally, in humans, a Zone IV is thought to exist in the base of the lung where compressed lung tissue is associated with increased pulmonary vascular resistance and low perfusion. Amis et al (1984) measured a vertical distribution of perfusion and ventilation in the equine lung consistent with zones I - III, as in humans zone I was at the top of the lung (dorsal region in the standing horse) and zone III was at the lowest point in the lung (ventral region) as illustrated in Figure 1.2. Thus both ventilation and perfusion increase towards the ventral regions of equine lungs. The horse in sternal recumbency is thought to have a similar distribution of blood flow to that seen in the standing horse (Amis et al 1984), i.e. higher perfusion in the lower lung than the upper lung. However, in lateral recumbency, instead of the blood flow being directed towards the lower lung, as expected from the analysis by West et al (1964),

blood flow was found to be higher in the uppermost lung in both anaesthetised and conscious horses (Staddon & Weaver 1981; Stolk 1982). The cause of this unusual distribution is unknown, although Stolk (1982) proposed that poor ventilation of the lower lung causes the redistribution of perfusion through the actions of the HPV response. However, there is no experimental evidence to support this hypothesis. Alternatively, mechanical compression of the lower lung by the viscera may occlude blood vessels and prevent blood flow. In support of this view, McDonell et al (1979) has presented radiographic evidence of marked compression of the lower lung in anaesthetised horses in lateral recumbency.

Dorsal recumbency was found to reduce pulmonary perfusion to a greater extent than lateral recumbency (Nyman et al 1987; Nyman & Hedenstierna 1989) possibly due to the compression of the caudal-dorsal regions of both right and left lung by the viscera due to the slanting diaphragm of the horse as illustrated in Figure1.3.

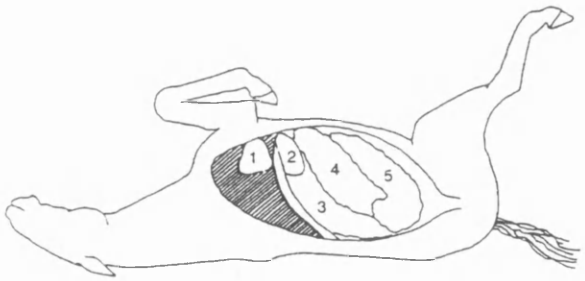


Figure 1.3: The horse in dorsal recumbency, illustrating the relationship between lungs, diaphragm and abdominal organs. Note that the slanting diaphragm allows the abdominal organs to press on the diaphragmatic lobes of the lung. 1 = heart; 2 = stomach; 3 = dorsal colon; 4 = ventral colon; 5 = caecum. Adapted from Nyman et al (1987).

1.1.4.4 Pulmonary perfusion during exercise

During exercise, cardiac output of the average thoroughbred increases from 30 $l \cdot min^{-1}$ to 180 - 190 $l \cdot min^{-1}$, approximately a 6 fold increase. This compares with a 3 - 4 fold increase in humans (Erickson 1993). One factor that contributes to the increased cardiac output in the horse is the mobilisation of the splenic reserve of red blood cells during exercise, which boosts the blood volume by 30% of its resting value and augments the oxygen carrying capacity of the blood. Pulmonary arterial

pressure increases markedly during exercise (> 80 mm Hg in horse vs < 37 mm Hg in human, Wagner et al 1986; Erickson et al 1992). As mentioned above, significant hypoxaemia and hypercapnia develop during exercise (Jones et al 1989) and while hypoventilation is thought to be the main cause, limited oxygen diffusion between the alveoli and the blood supply is also a significant factor (Bayly et al 1987; Fujimoto et al 1990; Wagner 1995). In summary, exercise induced arterial hypoxaemia in the horse may be due to the inadequate matching of increased cardiac output with ventilation rate.

1.1.5 Ventilation perfusion ratios across the equine lung

It has been recognised for several years that the degree of gas exchange in any lung unit is determined by the ratio of ventilation to blood flow (perfusion) (reviewed by West 1982). As described above, the ventilation and perfusion of the lung is region dependent, with the ventral region of the lungs being both better ventilated and perfused than the dorsal region. The ventilation perfusion ratio can vary between two extremes. At one extreme, non-ventilated alveoli are perfused (shunt) giving rise to a V/Q value of 0; at the other, the ventilated alveoli have no perfusion associated with them (dead space), giving a V/Q ratio of infinity. The method to assess the distribution of V/Q ratios throughout the lung is based on airway elimination of a series of inert gases (West 1977b). The analysis of the exhaled gases enables the allocation of ventilation and perfusion to 50 different compartments each with a specific V/Q ratio ranging from zero (shunt) to infinity (dead space). Between the two extremes are 48 compartments which are evenly distributed along a logarithmic axis from 0.005 to 100. An example of a normal human and equine V/Q distribution are shown in Figure 1.4 and Figure 1.5A. Comparison of the distributions associated with the two species suggests that there is a narrower distribution of V/Q values in the equine lung compared with humans. This has been confirmed by independent measurements (Amis et al 1984).

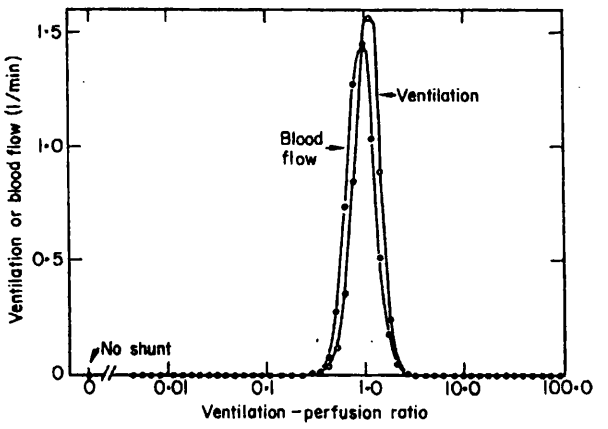


Figure 1.4: Ventilation perfusion ratio distribution in human lung (adapted from West 1977)

1.1.5.1 Effect of posture on ventilation perfusion ratio

There appear to be no reports of the ventilation perfusion distribution in the conscious recumbent horse, although a number of papers have been published examining the changes in ventilation and perfusion characteristics of the whole equine lung without information about distribution. McDonell & Hall (1974) showed that lateral recumbency caused a reduced perfusion of equine lung, particularly in the lower dependent lung and an even greater reduction in ventilation. However, it is unlikely that the laterally recumbent conscious horse develops a significant ventilation perfusion mismatch since only a small fall in alveolar PO_2 has been observed in the absence of general anaesthesia (Hall 1984; Rugh et al 1984).

1.1.5.2 Ventilation perfusion ratios during exercise

Arterial hypoxaemia is commonly observed during exercise in the horse (section 1.1.3.3). A common cause of hypoxaemia is a mismatch of ventilation and perfusion which would be indicated by an increased spread of the V/Q ratio distribution. Yet measurements by Wagner et al (1989) indicated that the narrow range of V/Q ratios observed at rest in the horse were not altered by exercise. A significant mismatch of ventilation and perfusion develops in humans during exercise. Equivalent measurements on exercising horses suggests a mismatch of ventilation and perfusion does not develop during exercise and therefore cannot be

the basis hypoxaemia in the exercising horse. Instead, these authors concluded that most of the exercise-induced hypoxaemia is caused by insufficient time for oxygen diffusion across the capillary wall.

1.2 Equine general anaesthesia

Most thoroughbreds undergo general anaesthesia at some time in their lives. A recent survey indicated that most equine operations involve a period of general anaesthesia between 30 and 90 minutes, but up to 4 hours is not uncommon (Johnston et al 1995). Prolonged periods of general anaesthesia in horse are produced largely by the administration of the gaseous anaesthetic agent halothane, although newer, more modern anaesthetic agents such isoflurane and enflurane are sometimes used.

1.2.1 Problems of equine general anaesthesia

There are many potential problems associated with equine general anaesthesia. The temperament and large size of the horse has, in the past, hindered the administration of anaesthetic drugs. Once anaesthetised, horses tend to become hypotensive and hypoxaemic. Recovery from anaesthesia is often complicated by post-anaesthetic myopathy. Not surprisingly, the rate of mortality after general anaesthesia is high compared to other species. These topics are discussed in more detail below.

1.2.1.1 Anaesthesia-induced hypotension

There are several causes of hypotension in horses undergoing general anaesthesia. Reduced blood volume due to dehydration or haemorrhage will significantly lower arterial blood pressure. Halothane, isoflurane and enflurane are known to depress cardiovascular function in the horse, in particular causing reduced cardiac output and arterial blood pressure (Steffey 1991). The cause of reduced cardiac output in the horse is a direct effect of halothane on the ventricular myocardium reducing the force of contraction leading to a reduced stroke volume (Bonagura & Muir 1991). Normal anaesthetic concentrations have been found to depress cardiac output by approximately 27% and may contribute to systemic and pulmonary hypotension and

hypoxaemia (Steffey & Howland 1978). The reduced systemic and pulmonary blood pressures may be due to markedly reduced cardiac output, anaesthetic agents may also act directly on the vasculature, baroreceptors and the cardiovascular control centre within the central nervous system (Soma 1980).

1.2.1.2 Arterial hypoxaemia in anaesthetised horses

Measurements of arterial PO_2 (PaO_2) in conscious standing horses breathing air ranged from 90 to 125 mm Hg (Hall et al 1968; Gillespie et al 1969; Nyman et al 1987). Within 30 minutes of the beginning of inhalational anaesthesia, a 1.2% halothane / 95% O_2 mixture, the PaO_2 rises towards 200 mm Hg (Gleed 1988). However, after approximately 30 minutes, PaO_2 returns towards normal levels, and in some cases below normal levels (arterial hypoxaemia, Hall et al 1968; Mitchell & Littlejohn 1974; Nyman & Hedenstierna 1989). While the term 'hypoxaemia' refers to reduced oxygen content of arterial blood, there is no generally accepted quantitative definition. During general anaesthesia, a patient is considered hypoxaemic when the PaO_2 falls below 60 mm Hg, i.e. when haemoglobin is less than 90% saturated with O_2. The lateral recumbent posture in conscious horses does not appear to cause significant hypoxaemia, with only slight falls in arterial oxygen reported (to 80 - 90 mm Hg, Hall 1984; Rugh et al 1984). Yet anaesthetised horses in either dorsal or lateral recumbency develop significant hypoxaemia despite the use of 95 - 100% O_2 in the inspired gases. This marked hypoxaemia was reversed when the horses were turned onto their sternum (Gleed 1988). These studies suggest that anaesthesia makes oxygen exchange in the equine lung extremely sensitive to posture; that is, lateral or dorsal recumbency in an anaesthetised horses can give rise to very large differences between alveolar and arterial oxygen tension ($P(A\text{-}a)O_2$) which can frequently result in arterial hypoxaemia. The physiological basis for these effects are still unknown.

1.2.1.3 Ventilation perfusion distribution in recumbent anaethetised horses

Nyman & Hedenstierna (1989) examined the ventilation perfusion distribution in conscious and anaesthetised horses. Their study clearly showed that the equine lung

develops perfused but unventilated regions when horses are anaesthetised and laterally recumbent. This is evident from the ventilation perfusion distributions illustrated in Figure 1.5. The degree of ventilation/perfusion mismatch was worse in dorsal recumbency. The authors concluded that anaesthesia caused collapse of alveoli during recumbency and that this was the main cause of hypoxaemia routinely observed in this situation.

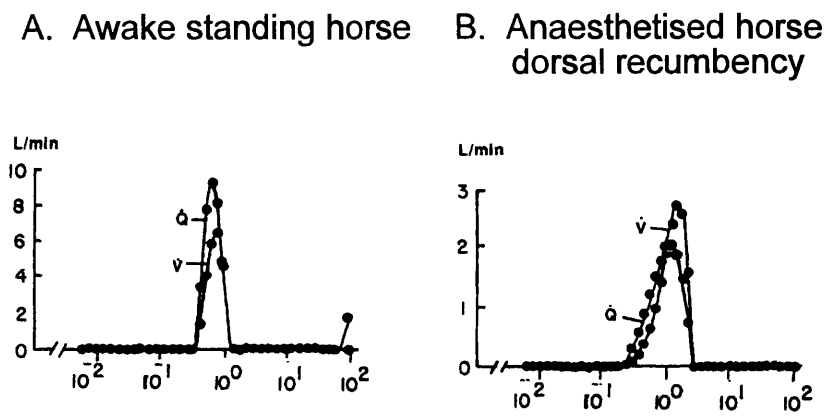


Figure 1.5: Ventilation perfusion (V/Q) ratio distribution in equine lung, **Panel A:** awake standing horse, **Panel B:** anaesthetised horse in dorsal recumbency. Adapted from Nyman & Hedenstierna (1989).

1.2.1.4 Effects of anaesthesia on hypoxic pulmonary vasoconstriction

As described in section 1.1.4.1, HPV aids in the regulation of ventilation (V) perfusion (Q) matching, ensuring that blood is redirected to better ventilated areas of the lungs (Dawson 1984). Studies on mammalian lungs, other than in horse, have shown that inhalational anaesthetic agents can blunt the HPV response (Fishman 1985) but the underlying mechanisms are unknown. Prior to the work described in this thesis, no *in vivo* or *in vitro* studies have been done to examine the sensitivity of the blood vessels of the equine lung to hypoxia or anaesthetic agents.

1.2.1.5 Strategies designed to alleviate arterial hypoxaemia

Several methods have been suggested to improve the PaO_2 in horses under general anaesthesia: (i) increase the inspired PO_2 to close to 100% (Klein 1990), (ii) mechanically assist or control ventilation (Shawley & Mandsager 1990), (iii)

increase cardiac output by administration of fluids, reduce anaesthetic dose, use of β-adrenoreceptor agonists (dopamine, dobutamine) (Daunt 1990), and/or (iv) change the horse's position to sternal recumbency (Muir 1991).

Recently, Gleed and Dobson (1990) reported that the β_2-adrenoceptor agonist clenbuterol markedly improved blood oxygenation in horses anaesthetised in dorsal recumbency, but not in sternal recumbency. β_2-adrenoceptor agonists, acting via adenylate cyclase, normally vasodilate systemic blood vessels, however, these actions may be modified in the presence of altered vascular tone and/or hypoxia (McCormack et al 1989). Since hypoxaemia can alter existing vascular tone, the mechanism of action of clenbuterol may be altered in the hypoxic pulmonary vasculature.

1.2.1.6 Post anaesthetic myopathy

In addition to the deleterious effects of hypotension during anaesthesia, Gandy et al (1987) have suggested arterial hypotension is the cause of post-anaesthetic myopathy. Post-anaesthetic myopathy is a potential complication of general anaesthesia and controversy surrounds its cause. The condition appears in the forelimb more often than the hind limb, suggesting damage to the radial nerve. Trim & Mason (1973) measured increased lactate and creatine phosphokinase levels in all cases of anaesthetic induced myopathy. This result suggests that myopathy is caused by ischaemic muscle damage secondary to inadequate blood flow.

1.2.1.7 Mortality associated with anaesthesia

The incidence of mortality and serious morbidity in a mixed population of horses associated with general anaesthesia is greater than 1% (Johnston et al 1995). An anaesthetic-related mortality rate as high as 10.5% has been reported in horses undergoing colic surgery (Ducharme et al 1983) with an average rate of 6.9% in a survey of mortality associated with emergency laparotomy (Hodgson & Dunlop 1990). This is considerably higher than in any other domestic species. Clarke & Hall (1990) carried out a survey at 53 small animal practices comprised of 41,881 general anaesthetic procedures. They found 0.15% healthy cats and dogs died

primarily as a result of anaesthesia. In man, the figure is considerably lower (0.01%) (Lunn & Mushin 1982). The relative importance of factors which influence the probability of mortality during anaesthesia is unknown (Young & Taylor 1993). However, it is apparent that the arterial hypoxaemia that commonly occurs during general anaesthesia depresses the ability of the cardiovascular system to maintain oxygen levels in such vital organs as the brain and the heart. Clearly a better understanding of equine pulmonary vascular physiology would aid the design of anaesthetic procedures with reduced mortality and morbidity.

1.3 Cellular physiology of the pulmonary artery

The changes in pulmonary vascular resistance described above are mediated by vasoconstriction or vasodilation of pulmonary arteries and arterioles. Vascular smooth muscle tone regulates vessel diameter and can be influenced by factors that affect the muscle directly or via effects on the endothelial cell layer within the tunica intima. As described below, a number of agents are released from the endothelial cell layer which act on the underlying smooth muscle to change vessel diameter and wall tone. In the next sections, vascular endothelial and smooth muscle cell function are discussed in the context of the mammalian pulmonary arterial system.

1.3.1 Vascular endothelial cell function

Moncada & Vane (1979) were the first to demonstrate a functional role for the endothelial cell layer of blood vessels They showed that the intimal layer released prostacyclin (PGI_2) which could subsequently vasodilate the blood vessel. Furchgott & Zadawzki (1980) demonstrated in a series of elegant experiments that the ability of acetylcholine (ACh) to relax vascular preparations was critically dependent on the presence of an intact endothelial layer. They speculated that ACh interacted with the endothelial layer stimulating the production of a factors (endothelium-derived relaxing factors, EDRFs) that caused the relaxation of the underlying smooth muscle. Subsequent experimental work, mainly on isolated vessels, revealed that endothelial cells release several regulatory substances in

response to a wide range chemical interactions between endothelial cells and white blood cells, platelets or constituents of plasma (reviewed by Furchgott 1983; Vanhoutte 1988; Vane & Botting 1993). Endothelial cells can be activated by amines, proteins, nucleotides, arachidonic acid and its metabolites as well as physical changes such as blood pressure and blood flow. The activation of endothelial cells is often mediated by specific receptors which cause relaxation by the release of either PGI_2 or nitric oxide (NO) or an as yet unidentified factor that mediates relaxation via the hyperpolarisation of smooth muscle cells (endothelium-derived hyperpolarising factor, EDHF). Vasoconstriction can be caused by a series of endothelial derived factors: so far endothelins (ETs), thromboxane A_2 (TxA_2), angiotensin II (AT II) and superoxide have been identified. The amount released from the endothelial cell layer and the sensitivity of the underlying smooth muscle vary across species. In this thesis, the release characteristics of some of these agents from equine and bovine pulmonary endothelial cells and their effects on isolated equine and bovine blood vessels have been studied. The characteristics of these agents are discussed in detail below.

1.3.1.1 Endothelial nitric oxide synthesis and release

The endothelium-derived relaxing factor first postulated by Furchgott & Zadawzki (1980) was identified as nitric oxide and reported simultaneously by two laboratories (Ignarro et al 1987; Palmer et al 1987). Interestingly, endothelial NO is released by many of the same stimuli which lead to the generation of PGI_2, in fact De Nucci et al (1988) have suggested that receptor triggered release of NO and PGI_2 are coupled. Nitric oxide is synthesised in endothelial cells from L-arginine by NO synthase (NOS), a NADPH-dependent dioxygenase (Bredt & Snyder 1990). NO (which has a half life of only a few seconds) diffuses into vascular smooth muscle where it binds to and activates the enzyme guanylate cyclase to cause an increase in intracellular levels of 3',5' cyclic guanosine monophosphate (cGMP). This second messenger system mediates relaxation of smooth muscle via actions on both the sarcoplasmic reticulum (SR) and myofilaments. Two distinct types of NOS have been identified. A constitutive, Ca^{2+}-calmodulin-dependent enzyme (cNOS) is

found in endothelium, neural tissue and platelets. The other, an inducible, Ca^{2+}-independent enzyme (iNOS), is found in macrophages, vascular smooth muscle cells, endothelial cells, myocytes and microglial cells (Moncada et al 1991; McCall & Vallance 1992). Endothelium-derived NO is strongly implicated in the local control of pulmonary flow, e.g. HPV response (section 1.4.4.2). Abnormal NO production is also implicated in a number of disease states, e.g. endothelium-dependent relaxation is impaired in isolated perfused lungs from patients with chronic lung disease (Cremona et al 1992) while inhaled NO selectively reverses pulmonary hypertension in humans (Frostell et al 1993), suggesting that NO release rather than its action on smooth muscle is abnormal. The cellular basis for chronic pulmonary hypertension is unknown, however Rengasamy & Johns (1991) showed that moderate chronic hypoxia inhibits the activity of an isoform of NO synthase in vascular endothelium. NO production can be effectively inhibited by the addition of compounds that compete with endogenous L-arginine for the binding site on both cNOS and iNOS (Pearson & Vanhoutte 1993), one such inhibitor is the compound N^{ω}-nitro-L-arginine methyl ester (L-NAME), which was used extensively in this study.

A series of factors modulate the release of NO from endothelial cells, the ones of interest in this study were bradykinin (Bk), ACh and oxygen . Bradykinin causes a marked stimulation of NO production from endothelial cells by initiating an increase in intracellular $[Ca^{2+}]$ and consequent stimulation of cNOS. The increase in intracellular $[Ca^{2+}]$ occurs through two main mechanisms: (i) the binding of Bk to specific membrane receptors (Bk_2) that mediate an increase in Ca^{2+} influx and (ii) the binding of Bk to specific receptors (Bk_2) which via GTP-binding protein (G protein) activates the enzyme phospholipase-C (PL-C). This enzyme catalyses the breakdown of phosphatidylinositol 4,5 di-phosphate to inositol triphosphate (IP_3), which binds to specific receptors of the endoplasmic reticulum and causes a release of Ca^{2+} into the cytosol. The net effect of both these actions is to increase intracellular $[Ca^{2+}]$ (Busse et al 1991) and hence stimulate NO production via cNOS (reviewed by Pearson & Vanhoutte 1993).

1.3.1.2 Synthesis and release of endothelins

The most potent vasoconstricting agents released from the endothelium are the endothelins (ETs). These peptides were originally isolated from the culture media of porcine vascular endothelial cells (Hickey et al 1985; Yanagisawa et al 1988) but are now found to be released from both systemic and pulmonary endothelial cells (Sakurai 1992). Three structurally different isopeptides of ET have been isolated (ET-1, ET-2, and ET-3) although ET-1 is the only one released by vascular endothelial cells (Vane & Botting 1993). Like many other biologically active peptides, ETs are produced by the breakdown of prepropeptide termed "big" ET by an endopeptidase called "endothelin-converting enzyme". Release of the peptide is regulated by synthesis which in turn is determined by post-translational regulation of "big" ET messenger RNA degradation, i.e. there appears to be no storage of the peptide within the cell. Factors that regulate the mRNA degradation include angiotensin II (Emori et al 1989), adrenaline (Yanagisawa et al 1988) and hypoxia (Highsmith et al 1988). The slow (over hours) stimulatory effects of these agents suggest the ET release is involved with the long term rather than short term regulation of vascular tone.

Release of ET-1 by endothelial cells causes the contraction of the underlying smooth muscle layer. Two distinct ET receptors have been characterised: ET_A and ET_B (Arai et al 1990; Sakurai et al 1990). Vascular smooth muscle possesses ET_A receptors that are selective for ET-1 and mediate contraction, while endothelial cells possess ET_B (nonselective) receptors that are thought to mediate release of endothelial derived relaxing factors. The mode of action of ET-1 in smooth muscle is described in detail in section 1.3.2.5. The activation of ET_B receptors in bovine aortic endothelial cells induces mobilisation of intracellular Ca^{2+} which can in turn cause the release of vasoactive agents such as NO and PGI_2 (Emori et al 1990; Hirata et al 1993). The role of ETs in elevating equine pulmonary artery pressure during normoxia or hypoxia is unknown. Recently, a role for ETs in the endothelium-dependent second phase of HPV in the rat has been suggested (Leach

et al 1994). Its effect on airway smooth muscle function in horses indicate that both ET_A and ET_B receptors are present within equine lung tissue (Sime et al 1993).

1.3.1.3 Endothelial synthesis and release of prostacyclin

Prostacyclin, also known as prostaglandin I_2 (PGI_2), is an unstable vinyl ether released from endothelial cells which was first identified by Moncada et al (1976). The biosynthesis of PGI_2 from arachidonic acid is catalysed by the cyclooxygenase activity of prostaglandin endoperoxidase synthase with the formation of the endoperoxides prostaglandin G_2 and prostaglandin H_2. PGH_2 is subsequently transformed into PGI_2 by the enzyme prostacyclin synthase (Gryglewski et al 1976). As with NO, a number of factors control the release of PGI_2 from endothelial cells including ACh and Bk. The two agents stimulate PGI_2 synthesis by causing an increase in the intracellular concentration of $[Ca^{2+}]$. This in turn activates the membrane bound enzyme phospholipase A_2 which generates arachidonic acid from the breakdown of membrane lipids. This route supplies arachidonic acid not only for PGI_2 synthesis, but also for the synthesis of TxA_2.

1.3.1.4 Endothelial synthesis and release of thromboxane

Thromboxane A_2 (TxA_2) is an eicosanoid synthesised from arachidonic acid in response to various stimuli and causes vasoconstriction, platelet aggregation and bronchoconstriction. Thromboxane A_2 has a short half life (1 - 2 min) and is rapidly hydrolysed to the stable, biologically inactive form TxB_2. As discussed above, PGI_2 has biological properties opposing the effect of TxA_2, i.e. it is a potent vasodilator and inhibitor of platelet aggregation, while TxA_2 is a vasoconstrictor and promoter of platelet aggregation. Stimulation of the endothelium by a number of agonists including ACh and ET-1 are thought to stimulate the release of both PGI_2 and TxA_2 (Barman et al 1989; Barnard et al 1991), and the ratio of release of these two compounds determines vascular tone (Barnard et al 1992).

Figure 1.6 schematically illustrates the endothelial factors described above and their respective intracellular pathways.

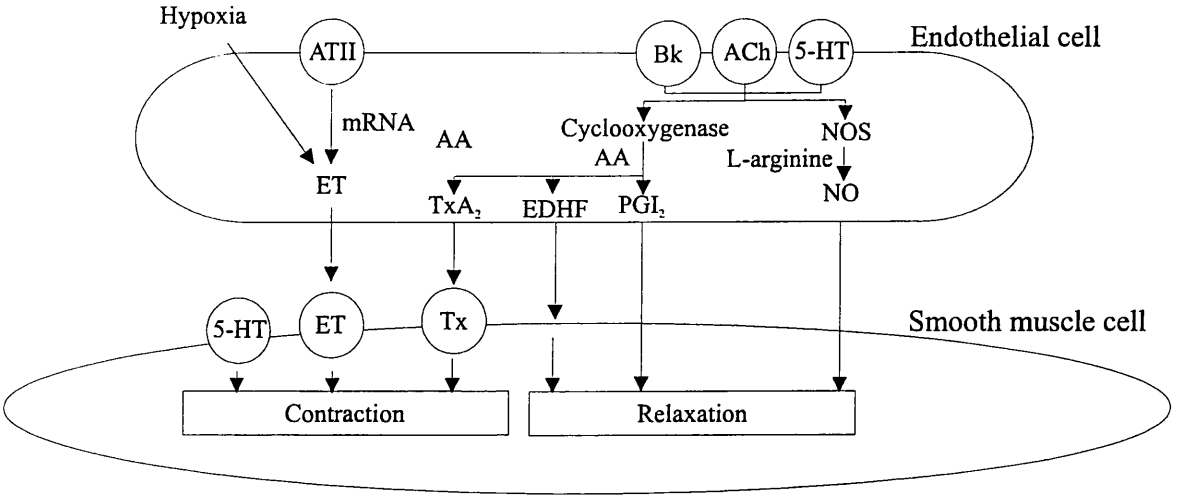


Figure 1.6: Schematic diagram of some of the neurohumoral factors that mediate the release of endothelium-derived factors.

1.3.2 Vascular smooth muscle cell function

Smooth muscle has a variety of functions throughout the body and the contractile properties of the muscle from different sites can be quite different. The most favoured system for classification was developed by Somlyo and Somlyo (1968; 1990) which classifies smooth muscle into either tonic or phasic muscles. Smooth muscle within large arteries is considered tonic, while smooth muscle of the resistance arteries, arterioles and veins is considered phasic. Tonic smooth muscle does not generate action potentials, but application of agonists or electrical stimulation causes a sustained depolarisation of the membrane potential and a sustained contraction. Smooth muscle from the circular layer of aorta or pulmonary artery of most species consists of tonic smooth muscle. Phasic smooth muscle generates frequent action potentials which give rise to phasic contractures. Application of agonists cause contractures that have both phasic and tonic components. Smooth muscle from the circular or longitudinal layer of portal vein in a number of species is an example of typical phasic muscle.

Contraction of smooth muscle is modulated by changes of intracellular calcium ion concentration ($[Ca^{2+}]$) and/or altered sensitivity of the contractile proteins to $[Ca^{2+}]$. Intracellular $[Ca^{2+}]$ is influenced by Ca^{2+} exchange across the sarcolemma and

sarcoplasmic reticulum (SR). Voltage gated Ca^{2+} channels or receptor operated channels are thought to control Ca^{2+} influx across the sarcolemma. Ca^{2+} release from the SR is mediated by agonists-induced second messengers acting at specific receptors. Other second messenger systems are also responsible for causing the altered responsiveness of contractile proteins to Ca^{2+}. The intracellular basis for the regulation of smooth muscle contraction is an active area of research (reviewed by Somlyo & Somlyo 1994) and is summarised below.

1.3.2.1 Control of contractile protein activity

An increase in intracellular [Ca^{2+}] is linked to contraction of smooth muscle via the formation of the calcium-calmodulin complex (CaCaM) and the consequent activation of myosin light chain kinase (MLCK). Once active, MLCK catalyses the phosphorylation of myosin light chain (MLC, Hai & Murphy 1989). In this form, myosin can interact with actin and generate force. Dephosphorylation of MLC and the subsequent inhibition of acto-myosin interaction occurs through the activity of myosin light chain phosphatase (MLCP, Cornwell & Lincoln 1989). Although this mechanism is common to all smooth muscle, the response of the contractile proteins to a sustained increase in [Ca^{2+}] can vary widely. The smooth muscle of the pulmonary artery (tonic smooth muscle) tends to respond with a maintained increase in MLC phosphorylation and tension, while in phasic muscle, phosphorylation and tension production is only transient (Kitazawa et al 1991). Thus the differing contractile behaviour of intact smooth muscle is, in part, due to distinct properties of the contractile proteins. Regulation of contraction by interactions on the actin filament may involve the proteins caldesmon (Marston & Smith 1985) and calponin (Winder & Walsh 1990). Phosphorylation of these proteins allows their disassociation from actin, and in this way the interaction of actin with phosphorylated myosin is increased. The control of caldesmon or calponin is not well understood but may be via either Ca^{2+}/calmodulin dependent or independent pathways (Adam et al 1992; Khalil & Morgan 1992). Recent work by Walsh et al (1994) has suggested that agonist-induced activation of protein kinase C may trigger a kinase cascade that eventually results in the activation of mitogen activated

protein kinase (MAP kinase). This kinase is responsible for the phosphorylation of calponin and the production of Ca^{2+} -independent force. These mechanisms are summarised in Figure 1.7.

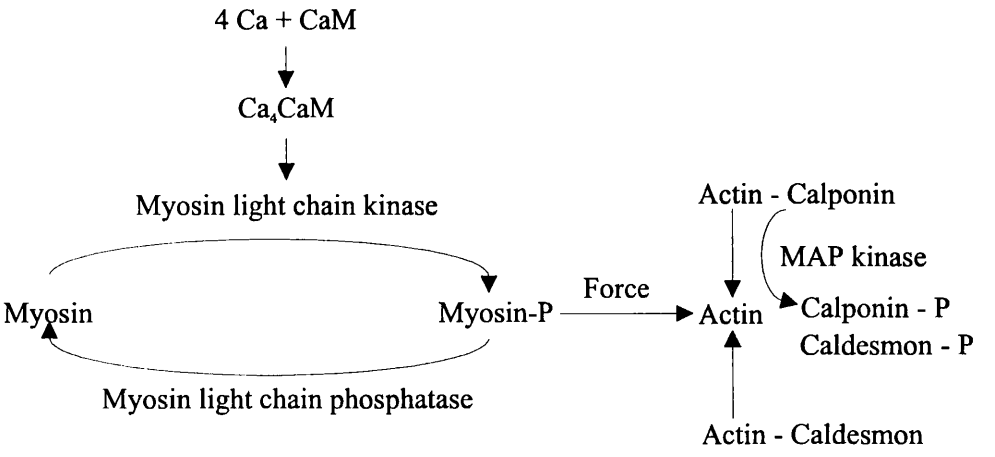


Figure 1.7: Schematic diagram of the events leading to smooth muscle contraction.

A variety of agonists have been shown to increase the responsiveness of the contractile proteins to Ca^{2+}. Receptors for some agonists (e.g. α_1-adrenoreceptors) activate phospholipase C (PL-C) via a membrane bound G-protein (Somlyo et al 1988). Stimulation of PL-C generates inositol (1,4,5) trisphosphate (Ins(1,4,5)P_3) and diacylglycerol (DAG) (Berridge & Irvine 1989). A rise in the DAG content of the sarcolemma along with a rise in intracellular [Ca^{2+}] causes the activation of protein kinase C (PKC) (Nishizuka 1984). Increased activity of this enzyme is thought to lead to the phosphorylation of the myofilaments in such a way as to increase their sensitivity to Ca^{2+}. These events are summarised in Figure 1.8.

A number of membrane bound receptors, including ß-adrenoreceptors, and prostanoid receptors, are linked via membrane bound G proteins to adenylate cyclase and through this system to the regulation of intracellular [cAMP]. Agonists linked to guanylate cyclase such as atrial natriuretic factor and NO, stimulate a rise in intracellular [cGMP]. An increase in the intracellular [cAMP] and [cGMP] in turn activate A-kinase and G-kinase respectively (although cross reactivity does occur, Jiang et al 1992); which leads to phosphorylation in a range of target proteins

in the cell including MLCK (Lincoln et al 1990). Phosphorylation decreases the affinity of MLCK for the CaCaM complex and therefore mediates relaxation, these events are summarised in Figure 1.9.

1.3.2.2 Control of intracellular [Ca^{2+}]

As discussed above, a range of receptors have been functionally linked to a G-protein to PL-C and Ins(1,4,5)P_3 production. Ins(1,4,5)P_3 can release Ca^{2+} from the SR by the activation of specific Ca^{2+} channels (Somlyo et al 1988) and the subsequent transient rise in [Ca^{2+}] can cause a transient increase in contractility. The activation of the inositol phosphate system is thought also to control Ca^{2+} influx across the plasma membrane. Although the processes that occur in smooth muscle are not fully understood, the compound Ins(1,3,4,5)P_4 (a metabolite of Ins(1,4,5)P_3) is thought to open Ca^{2+} channels in the plasma membrane and thereby control Ca^{2+} influx (Berridge 1993). Some workers have suggested that Ca^{2+} release from the SR may stimulate Ca^{2+} entry into the cell (capacitative Ca^{2+} entry; Putney 1986). A summary of the inositol phosphate system is shown in Fig 1.8.

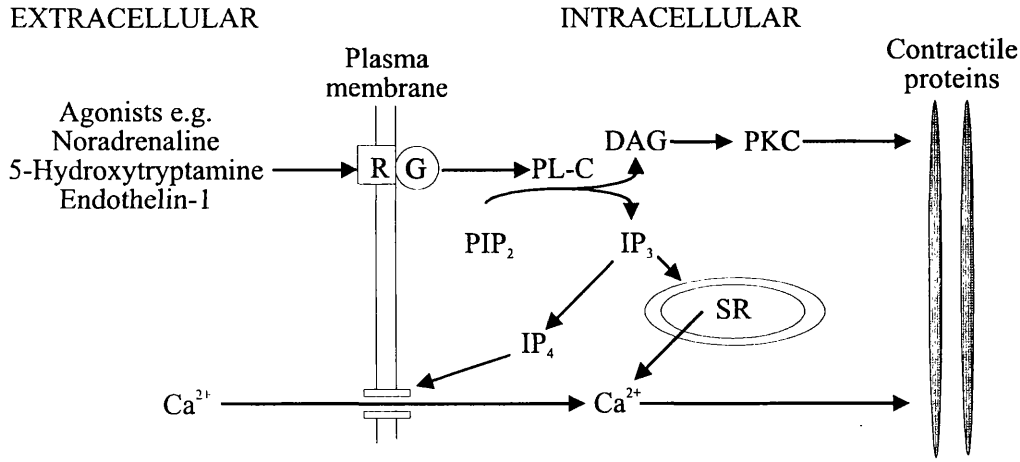


Figure 1.8: Summary of inositol phosphate system in smooth muscle.

Another mechanism that is thought to give rise to a sustained rise of intracellular [Ca^{2+}] is Ca^{2+} influx via voltage dependant Ca^{2+} channels on the surface membrane. These channels can be opened during the depolarisation caused by a train of action potentials. Of particular relevance to the smooth muscle of the pulmonary artery, is

the fact that some agonists are thought to be able to cause a graded depolarisation by binding to receptors that directly open non-specific cation channels. These receptor operated channels can therefore lead to a sustained Ca^{2+} influx via a sustained depolarisation of the surface membrane (Benham et al 1989). Conversely, hyperpolarising the cell membrane potential can reduce the Ca^{2+} influx through voltage dependent Ca^{2+} channels and cause relaxation. The as yet unidentified endothelial derived hyperpolarising factor (EDHF) is thought to mediate relaxation via this route. The most common means of hyperpolarising the surface membrane potential is by an increase in potassium permeability. As will be discussed in a later section, a subset of potassium currents are modulated by the metabolic state of the cell which in turn can control smooth muscle tone.

A decrease of intracellular [Ca^{2+}] can also occur by stimulation of Ca^{2+} re-uptake into the SR. Ca^{2+} uptake into the SR is mediated via the SR - Ca^{2+} pump, the activity of this pump is modulated by cytosolic [Ca^{2+}] and additionally controlled by the SR protein phospholamban (PLB). Phosphorylation of PLB causes an increased activity of SR - Ca^{2+} pump (Colyer & Wang 1991). Either A-kinase or G-kinase can phosphorylate PLB and thereby mediate an increased Ca-pump rate. Thus agonist induced increases in cAMP and cGMP concentrations, stimulates the SR - Ca^{2+} pump, decreases cytosolic [Ca^{2+}] and thereby mediates relaxation.

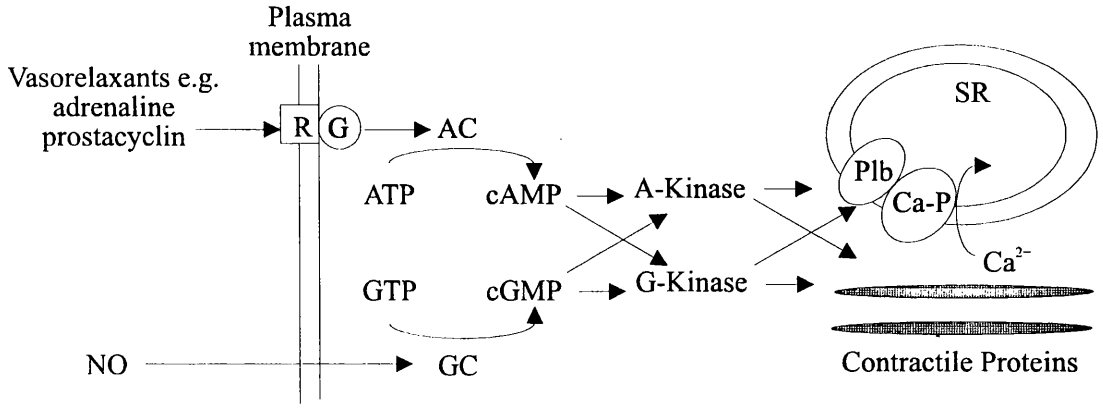


Figure 1.9: Schematic diagram of the intracellular pathways that mediate relaxation in smooth muscle.

A wide range of agonists use these common control pathways summarised in Figures 1.8 and 1.9. In the following sections, the actions of the drugs studied in this thesis are discussed in terms of the intracellular pathways described above.

1.3.2.3 Intracellular action of phenylephrine

Phenylephrine (PE) is an agonist for the α_1-adrenoreceptor. These receptors are normally activated by noradrenaline (NorAd) released from the nerve ending of the sympathetic nerves innervating blood vessels. Activation of α_1-adrenoreceptors mediate vasoconstriction via the activation of PL-C via an intermediary G-protein. As demonstrated by Kobayashi et al (1989), the vasoconstriction caused by activation of α_1-adrenoreceptors occurs via two pathways. The first is through the generation of $Ins(1,4,5)P_3$ and the release of Ca^{2+} from the SR. The subsequent increase in cytosolic $[Ca^{2+}]$ generates force. Secondly, the production of DAG (section 1.3.2.1) causes an increase in the sensitivity of the contractile proteins to Ca^{2+} and through this mechanism enhances force production (Danthuluri & Deth 1984). The increase of intracellular $[Ca^{2+}]$ is maintained by Ca^{2+} influx through the sarcolemma. The cellular mechanisms underlying this aspect of the response are poorly understood. As discussed above, activation of α_1-adrenoreceptors may depolarise the surface membrane and activate voltage sensitive Ca^{2+} channels (Nelson et al 1988). Ca^{2+} influx may also be increased by the action of $Ins(1,3,4,5)P_4$ on the sarcolemma. The majority of studies on the intracellular effects of α_1-adrenoreceptor activation have used systemic smooth muscle cells, but, similar mechanisms are thought to be applicable to smooth muscle from the pulmonary system (McGrath 1985).

1.3.2.4 Intracellular action of 5-hydroxytryptamine

5-hydroxytryptamine (5-HT) is a vasoconstrictor derived from the amino acid tryptophan. It is released from pulmonary neuroendocrine cells as well as platelets and abnormally high levels have been associated with pulmonary hypertension in the human (MacLean et al 1996). 5-HT causes vasoconstriction in both systemic and pulmonary vessels by activation of $5\text{-}HT_2$ receptors in smooth muscle. This

receptor has been linked via an intermediary G-protein to PL-C (Hoyer et al 1994). As described above activation of PL-C causes the generation of $Ins(1,4,5)P_3$ and the release of Ca^{2+} from the SR. Furthermore, the Ca^{2+} sensitivity of the contractile proteins may be enhanced via a DAG linked mechanism (section 1.3.2.1). Activation of 5-HT_2 receptors may also be associated with increased influx of Ca^{2+} across the sarcolemma, but like the α_1-adrenoreceptor response, the intracellular mechanisms of this aspect of the response are unclear.

While this description of 5-HT's action appears to apply to cattle, sheep, rabbit and rat pulmonary artery (MacLean et al 1993; Drummond & Wadsworth 1994; MacLean et al 1994; Klemm et al 1995), an alternative mechanism is associated with the contractile response in human pulmonary artery. Work by MacLean et al (1996) suggests that human pulmonary artery contracts via activation of 5-HT_1 receptors. These receptors are linked by an inhibitory G-protein (G_i) to adenylate cyclase. Thus activation of 5-HT_1 receptors would result in a decrease in cAMP levels within smooth muscle cells and in this way increase the vessel tone (Sumner & Humphrey 1990). As yet, the 5-HT receptor has not been characterised in equine pulmonary artery.

1.3.2.5 Intracellular action of endothelins

As described in section 1.3.1.2, endothelins, in particular ET-1, are released from vascular endothelial cells in response to a range of stimuli. ET-1 acts via the ET_A receptor in pulmonary vascular smooth muscle of rat, dog and pig (Watanabe et al 1991; Nakamichi et al 1992; Douglas et al 1993) to cause vasoconstriction. Activation of this receptor is thought to be linked to PL-C via an intervening G-protein. Thus via the pathways described above, activation of ET_A causes an increased intracellular [Ca^{2+}] and increased sensitivity of the myofilaments as summarised in Figure 1.8 (Marsden et al 1989). Ca^{2+} influx via voltage-dependant Ca^{2+} channels or non-selective cation channels is thought to contribute to the sustained phase of the ET-1 induced contraction in some smooth muscle types including pulmonary smooth muscle (Pearson & Vanhoutte 1993).

1.3.2.6 Intracellular action of thromboxane A_2

Vascular endothelial cells synthesise and release TxA_2 in response to a variety of stimuli including Bk and ACh (section 1.3.1.4). The thromboxane receptor on vascular smooth muscle has been identified as a prostaglandin (PGH_2) and TxA_2 binding protein (Furci et al 1991). As with other agonists, TxA_2 causes contraction of smooth muscle via activation of PL-C (Tod & Rubin 1992). Thus contraction involves Ca^{2+} release from the SR, Ca^{2+} influx via the sarcolemma and increased Ca^{2+} sensitivity of the myofilaments (Schror 1993).

1.3.2.7 Intracellular action of nitric oxide

The intracellular basis for the ability of NO to relax smooth muscle is thought to be the NO mediated stimulation of guanylate cyclase (reviewed by Ignarro & Kadowitz 1985). Nitric oxide binds to haem groups within smooth muscle and the NO-haem complex can in turn activate soluble guanylate cyclase (Ignarro et al 1981; Ignarro 1989). As described above, the increased cGMP levels mediate relaxation by the activation of G-kinase and the consequent phosphorylation of myofibrillar MLCK and SR bound PLB. These two events lead to a decreased Ca^{2+} responsiveness of the contractile proteins and a decrease in cytosolic [Ca^{2+}]; both will contribute to relaxation of the smooth muscle. These pathways are shown schematically in Figure 1.9.

1.3.2.8 Intracellular action of prostacyclin

Bk and ACh are two of a number of agents that stimulate PGI_2 release from endothelial cells. The potent vasodilator effect of PGI_2 is thought to be mediated by a surface receptor via a stimulatory G protein (G_s) linked to the enzyme adenylate cyclase, though little structural information is available about the PGI_2 receptor. As described above, activation of adenylate cyclase causes an increase in intracellular [cAMP]. This second messenger activates protein kinase A (PKA), which in turn phosphorylates the same intracellular targets as protein kinase G (PKG), i.e. the myofilaments and the SR causing relaxation. These events are shown schematically in Figure 1.9. PGI_2 has also been shown to hyperpolarise the smooth muscle

membrane by increasing the potassium permeability of the membrane. This effect would reduce Ca^{2+} influx into the cell and aid relaxation, however the intracellular basis for this effect is not known (Schror 1993).

1.4 The cellular basis for hypoxic pulmonary vasoconstriction

As mentioned previously, the HPV response is an important component in the control system that matches ventilation and perfusion to maintain arterial PO_2. The cellular basis for this response has been the subject a large amount of experimental work over the past 30 years. The fact that a consensus has yet to be reached reflects the complexity of the response and the apparently wide variation across species. This section will summarise the main features of *in vitro* studies of HPV with reference to the cellular physiology of the endothelial cell layer and underlying smooth muscle discussed in previous sections.

1.4.1 Characteristics of the contractile response of pulmonary arteries to hypoxia

There are two main preparations used to study HPV *in vitro*; (i) the isolated perfused lung preparation, practical for the lungs of small mammals. In this system the composition of the gases within the lungs can be altered while monitoring pulmonary perfusion pressures or pulmonary perfusion and (ii) the isolated pulmonary artery. Tension measurements are commonly used to measure the contractile response of a section of pulmonary artery which is bathed in a physiological saline solution; hypoxia is simulated by reducing the PO_2 of the gases equilibrated with the saline. While this is the least physiological of the two preparations, it has advantages in that sections of arteries from different lung regions and different parts of the vascular tree can be studied from mammals of all sizes.

1.4.2 Hypoxic response in the perfused lung

Two main forms of the vascular response to hypoxia have been noted in isolated lung preparations. In isolated rat lungs hypoxia ($\leq 2\%$ O_2) caused a slow rise in

pulmonary perfusion pressure which reached a plateau after approximately 5 minutes (Robertson et al 1989). A similar finding has been published for rabbit (Clarke et al 1993). In contrast, McMurty et al (1978) observed a transient HPV response in isolated rat lung and similar transient responses have been observed in ferrets, dogs, cats, calves and pigs (Rudolph & Yuan 1966; Barer et al 1970; Sylvester et al 1980; Wiener et al 1995). Both forms of pressor response are thought to represent HPV, yet they have clearly different phases and time courses. This illustrates the difficulties in comparing HPV responses between studies; some of these differences may represent differences in the experimental systems whilst there may be also significant differences in the form of the HPV response across species. Note that with the isolated lung preparation, the HPV response is observed in the absence of agonist stimulation.

1.4.3 Hypoxic response in the isolated pulmonary artery

1.4.3.1 Response to ≤ 2% O_2

The range of responses to hypoxia vary to an even greater degree in isolated vessel studies. As in the review of isolated lung work, hypoxia will be considered as ≤ 2% O_2. However a number of studies have used O_2 levels greater than 2% and these results will be considered separately. In general, the HPV response in unstimulated small pulmonary vessels (i.e. respiratory arterioles, ≤ 0.3 mm i.d.) is small or absent, while significant responses are seen in unstimulated large vessels (≥ 2.0 mm i.d., representing first or second order pulmonary arteries) (Kovitz et al 1993; Leach et al 1994). In the majority of studies, the HPV response is studied after a substantial amount of tone has been developed by perfusion with potassium chloride (KCl) or an agonist. Vessels isolated from the rat lung that represent large pulmonary arteries contract in a multiphasic manner to hypoxia. In these studies there is an initial transient contracture (within 5 min) followed by relaxation before a second phase of contraction occurs over a slower time course (20 - 30 min, Rodman et al 1989). Several authors have studied the response in pulmonary vessels using a range of agonists: Bennie et al (1991) using KCl; Greenberg & Kishyama (1993)

using PE; Leach et al (1994) using no stimulation or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Similar multiphasic responses were observed in porcine coronary arteries (Kovitz et al 1993). Large pulmonary arteries from pigs, sheep and humans appear to respond with a slow monophasic contracture in the absence of an agonist (Demiryurek et al 1994). Rabbit large pulmonary artery has been reported to respond to hypoxia with a variety of responses such as a transient contracture followed by relaxation in PE (Demiryurek et al 1994) or to a monophasic contraction in histamine (Detar & Gellai 1971). Human pulmonary artery responds with a sustained response in the presence of tone generated by histamine or KCl (Hoshino et al 1988).

Small pulmonary arteries do not respond significantly to hypoxia in the absence of an agonist, and their response in the presence an agonist is different from large arteries. Leach et al (1994) noted a prominent phasic response to hypoxia but only a very small sustained phase in small pulmonary artery from the rat precontracted with $PGF_{2\alpha}$. A transient response followed by pronounced relaxation was observed in small pulmonary arteries from pigs (Kovitz et al 1993) and ferrets (Wiener et al 1995). In the presence of a sustained contraction, sheep small pulmonary artery responds with a phasic response to hypoxia in 5-HT stimulated vessels (Demiryurek et al 1991). As with the work on perfused lung, some of these differences undoubtedly arise for technical reasons (e.g. rate of equilibration of hypoxic mixture, temperature, means of measuring contractile activity), while the presence and class of the agonist may also be important (Rodman et al 1989). Two other factors may also contribute to the range of responses described above. Firstly, the response of a blood vessel to hypoxia may depend on the type of smooth muscle within the vessel wall. Resistance arteries and arterioles have phasic smooth muscle within the tunica media while large arteries have tonic smooth muscle (section 1.3.2). Secondly, there may be significant differences in the response to hypoxia between species.

1.4.3.2 Response to 4% - 25% O_2

In a number of studies, O_2 values greater than 2% were used to simulate hypoxia. Under these conditions, isolated vessels failed to show a significant HPV response,

instead a graded relaxation was observed. In one study, porcine pulmonary arteries (> 3 mm i.d.) showed a maximal relaxation in 4% O_2; below this level, hypoxia caused a contraction as described above (Kovitz et al 1993). However, small pulmonary arteries (1 - 2 mm i.d.) showed profound dilatation to 4% O_2, unlike the response of the larger arteries, the small vessels dilated in < 2% O_2 but this was preceded by a transient vasoconstriction (see above, Kovitz et al 1993). Similar behaviour was observed in ferret pulmonary arteries. However, only a graded vasoconstriction was observed in rat pulmonary arteries (Rodman et al 1990). One of the few studies on isolated equine pulmonary arteries indicated that 2% O_2 caused a significant vasodilation after precontraction with histamine (Tomasic 1996). These vessels were 1 - 2 mm wide but were classified as 'pulmonary arteriole rings'.

Therefore, hypoxia (< 2% O_2) causes a transient and/or sustained contraction in large pulmonary arteries in most species studied. In small vessels (i.e. resistance arteries), < 2% O_2 causes a transient contraction, but the steady state response is a net vasodilation. Oxygen at > 2% commonly causes a small transient vasoconstriction followed by vasodilation, though a graded hypoxic constriction has been observed in rat pulmonary artery.

1.4.4 The role of the endothelium in the response to hypoxia

As is evident from the preceding sections, the HPV response may have several phases, and the role of the endothelium in mediating any of these phases remains controversial.

1.4.4.1 Removal of the endothelium

The transient and sustained phases of the hypoxic vasoconstrictor response are abolished by mechanical removal of the endothelium in porcine and ovine pulmonary arteries (Demiryurek et al 1991; Kovitz et al 1993). Only a monophasic hypoxic vasoconstrictor response was observed by Holden & McCall (1984) in porcine pulmonary arteries, yet this was completely abolished by removal of the endothelium. Similarly, removal of the endothelium completely abolished the

sustained hypoxic vasoconstrictor phase in rat pulmonary artery and depressed the transient phase (Rodman et al 1990; Leach et al 1994). Yet Bennie et al (1991) concluded that while the transient phase of the HPV was endothelium dependant, the sustained phase was independent of the endothelium, in agreement with studies on sheep (Demiryurek et al 1991), human (Demiryurek et al 1993) and rabbit (Johns et al 1989). In summary, both the transient and sustained phases of the hypoxic vasoconstrictor response appears to have significant endothelium-dependence, although the degree to which this occurs appears to vary across species. In most species studied, the transient phase of the vasoconstrictor response appears to be completely endothelium dependent, while in most (but not all) species, a significant component of the sustained response to hypoxia is endothelium independent.

1.4.4.2 Release of nitric oxide

In porcine pulmonary arteries, the transient vasoconstriction appears to be abolished by L-NAME (an inhibitor of NO synthase), but the sustained phase persists. Only the combination of indomethacin and L-NAME appears to inhibit the endothelium dependent component of the sustained contraction (Kovitz et al 1993). In agreement with these results, the transient vasoconstrictor response seen in small pulmonary vessels of the sheep was abolished by L-NAME, whereas the sustained HPV response observed in large pulmonary vessels was unaffected by L-NAME (Demiryurek et al 1991). The consensus is that the transient phase of the HPV response observed in most pulmonary arteries is abolished with L-NAME, and results from a transient decrease in the production of NO from pulmonary endothelial cells (Warren et al 1989), while the sustained vasoconstrictor response is not significantly affected by NO production from the endothelium.

1.4.4.3 Release of prostanoids

The ability of hypoxia to alter PGI_2 release from vascular endothelium has been the focus of a number of studies. Initial work with the cyclooxygenase inhibitor indomethacin has yielded mixed results. The HPV response in isolated human pulmonary artery was enhanced by indomethacin (Jin et al 1992) but the inhibitor

was ineffective in rat pulmonary vessels (Rodman et al 1989). In contrast, the role of PGI_2 in the hypoxic contraction of systemic vessels seems more consistent (Pearson & Vanhoutte 1993). The basal release of PGI_2 from sheep coronary arteries was reduced by 50% during hypoxic vasoconstriction (Amatyra et al 1989). Using rat coronary arteries, Okada (1991) showed that there was a decrease in PGI_2 release during hypoxic vasoconstriction and an increase release of PGI_2 during the phase of hypoxic vasoconstriction. But in both these studies, it was unclear whether PGI_2 was released from endothelial cells or smooth muscle cells of the vessel. Cultured cell studies have indicated that PGI_2 release from ovine pulmonary smooth muscle cells was reduced by hypoxia but PGI_2 release from pulmonary endothelial cells is insensitive to hypoxia (Rabinovitch et al 1989). In contrast, PGI_2 release was significantly reduced when cultured bovine pulmonary endothelial cells were exposed to hypoxia (Martin et al 1992). In summary, it is clear that hypoxia can affect PGI_2 release from endothelial cells, and this vasoactive agent may have a role in the hypoxic vasoconstrictor response in the pulmonary circulation. However, the relative contributions of smooth muscle-derived and endothelial cell-derived PGI_2 is unclear.

1.4.4.4 Release of endothelins

The endothelium-dependent phase of the sustained hypoxic contraction observed in some species may be mediated by increased activity or increased release of an endothelium-derived contracting factor, in particular ET-1. However, the ET_A selective antagonist BQ123 or the ET_B selective antagonist sarafotoxin S6c did not affect the hypoxic vasoconstrictor response in dog pulmonary artery (Douglas et al 1993) or sheep pulmonary artery (Demiryurek et al 1994). Work on cultured bovine coronary endothelial cells indicates that hypoxia promotes the release of ET (Hieda & Gomez-Sanchez 1990) but the time course of this response was very slow, with significant increases in ET production only obvious after 24 h exposure to hypoxia. Similar results were observed using human umbilical venous endothelial cells (Gertler & Ocasio 1993) and bovine pulmonary artery endothelial cells (Hassoun et al 1992). The slow time course of increased ET production suggests that this

response is involved with the chronic, rather than the acute, response of blood vessels to hypoxia. Experiments on coronary blood vessels suggest that a sustained hypoxic vasoconstrictor response occurs due to the release of a diffusable constrictor substance from the endothelium. This substance has yet to be identified, but does not have the properties of ET (Rubanyi & Vanhoutte 1985). Recently evidence has emerged that leukotrienes (derived from arachidonic acid) are produced in response to hypoxia and mediate vasoconstriction in ferret pulmonary vessels (Tseng et al 1990), their exact role in the vasoconstrictor response needs further examination.

1.4.5 Direct effects of hypoxia on pulmonary vascular smooth muscle

From the analysis of the literature described above there appear to be two distinct effects of hypoxia on smooth muscle. There is a vasodilatory effect of mild hypoxia on smooth muscle in pulmonary arteries of most species, while more profound hypoxia (< 2% O_2) causes vasoconstriction. Furthermore, in some animals (sheep and rat), a significant component of the sustained phase of hypoxic vasoconstriction appears to be endothelium independent (Rodman et al 1990; Bennie et al 1991; Demiryurek et al 1991). Cultured smooth muscle cells from bovine pulmonary artery contract when exposed to 3% O_2 (Murray et al 1990). Freshly dissociated smooth muscle cells from cat pulmonary artery contract in response to hypoxia (Madden et al 1985) but it is difficult to extrapolate from results on isolated cells to intact vessels.

The cellular basis for hypoxia-induced contraction of smooth muscle is the subject of a considerable amount of research. On the basis of the description of smooth muscle function (section 1.3.2), hypoxia could (i) increase Ca^{2+} influx into the cell by direct activation of Ca^{2+} channels (ii) depolarise the membrane potential and therefore increase Ca^{2+} influx via voltage-gated Ca^{2+} channels (iii) release Ca^{2+} from internal stores and/or (iv) alter the responsiveness of the myofilamants to Ca^{2+}. Current experimental work favours mechanism (ii). Harder et al (1985) have shown a depolarisation of smooth muscle cells and increased frequency of Ca^{2+} dependent

action potentials in cat pulmonary arteries exposed to hypoxia. This depolarisation is thought to be caused by reduced K^+ currents (Yuan et al 1993). However, smooth muscle cells have a range of types of K^+ channels, and the characteristics of the channel involved in the response to hypoxia remain controversial, with some studies suggesting a specific hypoxia-induced current (Post et al 1995; Osipenko et al 1997).

Less controversy surrounds the intracellular mechanisms underlying the hypoxia induced vasodilation seen in systemic vessels and larger pulmonary vessels. The activation of the K_{ATP} channel is thought to underlie the response (Rodman et al 1990; Greenberg & Kishiyama 1993; Kovitz et al 1993; Demiryurek et al 1994). This channel is activated by reduced ATP and increased ADP within the cell which mediates hyperpolarisation, leading to reduced Ca^{2+} influx and smooth muscle relaxation.

1.5 Primary isolation of vascular endothelial cells

Endothelial cell culture is now a common experimental technique, and primary endothelial cell cultures from many animal species have generated valuable information on the role of the endothelium in the release of mediators which have been implicated in pathological conditions. There are few data on endothelial cell culture using equine tissues, and yet damage and/or altered function of the endothelium may be implicated in many equine disease states e.g. laminitis (Hood et al 1993), endotoxaemia (Moore 1994) and thrombosis (Weiss et al 1994). There are only three reports in the literature of the use of primary equine endothelial cell cultures (Lamar et al 1986; Turek et al 1987; Bochsler et al 1989), although Bottoms et al (1985) described the measurement of eicosanoid release from dispersed equine vascular endothelial cells (30 replications) exposed to endotoxin. Lamar et al (1986) reported a basic characterisation of equine aortic and pulmonary artery endothelial cells in culture grown on gelatin-coated petri dishes, and indicated differences in the propensity of growth supplements and inhibitors to alter endothelial and smooth muscle cell growth in equine cells compared to similar cells

from other species. Turek et al (1987) exposed passaged equine pulmonary and aortic endothelial cells to endotoxin and measured cell damage. Bochsler et al (1989) isolated and determined optimal growth conditions for omental microvascular endothelial cells. In this thesis, modifications to these techniques were made in order to develop a simple and reliable method for obtaining confluent monolayers of equine endothelial cells in culture. These cultured cells were used to measure the release of vasoactive substances during normoxia and hypoxia.

1.6 Aims of the thesis

The purpose of the experimental work in this thesis was to characterise the response of equine pulmonary endothelial cells and isolated pulmonary arteries under normoxic and hypoxic conditions. Experiments on equine pulmonary endothelial cells were designed to monitor the release of vasoactive agents which are involved in the response of vessels to hypoxia. Measurements were also made from endothelial cells isolated from equine aorta and bovine pulmonary artery for comparison. The reactivity of equine pulmonary artery was compared to bovine vessels using isometric measurements from rings of isolated vessels. These experiments were designed to compare the characteristics and magnitude of the contractile response to common agonists and hypoxia. This work will determine the similarities and differences between equine and bovine pulmonary vessels and may provide an explanation for some of the unusual aspects of equine pulmonary function.

Chapter 2: Materials and Methods

2.1 Pulmonary histology

Histological techniques were applied to sections of equine and bovine lung parenchyma to study the structure of small arteries (2 - 3 mm o.d.). Haematoxylin and Eosin (H & E) stain was used to identify the structures in equine and bovine lung parenchyma. The basic dye, haematoxylin, stains acidic structures a purplish blue colour, specifically nuclei, which have a strong affinity for the dye due to their high DNA content. Eosin, an acidic dye, stains basic structures such as cytoplasm and connective tissue pink or pinkish-red. In a separate series of sections, Verhoeff's haematoxylin for elastic fibres was used to compare and contrast the composition of equine and bovine pulmonary arteries. This stains elastic fibres and nuclei black, and the remaining tissue is counterstained red for collagen and yellow for smooth muscle. Factor VIII (von Willebrand factor) stains the endothelial cells dark brown. This was used to indicate the extent to which the endothelium was removed by rubbing with a roughened match stick.

2.2 Endothelial cell culture and characterisation

2.2.1 Primary isolation of vascular endothelial cells

The pulmonary artery and aorta were removed from adult horses euthanased on clinical grounds (Dept of Veterinary Pathology Post Mortem Room, University of Glasgow and Grayshill Abattoir, Kilsyth). Tissues were removed within 10 minutes of death and were not obtained from horses with clinical or pathological evidence of cardiopulmonary disease or endotoxaemia. Bovine pulmonary arteries were acquired from healthy cattle post mortem (Sandyford Abattoir Co. Ltd., Paisley, Scotland). Both equine and bovine pulmonary arteries were sectioned from the base of the heart to include the branches of the main pulmonary artery. Approximately 20 cm of the equine aorta was removed from the thoracic cavity. The vessels were rinsed with sterile phosphate buffered saline pH 7.4 solution (PBS, Unipath, Hampshire, England) in order to remove any blood, placed in chilled PBS solution

supplemented with 200 i.u.·ml^{-1} penicillin and 200 µg·ml^{-1} streptomycin (pen/strep, Gibco BRL, Paisley, Scotland) and returned to the laboratory.

The vessels were cleaned of excess fat and connective tissue under sterile conditions in a laminar flow hood. One end of the vessel was clamped shut with a sterile bowel clamp and rinsed three times with sterile PBS plus pen/strep. The initial dissociation solution contained 0.1% collagenase (Type II, 1.7 units·mg^{-1} solid, Sigma, Dorset, England) dissolved in DMEM (Dulbecco's modified Eagle's medium) supplemented with 20 mM HEPES (Gibco BRL, Paisley, Scotland). Approximately 20 ml of this solution (warmed to 37°C) was added to the open end of the blood vessels and this was clamped shut with a sterile bowel clamp after removing all the air. The vessels were wrapped in plastic film and incubated at 37°C for 30 - 40 minutes during which time they were massaged gently on two occasions for 5 - 10 seconds, to aid detachment of endothelial cells from the tunica intima. After removal from the incubator, the vessels were gently massaged again and the endothelial cell-collagenase solution was poured off into a sterile 50 ml plastic tube. The pulmonary artery and aorta were rinsed with 10 ml sterile PBS + pen/strep three times, and the solution was poured into a 50 ml sterile tube together with the cells. The tubes were then centrifuged for 5 minutes at 390 x g. The supernatant was discarded and 20 ml of DMEM plus supplements (10% heat-inactivated foetal calf serum, 10% heat-inactivated newborn calf serum, Gibco BRL, Paisley, Scotland; and 2% pen/strep) were added to the tube, the pellet and medium were gently pipetted with a sterile pipette to resuspend the cells and the mixture centrifuged at 390 x g for 5 minutes. The supernatant was discarded and the procedure repeated twice more to wash the cells thoroughly. The cells were resuspended in 40 ml of supplemented medium and 1 ml aliquots were added to each well of a sterile irradiated polystyrene 24-microwell plate (Greiner Labortechnik, Gloucester, England) and the plates were placed in a humidified incubator (LEEC, Nottingham, England) at 37°C, 5% CO_2/95% air. Cells were not added to 4 wells in each microwell plate, these were used as experimental controls. The endothelial cells initially plated down as aggregates, from which cells

replicated. Collagenase levels were progressively increased in an attempt to isolate individual cells from the aggregates, but this technique proved unsuccessful. The formation of aggregates is a recognised phenomenon in endothelial and epithelial cell culture (Freshney 1994) and prevents accurate cell counting. The cells were washed with sterile PBS + pen/strep and new supplemented medium was added every second day until the cells became confluent, which in equine cells took 5 - 8 days and in bovine cells, 4 - 6 days. Upon reaching confluency, the cells were used in the experimental protocols described in section 2.2.5.

2.2.2 Immunofluorescence studies

2.2.2.1 Endothelial cell membrane marker, CD31

CD31 is a glycoprotein of the immunoglobulin superfamily, which is expressed on the surface of endothelial cells, platelets and leukocytes. Endothelial cells isolated by the method described above were grown on sterile 22 mm^2 glass coverslips in culture medium with added supplements until confluent (5 - 8 days). A recognised marker of endothelial cells is the surface membrane protein CD31 (Hewett & Murray 1993). Initial studies were made to verify the phenotypic nature of the equine endothelial cells by probing for CD31 using the primary antibody mouse anti-human monoclonal JC/70A (DAKO, High Wycombe, England). This antibody reacts with endothelial cells in non-malignant tissue was recommended by DAKO as positive for horse and elephant cells. The fluorescent-labelled rabbit anti-mouse immunoglobulin was used as the secondary antibody (DAKO, F 0313). This technique was tried on several occasions, with different methods of fixation: cold methanol (- 20°C), acetone at room temperature and finally 4% buffered neutral formalin (BNF) for 30 minutes at room temperature. BNF proved the best means to fix the cells, since alternative methods caused the cells to slough off the coverslips. The primary antibody was used in dilutions of 1:5, 1:20, 1:50 and1:500. The secondary antibody was used in dilutions of 1:10, 1:20 and 1:100. Two drops of a fluorescent preservative mounting agent (Fluorsave, Calbiochem, Nottingham, England) were placed onto the cells and the coverslips were mounted on microscope

slides. Cells were viewed using an Olympus BH2 microscope, under ultraviolet light (UV filter KB-4). Despite the range of conditions investigated, no staining was evident, so another method was used.

2.2.2.2 von Willebrand factor

An alternative means of confirming the phenotype of endothelial cells has been used by a number of workers (Jaffe et al 1973; Smith et al 1996). This involves labelling of a protein, von Willebrand factor (Factor VIII), a glycoprotein synthesised by endothelial cells which is not found in cultured smooth muscle or fibroblasts. For this technique, endothelial cells were grown on coverslips until confluent, as described above, and then the cells were fixed in 4% BNF for 30 minutes at room temperature. After fixation the coverslips were rinsed with PBS and allowed to air dry. The primary antibody (rabbit anti-human von Willebrand Factor, DAKO) was diluted in PBS (1:500). Approximately 40 µl of the solution was placed on each coverslip and left to incubate for 24 hours at 4°C. The coverslips were rinsed with PBS three times, the secondary antibody was added (FITC conjugated swine anti-rabbit Ig, DAKO) at a 1:20 dilution, and the coverslips were incubated for 24 hours at 4°C. The coverslips were rinsed with PBS and allowed to air dry. A control slide was prepared using the same procedure described above, except omitting the primary antibody. The cells were mounted on to microscope slides as described above and viewed under ultraviolet light. This method proved successful, and photographs were taken using 400 ASA Fujichrome slide film.

2.2.3 NADPH-diaphorase staining

Equine and bovine endothelial cells were further characterised using nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase staining for NO synthase, using a method similar to that of Valtschanoff et al (1992). Pulmonary artery endothelial cells were grown on coverslips as described in section 2.2.2.1. To stimulate the release of NO in endothelial cells, 10^{-9} M Bk (final concentration) was added for 15 minutes. The cells were then fixed in 4% paraformaldehyde for 30 min. Each coverslip was incubated in 0.1 M phosphate buffer (PB) + 0.25%

Triton-X 100 (pH 7.3) for 5 - 10 minutes, before being transferred to a reaction solution of 0.1 M PB/Triton solution + 0.1 $mg \cdot ml^{-1}$ nitro blue tetrazolium (NBT, Sigma) + 0.2 $mg \cdot ml^{-1}$ NADPH-β (Sigma). The coverslips were covered with foil and placed on a shaker at room temperature for 5 minutes. They were then transferred to an incubator at 37°C for 18 hours. Subsequently, the coverslips were dehydrated in increasing concentrations of ethanol, starting with 70%, 80%, 90 %, and 100% (3 times), and placed into a clearing agent, Histoclear (BS&S, Scotland, Ltd.) for 5 minutes, 3 times and then fixed in Histomount (BS&S, Scotland, Ltd.). The coverslips were mounted onto glass microscope slides. Photographs were taken on a Leitz microscope with Kodak technical pan film.

2.2.4 Measurement of intracellular calcium concentration

Endothelial cells were plated onto glass coverslips (22 mm^2) and were grown to confluence as described in section 2.2.2.1. Cells were then washed in PBS and left in SFM for 60 minutes. The fluorescent Ca^{2+}-indicator Fura-2 was loaded into the cells by incubation with 5 μM of the acetoxymethyl ester form (Fura-2AM, Calbiochem, UK) and dimethylsulphoxide (DMSO, 5% v/v) for 20 minutes at room temperature. The coverslips were placed individually in a heated chamber and perfused with SFM (37 ± 0.5°C). The chamber was mounted on an inverted microscope (Nikon Diaphot) and the fluorescence from the intracellular Fura-2 was measured using standard microfluorescence techniques (Grynkiewicz et al 1985). The ratio of the fluorescence on illumination at 340 nm and 380 nm is a function of intracellular calcium concentration ($[Ca^{2+}]$), and an increase in intracellular $[Ca^{2+}]$ is indicated by an increase in the fluorescence ratio. Calibration of the fluorescent signal requires the cytoplasm to be equilibrated with a series of calibration solutions. This is difficult to achieve because rendering the cell membrane permeable to the calibrating solutions will also cause the loss of intracellular Fura-2 (Highsmith et al 1986). Consequently, experimental records are presented as fluorescence ratios.

2.2.5 Experimental protocols for cultured cells

2.2.5.1 Cell culture preparation

For all protocols, cells were grown to confluency as described in section 2.2.1. Upon reaching confluency, the cells were rinsed three times with sterile PBS with pen/strep, 0.5 ml of serum-free medium (SFM, Sigma, Dorset) was added to each well and the plates were returned to the incubator for 1 hour prior to the beginning of the experimental procedure. This was to allow the cells to equilibrate in the SFM before stimulating the cells. This is common practise prior to measurements from cultured cells since serum containing medium may interfere with the assay systems (Gorfien et al 1993). All experiments were run in duplicate and samples were taken at 1, 2 and 4 hour time periods. The effects of hypoxia versus normoxia, with or without the addition of indomethacin or L-NAME, on the rate of ET, PGI_2 or NO release were quantified. A preliminary study was made on the effects of halothane on ET release.

2.2.5.2 Hypoxic studies

After 1 hour pre-incubation with SFM, the medium was removed from each well and 0.5 ml of 10^{-5} M indomethacin or 10^{-4} M L-NAME (final concentration in SFM), or vehicle (SFM) only was added. All assays were run under normoxic conditions (control) by adding the above mentioned agonists to trays which were placed in the LEEC incubator at 37°C, and gassed with 5% CO_2 / 95% air. Incubations under hypoxic conditions were carried out by placing the cells in an anaerobic incubator (MK3 Anaerobic Work Station, Don Whitley Scientific, Ltd, West Yorkshire, England). Anaerobic conditions were maintained within the incubator by using a 95% N_2 / 5% CO_2 gas mixture. The partial pressure of oxygen (PO_2) in a test solution of medium was monitored during the incubation using an oxygen electrode (Strathkelvin Instruments, Bearsden, Scotland). The PO_2 reading immediately after the trays were placed in the anaerobic incubator was > 200 mm Hg . After 1 hour the PO_2 had decreased to 20 $\pm$ 3 mm Hg, after 2 hours to 10 $\pm$ 3 mm Hg and 4 hours to 5 $\pm$ 4 mm Hg. After incubation with agonists, 0.5 ml

samples were taken, placed in 1.5 ml Eppendorf tubes and immediately frozen in the -70°C freezer, where they were stored until used in the ET, PGI_2 or nitrite assays within a few months.

2.2.5.3 Halothane studies

Equine pulmonary artery endothelial cells were exposed to 2% halothane over a 4 h period. Halothane was vapourised in a Fluotec MK II vapouriser with 5% CO_2 / 95% O_2. The trays of cells were placed in a purpose built perspex tray (Figure 2.1) and placed in a 37°C incubator. A control plate was run in the LEEC 5% CO_2 / 95% air incubator. These cells were incubated in 0.5 ml SFM with 10^{-4} M L-NAME, 10^{-5} M indomethacin or vehicle (SFM) only. Samples were taken at 1, 2 and 4 hours and stored at -70°C until assayed for ET concentration.

2.3 Assays used to measure endothelial-derived agents

The cells were grown in DMEM with added supplements as described in section 2.2.1, until confluent. Samples of medium (0.5 ml) were taken 1, 2 and 4 hours after the addition of serum-free medium, put into 1.5 ml Eppendorf tubes and frozen immediately, and were stored at -70°C until assayed. Samples were thawed and kept on ice when used in the assay. Assays were used to measure the release of the vasoactive compounds ET, PGI_2, TxA_2 and NO. For ET, a non-specific radioimmunoassay and specific ET-1 enzyme-linked immunosorbent assay was used. The analysis of PGI_2 was performed with a specific radioimmunoassay and specific enzymeimmunoassay. All samples and standards were measured in duplicate. The measurement of nitrate and nitrite was performed with two methods of Griess reagents, run in triplicate. Radioimmunoassay results are reported as counts per minute (cpm).

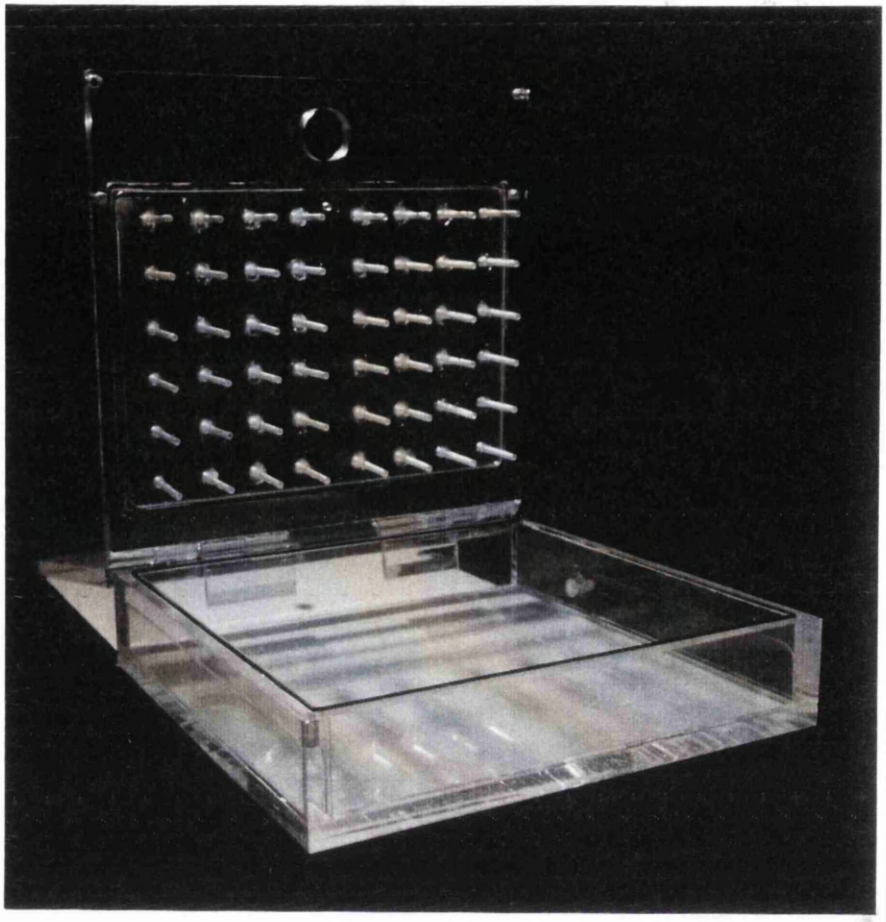


Figure 2.1: Photograph of perspex incubator used to expose cultured endothelial cells to halothane. The incubator was designed to house 2 x 24 well culture trays. Individual cannulae, in the lid, were adjusted to direct halothane-containing gases over the surface of all 48 wells.

2.3.1 Measurement of endothelin production by cultured endothelial cells

2.3.1.1 Endothelin immunoreactivity assay

Endothelin release by endothelial cells in culture was measured by radioimmunoassay using a kit supplied by Peninsula Laboratories (St Helens, UK). The assay is based upon the competition of labelled ^{125}I-ET and unlabelled ET (either standard or unknown) binding to a limited quantity of specific antibodies. As the concentration of unlabelled ET in the reaction increases, the amount of ^{125}I-ET bound to the antibody decreases. By measuring the amount of ^{125}I-ET bound as a function of the concentration of the unlabelled ET in standard reaction mixtures, a standard curve is constructed from which the concentration of ET in samples can be determined. The specificity of this assay was 100% cross-reactivity with ET-1, ET-2, big ET-1 and 70% cross-reactivity with ET-3. Eight standards were run in the range of 10 - 1280 pg ET·ml^{-1}. The samples were counted on the Minaxi Auto-Gamma counter (500 Series, United Technologies, Packard, Berkshire). This assay system performed consistently with repeatable calibration curves: the mean $\pm$ s.e.m. total binding for the assays was 41.9% $\pm$ 1.7% and EC_{50} was 213.6 $\pm$ 21.6 pg·ml^{-1}. Figure 2.2 shows a typical standard curve for the radioimmunoassay.

2.3.1.2 Endothelin-1 specific assay

A solid-phase enzyme-linked immunosorbent assay (ELISA) was used to measure ET-1 from cultured equine and bovine endothelial cells (R & D Systems Europe, Abington, UK). The assay involves the simultaneous reaction of ET-1 present in the sample or standards with two antibodies directed against different epitopes of the ET-1 molecule. One antibody is coated onto the surface of the wells of a microtitre plate and the other is conjugated to the enzyme horseradish peroxidase. Any ET-1 present forms a bridge between the two molecules. After removal of unbound material by aspiration and washing, the amount of conjugate bound to the well is detected by reaction with a substrate specific for the enzyme which yields a

coloured product proportional to the amount of conjugate and thus ET-1 in the sample. Six standards ranged from 0 to 126 pg ET-1·ml^{-1}. The coloured product was quantified photometrically with an ELISA reader (EL 312 Microplate Reader, Bio-Tek Instruments) by reading at 450 nm with a correction wavelength of 650 nm. The standard curve was obtained by plotting the optical density against the linear concentration of ET-1 (Figure 2.3). This assay measures ET-1, with cross-reactivities to big ET-1 of < 1%, sarafatoxin < 2%, ET-2 45%, and ET-3 14%. The advantage of this system is that it is more selective for ET-1, has a sensitivity of less than 1.0 pg·ml^{-1} (approximately 0.25 pg·ml^{-1}) and takes only 90 minutes. Unfortunately, this method gave anomalously high and variable readings in the blank sample, suggesting contamination with ET-1 or unrelated substances that interfered with the assay. Therefore its use was discontinued.

2.3.2 Measurement of prostacyclin production by cultured endothelial cells

2.3.2.1 Prostacyclin radioimmunoassay

This radioimmunoassay measured the stable metabolite of PGI_2, 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$). The protocol for the 6-keto-$PGF_{1\alpha}$ assay was as described in the radioimmunoassay kit from Amersham International plc (Little Chalfont, England). The assay is based on the competition between unlabelled 6-keto-$PGF_{1\alpha}$ and a fixed quantity of the tritium labelled compound for binding to an antibody with a high affinity for 6-keto-$PGF_{1\alpha}$. The assay has a limit of detection of 140 pg·ml^{-1}. The samples were counted on the beta 1600 TR Liquid Scintillation Analyzer (Packard, Berkshire). The cross reactivity of the 6-keto-$PGF_{1\alpha}$ antiserum and other prostaglandins in this kit was low: the highest cross-reactivity was 5.1% with prostaglandin E_2. Figure 2.4 shows a typical standard curve for this method. Several of these kits were used in measuring the release of 6-keto-$PGF_{1\alpha}$. However, in January 1996, without prior notice, Amersham stopped producing this kit and no replacements were available, therefore not all samples were assayed.

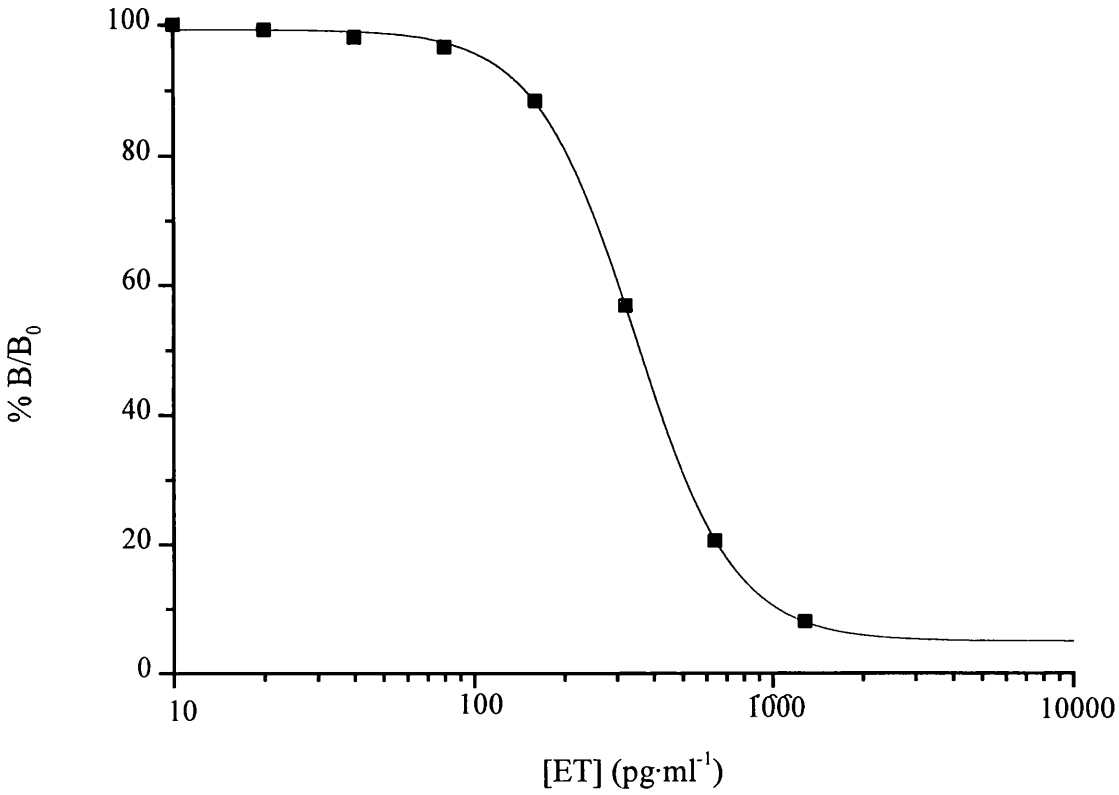


Figure 2.2: Typical radioimmunoassay calibration curve for endothelin (ET). Solid line is the best fit logistic sigmoidal relationship between the normalised percentage bound drug ($\%B/B_0$) and [ET] ($pg \cdot ml^{-1}$) using the equation:

$$\%B/B_0 = (B_{max} - B_{min})/(1+([ET]/EC_{50})^n) + B_{min}$$

Where $B_{max} = 99.3 \pm 0.3$ %; $B_{min} = 5.00 \pm 0.92$ %; $EC_{50} = 345.4 \pm 4.9$ $pg \cdot ml^{-1}$ and $n = 2.61 \pm 0.08$.

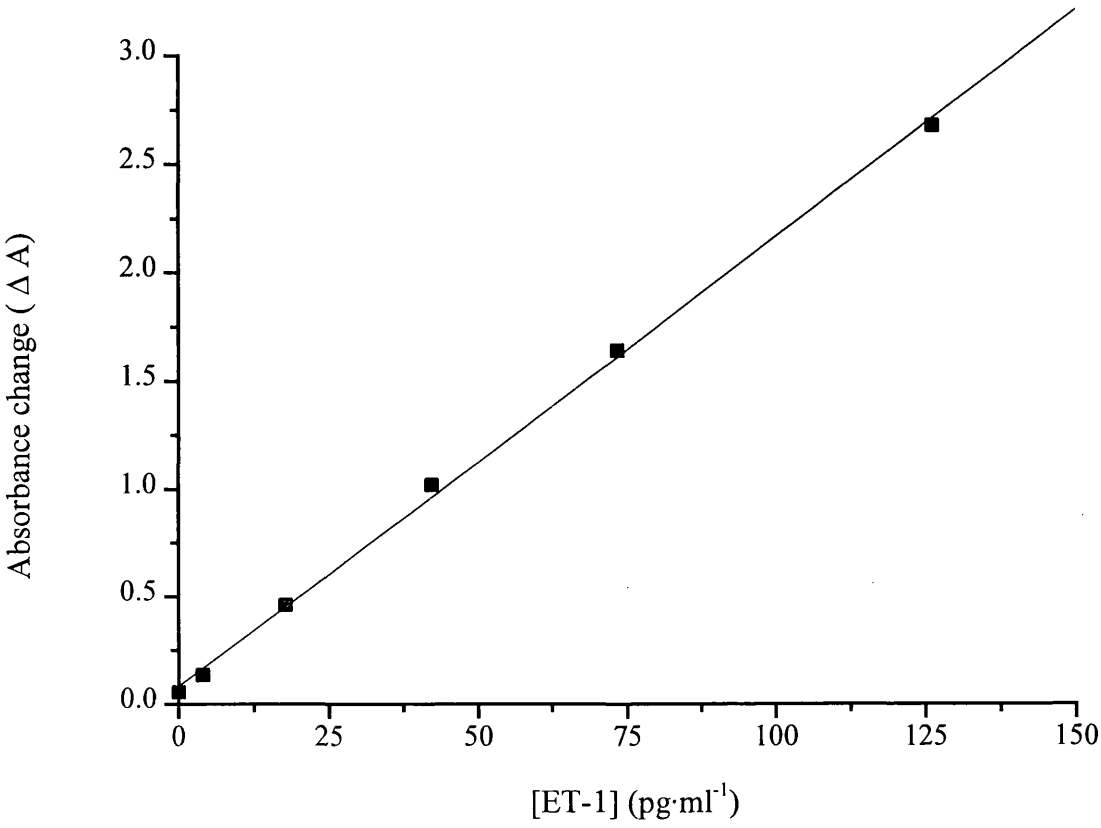


Figure 2.3: Typical endothelin-1 (ET-1) ELISA calibration curve. Solid line is the best fit straight line relationship between absorbance change (ΔA) and [ET-1] ($pg \cdot ml^{-1}$) using the equation:

$$\Delta A = K \times [ET\text{-}1] + c$$

Where $K = 0.02 \pm 0.0004$ $ml \cdot pg^{-1}$ and $c = 0.083 \pm 0.025$.

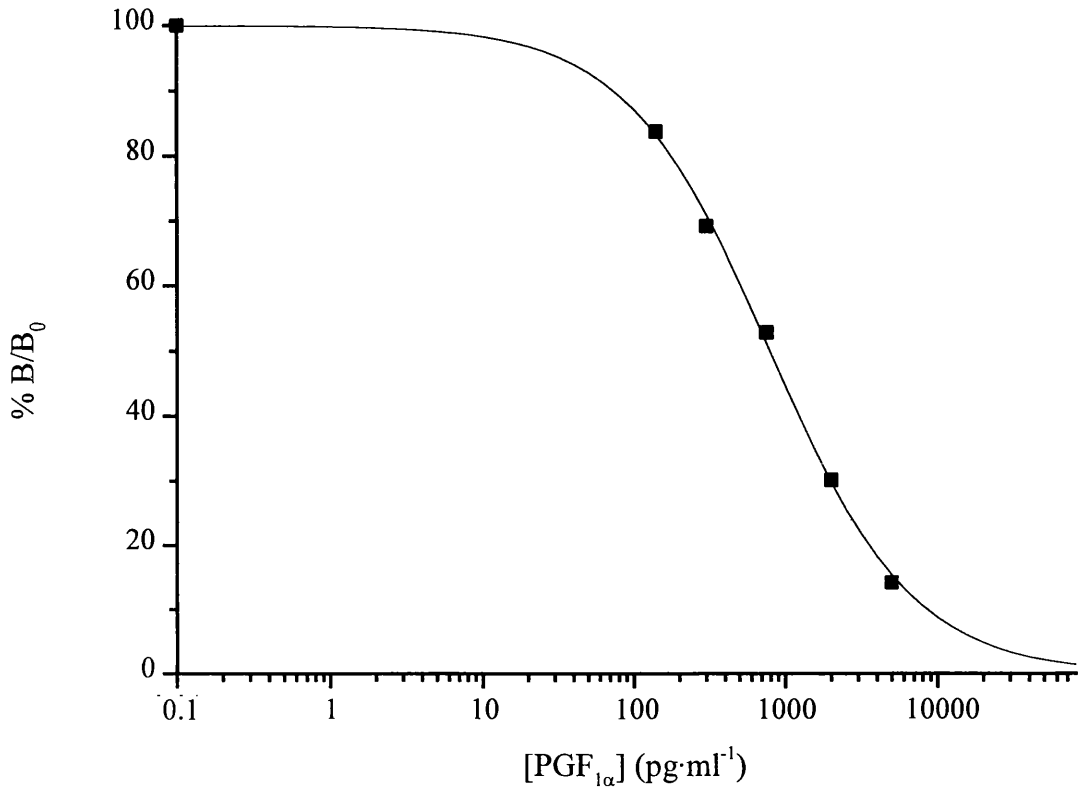


Figure 2.4: Typical radioimmunoassay calibration curve for prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$). Solid line is the best fit logistic sigmoidal relationship between normalised percentage drug bound (%B/B_0) and [PG $F_{1\alpha}$] (pg·ml^{-1}) using the equation:

$$\%B/B_0 = (B_{max} - B_{min})/(1+([PG\ F_{1\alpha}]/EC_{50})^n) + B_{min}$$

Where B_{max} = 100.2 ± 1.5 %; B_{min} = 0.60 ± 0.41 %; EC_{50} =789.7 ± 27.6 pg·ml^{-1} and n = 0.93 ± 0.03.

2.3.2.2 Prostacyclin enzyme-immunoassay

An enzyme-immunoassay (EIA) was also used to measure basal release of 6-keto-$PGF_{1\alpha}$ (Amersham International plc, Little Chalfont, England) in cultured equine and bovine endothelial cells. This EIA was chosen for its high sensitivity of approximately 0.2 $pg \cdot ml^{-1}$, a short (2.5 hour) protocol and because it did not involve the use of radioactive isotopes. The specificity for this assay was 100% cross-reactivity for 6-keto-$PG_{1\alpha}$, 10.5% with 2,3-dinor-6-keto-$PGF_{1\alpha}$, 9.2% with 6-keto-PGE_1 and less than 1% with other prostaglandins, TxA_2 and arachidonic acid. The assay is based on the competition between labelled 6-keto-$PGF_{1\alpha}$ and a fixed quantity of peroxidase labelled 6-keto-$PGF_{1\alpha}$ for a limited number of binding sites on a 6-keto-$PGF_{1\alpha}$ specific antibody. Six standards ranged from 0 to 64 $pg \cdot ml^{-1}$. The resultant colour was measured photometrically at 450 nm with the EL 312 Microplate ELISA reader. The standard curve was obtained by plotting the optical density against concentration of 6-keto-$PG_{1\alpha}$ (Figure 2.5). For reasons that are not known, the greatest normalised percentage bound drug measured during calibration (at 0.5 $pg \cdot ml^{-1}$) was routinely about 70% of the total bound. The data from this EIA system showed very large inter- and intra-assay differences, so they were not used to calculate sample values.

2.3.3 Measurement of thromboxane production by cultured endothelial cells

The radioimmunoassay for measuring TxA_2 was based on the protocol by Higgins and Lees (1984), and had been previously validated in this laboratory. The buffer solution was prepared by adding 0.1% bovine serum albumin (BSA, Sigma, UK) and 0.1% sodium azide to sterile PBS, pH 7.4 and stirring slowly until dissolved. The drug bound to antibody was separated from unbound drug using charcoal/dextran solution, 0.1% dextran / 2% charcoal at 4°C. Charcoal and antibody dilution curves were created to identify optimal dilutions for the assay. Figure 2.6 is a typical charcoal dilution curve used to determine the minimum

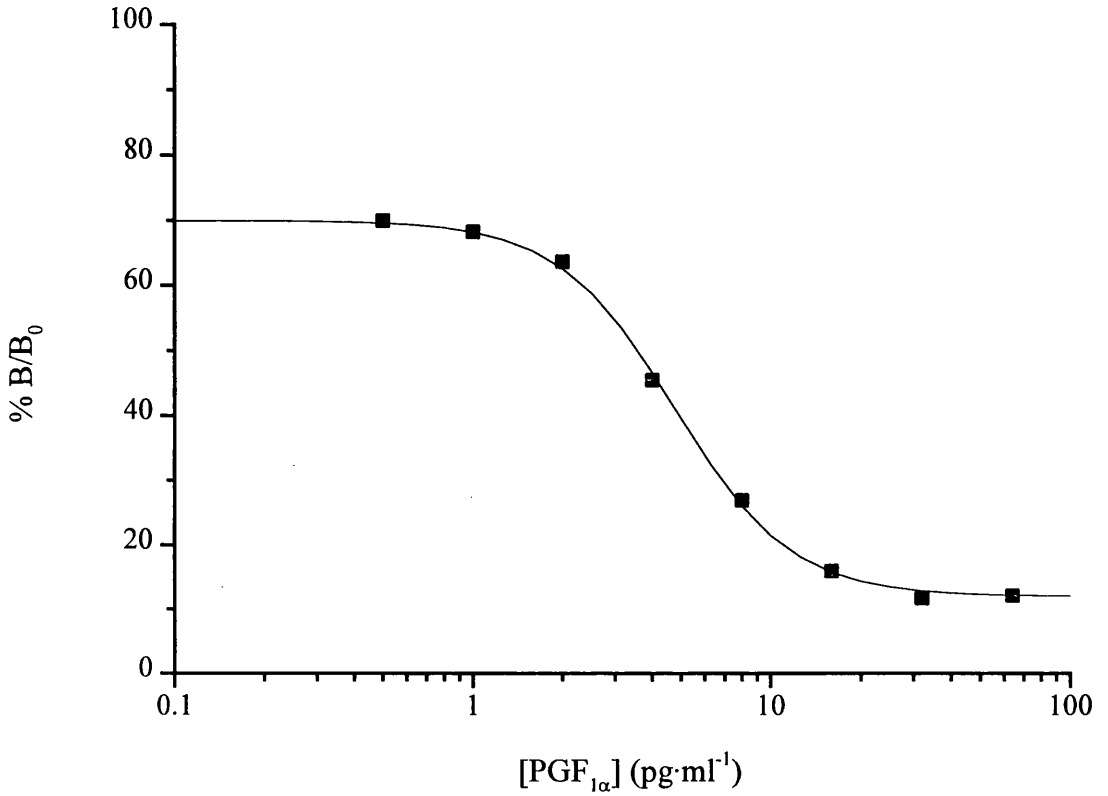


Figure 2.5: Typical calibration curve for 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) enzyme-immunoassay. Solid line is a best fit sigmoidal relationship between normalised percentage bound drug ($\%B/B_0$) and [6-keto-$PGF_{1\alpha}$] ($pg \cdot ml^{-1}$) using the equation:

$$\%B/B_0 = (B_{max}-B_{min})/(1+([PGF_{1\alpha}]/EC_{50})^n) + B_{min}$$

Where $B_{max} = 70.7 \pm 0.9$ %; $B_{min} = 11.4 \pm 0.7$ %; $EC_{50} = 4.76 \pm 0.16$ $pg \cdot ml^{-1}$ and $n = 2.11 \pm 0.13$.

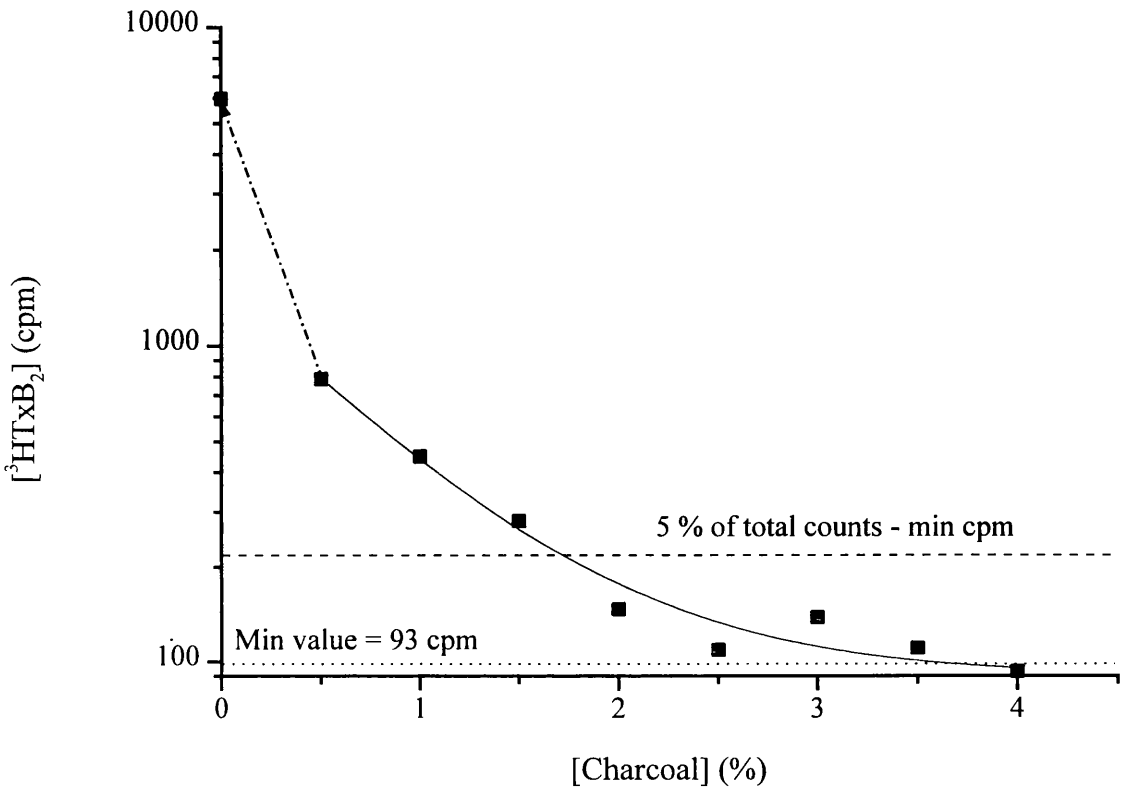


Figure 2.6: Charcoal dilution curve for thromboxane B_2 (TxB_2) radioimmunoassay. Value at 0% charcoal represents total counts. 5% of total counts is indicated by dashed line. Background or minimum level is estimated at 93 cpm and is shown with a dotted line.

charcoal concentration needed to bind greater than 95% of the free tritiated TxB_2 (2%).

A 25% antiserum, Anti-TxB_2 (Sigma, UK), was made up in standard buffer and used as a working stock. Based on an antibody dilution curve performed before each assay, dilutions of the order of 10 fold were made depending on the concentration of TxB_2 in the samples. On average a 2% antiserum solution gave close to 50% binding of ^3H-TxB_2, a value considered optimal for assay design (Chard 1990) (Figure 2.7). The tracer was prepared by adding 6 µl of tritiated TxB_2 (Amersham, UK) to 10 ml of buffer (sufficient for 100 tests) and this solution was stored at -20°C. A sample calibration curve for TxB_2 is shown in Figure 2.8.

2.3.3.1 Arachidonic acid stimulated thromboxane A_2 release

In a limited set of experiments, arachidonic acid was used to stimulate TxA_2 release from bovine endothelial cells. Based on experiments by Feddersen et al (1990), arachidonic acid (Sigma, UK), was added to cultured bovine endothelial cells to give final concentrations of 50µg·ml^{-1} and 200 µg·ml^{-1}. The levels of the stable metabolite TxB_2 were measured after incubation with or without arachidonic acid for 1 hour.

2.3.4 Determination of nitrite levels

Nitric oxide, once formed, has a half-life of approximately 3 seconds. It reacts with aqueous solutions to form the stable metabolites, nitrate and nitrite. These metabolites can be measured spectrophotometrically using commercially available assay systems.

2.3.4.1 Griess-Ilosvay's reagent

Nitrite levels in endothelial cell samples incubated for 1, 2 and 4 hours in serum free medium (SFM) were measured by using the Griess-Ilosvay's reagents (BDH Ltd, Poole, England) (Green et al 1982). This method depends on the diazotisation of sulphanilic acid by nitrous acid. The compound formed is then coupled with 1-naphthylamine-7-sulphonic acid to produce a red azo dye. In this method the

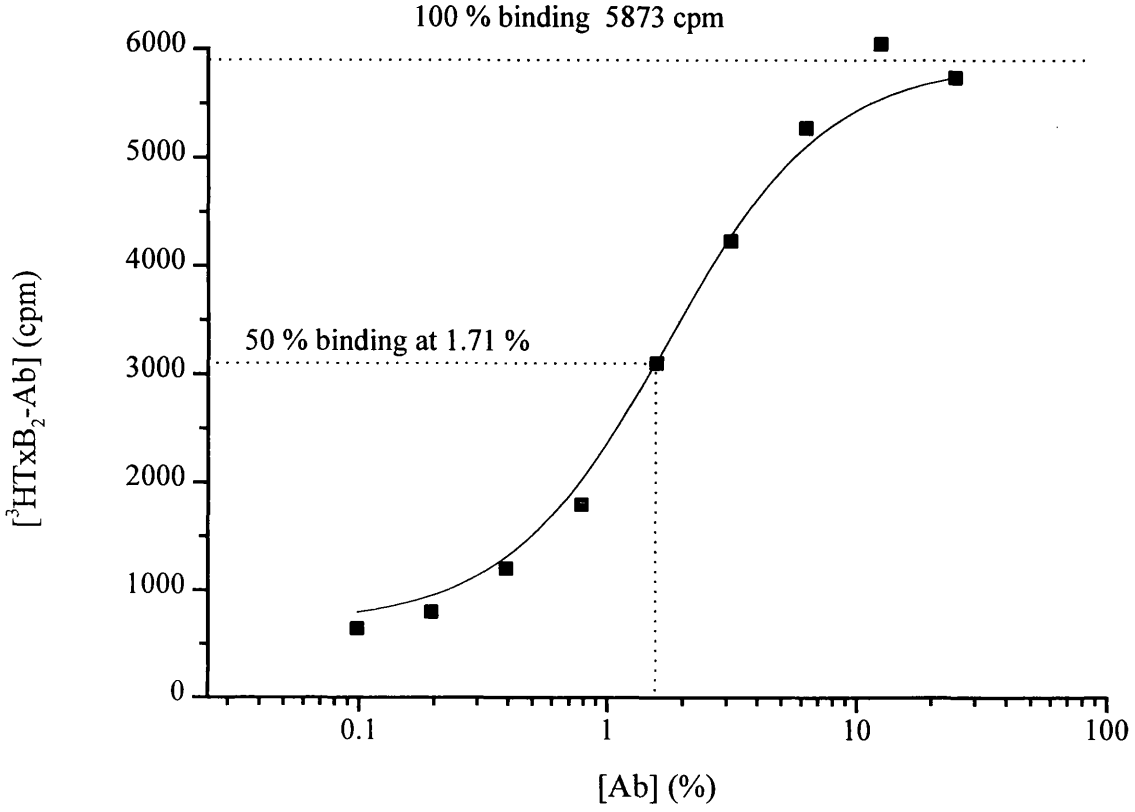


Figure 2.7: Antibody dilution curve for tritiated thromboxane B_2 ($^3HTxB_2$) radioimmunoassay: Solid line is the best fit sigmoidal relationship between $^3HTxB_2$ - antibody complex ([$^3HTxB_2$-Ab]) (cpm) and the concentration of antibody ([Ab]) (%). The 50% binding level = 1.71%.

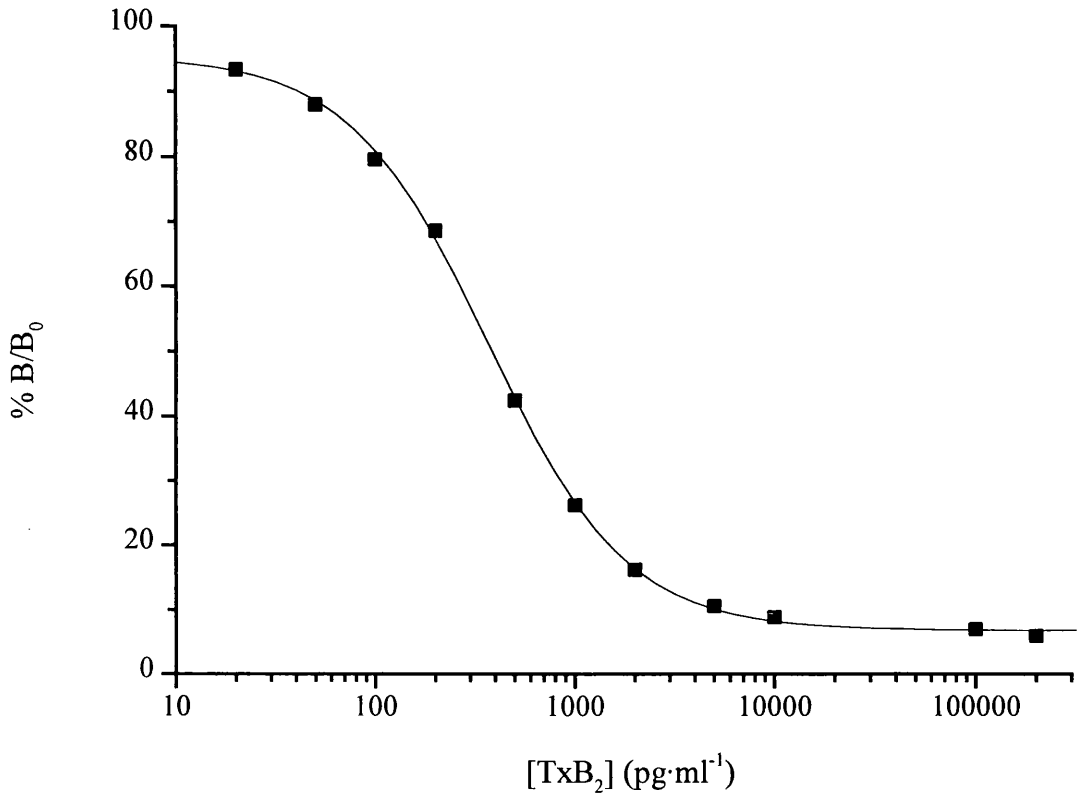


Figure 2.8: Typical radioimmunoassay calibration curve for thromboxane B_2 (TxB_2). Solid line is a best fit sigmoidal relationship between normalised percentage bound drug ($\%B/B_0$) and [TxB_2] ($pg \cdot ml^{-1}$) using the equation:

$$\%B/B_0 = (B_{max}-B_{min})/(1+[TxB_2]/EC_{50})^n) + B_{min}$$

Where $B_{max} = 95.4 \pm 0.9$ %; $B_{min} = 6.80 \pm 0.50$ %; $EC_{50} = 370 \pm 11$ $pg \cdot ml^{-1}$ and $n = 1.25 \pm 0.04$.

Griess-Ilosvay's Reagent No. 1, sulphanilic acid was mixed with equal parts of the Griess-Ilosvay's Reagent No. 2, naphthylamine sulphonic acid. This was added to the sample of serum-free medium in a ratio of 2 parts Griess reagent to 1 part sample. A nitrite standard curve was prepared (10^{-2} to 3 x 10^{-9} M) with the SFM used as the blank. The absorbance of the samples was measured spectrophotometrically at 540 nm on a Pye Unicam SP8-500 UV/VIS spectrophotometer (Cambridge, England). The limit of detection of this method was 10^{-5} M nitrite (Figure 2.9). However, this assay failed to detect nitrite above this value in any of the samples. One method to increase the sensitivity of the assay is to convert the nitrate in the solution to nitrite, which is described in the next section.

2.3.4.2 Modified Griess reagent

A second spectrophotometric method was used which involved measuring the accumulation of the stable degradation products of NO, nitrite and nitrate, as described by Schmidt (1995). Nitrate reduction to nitrite was performed in light sensitive polypropylene tubes by incubating the samples (150 µl) with 0.1 units·ml^{-1} nitrate reductase (Boehringer Mannheim, UK), 50 µM NADPH (Sigma) and 5 µM flavin adenine dinucleotide (FAD, Sigma) for 15 minutes at 37°C in a final volume of 160 µl. Because NADPH absorbs light, to avoid interference with nitrite determination, NADPH was oxidised by incubating the samples with 10 units·ml^{-1} lactate dehydrogenase (Boehringer Mannheim) and 10 nM sodium pyruvate (Boehringer Mannheim) for 5 minutes at 37°C in a final volume of 170 µl. Total nitrite was determined photometrically with the EL 312 Microplate ELISA reader, by using a stepwise addition of the Griess reagent. Samples were cooled to 4°C, sulphanilamide (1 mM end concentration) was added followed by HCl (0.1 M end concentration) to a final volume of 200 µl. The samples were centrifuged at 1000 x g for 15 minutes at 4°C. Finally, an aliquot of supernatant (150 µl) was transferred to a 96 well microtitre plate and read at 540 nm. From the sample absorbency was subtracted the absorbance of 150 µl of SFM to give the absorbance value A_1

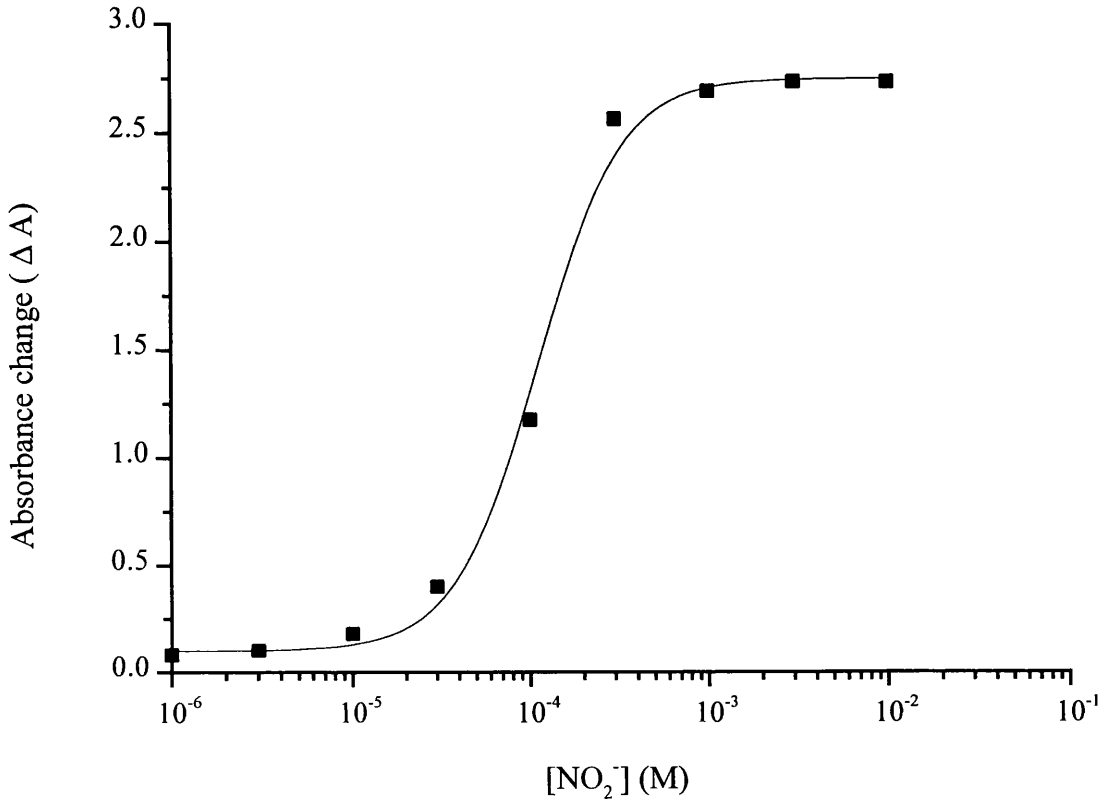


Figure 2.9: Typical Griess-Ilosvay calibration curve: Solid line is the best fit to a sigmoidal logistic relationship between the absorbance change (ΔA) and nitrite concentration $[NO_2^-]$ (M) using the equation:

$$\Delta A = (\Delta A_{max} - \Delta A_{min})/(1+([NO_2^-]/EC_{50})^n)) + \Delta A_{min}$$

Where $EC_{50} = 1.1 \times 10^{-4} \pm 7.4 \times 10^{-6}$ M; $n = 1.86 \pm 0.22$; $\Delta A_{max} = 2.75 \pm 0.14$; $\Delta A_{min} = 0.10 \pm 0.005$.

(blanks). Then 10 µl of 1 mM naphthylethylene-diamine was added to all the samples (including the SFM blanks) and incubated at room temperature for 10 minutes before reading again at 540 nm. The blanks were subtracted from the second reading to give the absorbance value A_2. The calibration curve was obtained by calculation of the absorbance change ($\Delta A = A_2 - A_1$) for a range of nitrite/nitrate standards. This method gives a linear calibration curve for NO (Figure 2.10) with a detection limit of 30 pmol NO/50 µl sample volume.

2.3.5 Protein determination

After SFM samples were taken from the assay incubations, the cells in each tray were removed, spun down (1000 x g for 10 min) and frozen at -70°C. These were later used in a protein assay to measure the amount of protein in each well. Originally, the cells were removed from the trays by using 0.25% trypsin-EDTA (Gibco, BRL, Paisley, Scotland). However, this appeared to completely destroy the cells. Subsequently, the cells were mechanically removed by scraping the cells out of each well and rinsing with sterile PBS, repeated three times.

2.3.5.1 Lowry protein determination

The two assays used were the Lowry assay and the Coomassie Blue assay. The Lowry protein assay kit (Sigma, Poole, England) is based on Peterson's modification of the micro-Lowry method (Peterson 1977) and utilises sodium dodecylsulphate, included in the Lowry Reagent to facilitate the dissolution of the relatively insoluble lipoproteins. Chemicals such as Tris, EDTA, amino acid and peptide buffers can interfere with the direct Lowry procedure. Because trypsin-EDTA was used to remove the cells from the cell culture trays, the protein precipitation procedure using deoxycholate (DOC) and trichloroacetic acid (TCA), was employed to eliminate these interferences. The principle of the assay is that an alkaline cupric tartrate reagent complexes with the peptide bonds and forms a purple-blue colour when the phenol reagent is added. Standards were made from bovine serum albumin (25 - 400 $\mu g \cdot ml^{-1}$). Absorbance was read at 740 nm on the

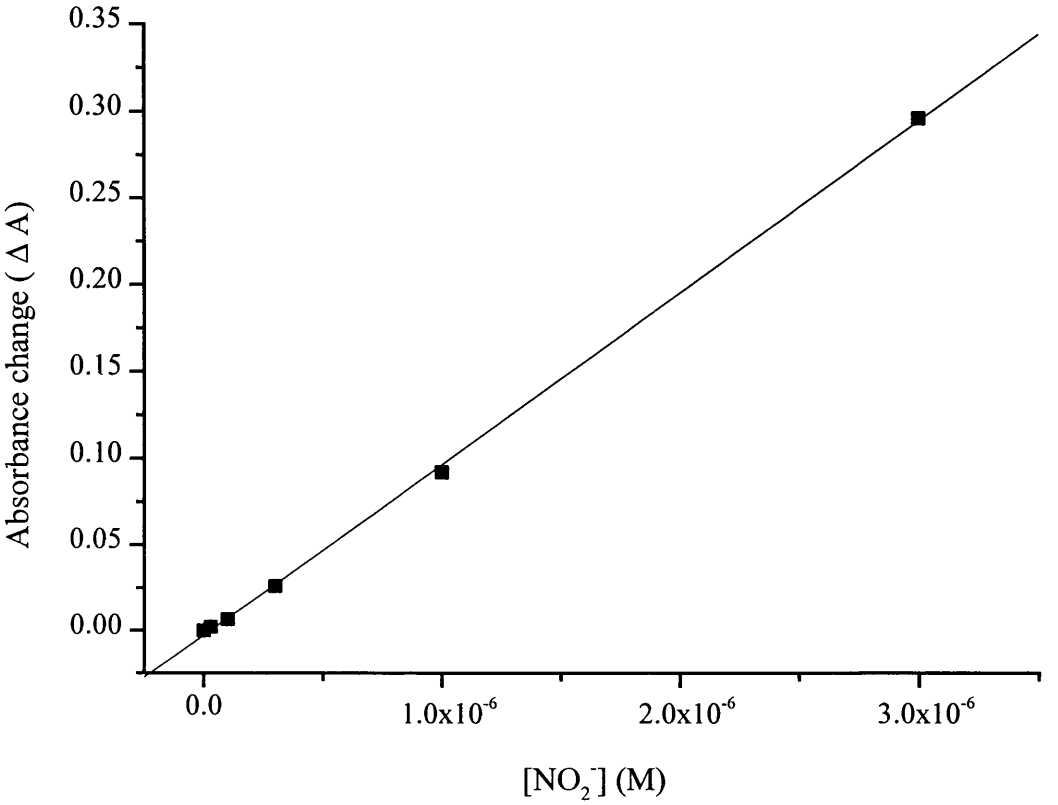


Figure 2.10: Typical modified Griess calibration curve: Solid line is the best fit straight line relationship between absorbance change (ΔA) and nitrite concentration[NO_2^-] (M) using the equation:

$$\Delta A = K \times [NO_2^-]$$

where $K = 9.90 \pm 0.11 \times 10^4\ M^{-1}$.

Pye Unicam SP8-500 UV/VIS spectrophotometer (Cambridge, England). The protein was determined from a calibration curve (Figure 2.11). Due to a high interassay protein level variability using this method, a second more reliable method was tried.

2.3.5.2 Coomassie blue for protein determination

The second method used was the Coomassie protein assay (Sedmak & Grossberg 1977) purchased in kit form from Pierce & Warringer Ltd, Chester, England. The cells were removed from the trays with a cell scraper instead of trypsin-EDTA and frozen to -70°C. Just before the assay, the cells membranes were mechanically lysed by an ultrasonic probe (VibraCell, Sonics & Materials, Connecticut, USA) and brought up to a final volume of 1.5 ml with sterile PBS. The Coomassie protein assay is based on the absorbance shift from 465 nm to 595 nm that occurs when Coomassie Brilliant Blue G-250 binds to proteins in an acidic solution. The dye consists of Coomassie brilliant blue G-250, phosphoric acid, methanol and solubilising agents. Standards made from bovine serum albumin (Pierce & Warringer Ltd, Chester, England) ranged from 1.56 $\mu g \cdot ml^{-1}$ to 2 $mg \cdot ml^{-1}$. The resultant colour change was read on the EL 312 Microplate ELISA reader at 595 nm. Figure 2.12 shows a standard curve from this method.

2.3.6 Calculations and statistics

2.3.6.1 Radioimmunoassays

The standard curves for ET, 6-keto-$PGF_{1\alpha}$, TxB_2 radioimmunoassays and 6-keto-$PGF_{1\alpha}$ EIA, were obtained by plotting percentage binding ($\%B/B_0$) against the log concentration of the compound. To calculate the zero standard (B_0), the mean counts per minute (cpm) attributed to non specific binding (NSB) was subtracted from the mean cpm due to maximal binding of the drug (TB) for each assay (Equation 1). To calculate $\%B/B_0$, the mean NSB was subtracted from the mean cpm of standard (S), divided by B_0 and multiplied by 100. This is shown in Equation 2:

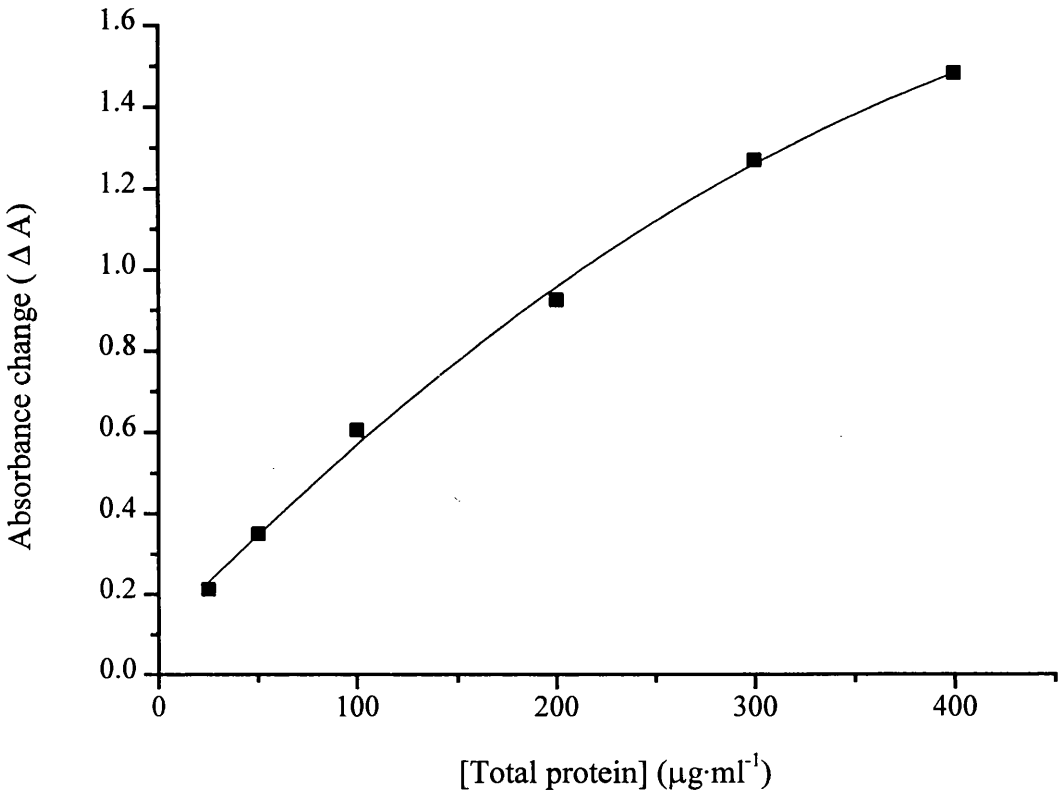


Figure 2.11: Typical calibration curve for the Lowry protein assay: Solid line is a exponential relationship between absorbance change at 750 nm (ΔA) and [total protein] ($\mu g \cdot ml^{-1}$) using the equation:

$$\Delta A = \Delta A_{max} \times (1-e^{(-K \times [\text{total protein}])})$$

Where $\Delta A_{max} = 1.92 \pm 0.17$ and $K = 3.60 \pm 0.56 \times 10^{-3}$.

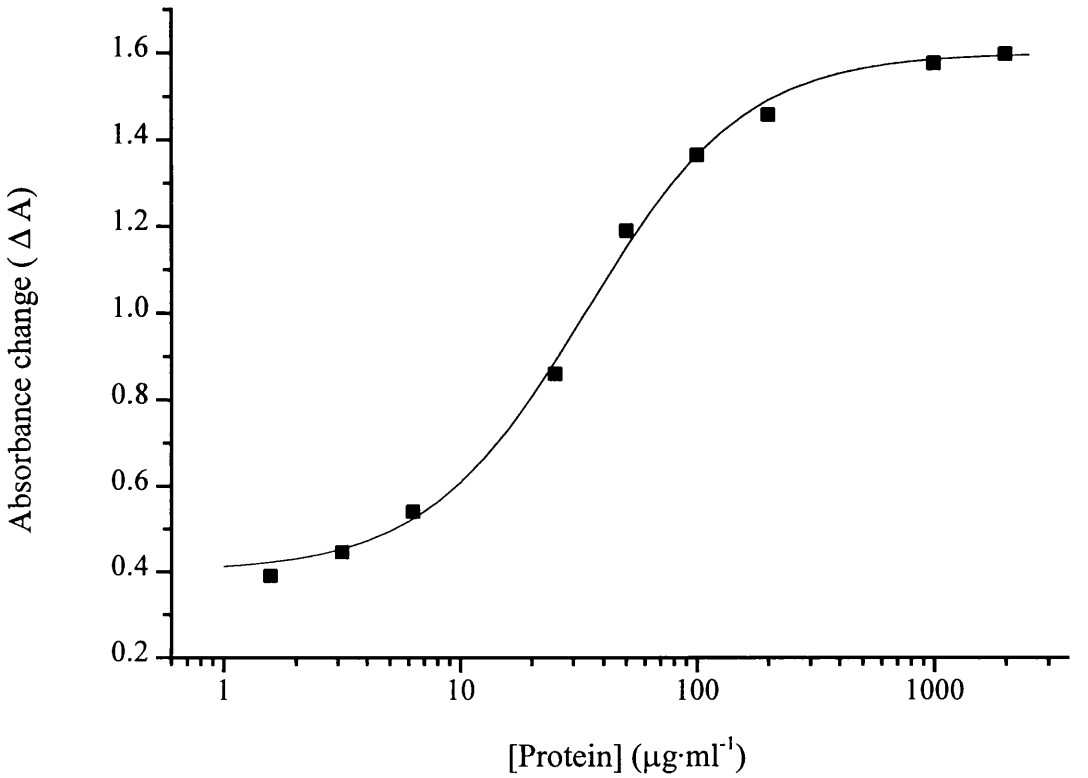


Figure 2.12: Typical calibration curve for the Coomassie protein assay: Solid line is a best fit logistic sigmoidal relationship between absorbance change at 570 nm (ΔA) and [total protein] ($\mu g \cdot ml^{-1}$) using the equation:

$$\Delta A = (\Delta A_{max} - \Delta A_{min})/(1+([\text{total protein}]/EC_{50})^{n})) \times + \Delta A_{min}$$

Where $EC_{50} = 33.5 \pm 1.5\ \mu g \cdot ml^{-1}$; $n = 1.29 \pm 0.07$; $\Delta A_{max} = 1.60 \pm 0.08$ and $\Delta A_{min} = 0.40 \pm 0.04$

$B_0 = TB - NSB$ Equation 1

$\%B/B_0 = 100 \times (S\text{-}NSB)/B_0$ Equation 2

The relationship between $\%B/B_0$ and concentration of the compound (D) was fitted to a logistic sigmoidal relationship (equation 3) using the program Fig P (Biosoft, Oxford, UK):

$$\%B/B_0 = (B_{max} - B_{min})/(1+([D]/EC_{50})^n) + B_{min} \quad \text{Equation 3}$$

B_{max} and B_{min} are the maximum and minimum values of the assay curve (which should be close to 100 and 0 respectively). EC_{50} is the concentration of the compound that gives a $\%B/B_0$ of 50%. The value of the exponent (n) describes the maximum steepness of the relationship between $\%B/B_0$ and the [D]. The FigP program reports the best estimate ($\pm$ s.e.m.) of values B_{max}, B_{min}, EC_{50} and n that fit the calibration data. The general form of the logistic equation described above provided a satisfactory fit ($p < 0.05$) to a range of calibration data. Most assay protocols suggest that the experimental values are determined by reading the values from the best fit calibration curve. An alternative method is to solve the logistic equation for the concentration of the drug ([D]) and use the constants from the best fit curve (calculated with FigP) as shown in Equation 4:

$$[D] = EC_{50} \times [(B_{max} - B_{min})-(\%B/B_0 - B_{min})/(\%B/B_0 - B_{min})]^{1/n} \quad \text{Equation 4}$$

The advantage of this latter method is that the accuracy of the estimates of the experimental values are not limited by the resolution of the graph paper and care with which the calibration curves and lines are drawn.

2.3.6.2 ELISA

With this assay system, the standard curve was obtained by plotting the absorbance change (ΔA) against the concentration of drug (D). This relationship is normally linear and the data was fitted to a straight line relationship using the program FigP (Equation 5).

$$\Delta A = K \times [D] + c \qquad \text{Equation 5}$$

Where K is the gradient of the line and c is the intercept. As with the logistic relationship, the K and c values are reported ± s.e.m (an estimate of the precision of each parameter used by the software program Origin). The inverse of this relationship was then used to calculate the concentration of the drug in the experimental solutions.

2.3.6.3 Protein assay

The Lowry protein assay calibration was fitted to an exponential curve, Equation 6, where ΔA is absorbance change, ΔA_{max} is the maximum absorbance change, and K is a constant.

$$\Delta A = \Delta A_{max} \times (1-e^{(-K \times [\text{total protein}])}) \qquad \text{Equation 6}$$

The total protein concentration could then be calculated from optical density measurements using Equation 7.

$$[\text{total protein}] = -(1/K) \times \ln (\Delta A_{max} - \Delta A)/ \Delta A_{max} \qquad \text{Equation 7}$$

The Coomassie method produced a calibration curve that was best fitted to a logistic sigmoidal curve of similar form to Equation 3.

2.3.6.4 Analysis of variance

The purpose of the experiments using cultured endothelial cells was to monitor the release of various vasoactive compounds over a 4 h period under normoxic and hypoxic conditions. The data take the form of values of concentration at 1, 2 and 4 h from equine and bovine cells under normoxic and hypoxic conditions and in the presence of inhibitors (L-NAME and indomethacin). Examining the data for statistical significance requires a technique that allows multiple comparisons to be made. One approach would be to use the Student's t test to compare each pair of groups. This has two potential flaws. Firstly if there are many comparisons to be made there is an unacceptably high probability that a significant result will be falsely reported. Secondly, when the sample size is small the estimate of the

variance will be poor. The use of the analysis of variance (ANOVA) method overcomes these two difficulties by adjusting the significance level to allow for the multiple comparisons and using all the data to estimate the variance, hence allowing more accurate comparisons. For this reason, ANOVA methods were used through out this thesis, except when a comparison of two data sets permitted the use of the Student's t test. On the advice from members of staff from the Department of Statistics (University of Glasgow), the General Linear Model (GLM) form of ANOVA was used in the form implemented in the program Minitab (version 10, Minitab Inc, PA USA). The variables used in each experiment were grouped into two forms: dependant variables - the measured variable i.e. [ET] and independent variables i.e. variables fixed by experimental design, e.g. hypoxia, L-NAME or indomethacin. Time (1, 2 and 4 h) was used as a covariate, i.e. a variable that alters the dependant variable in a set direction. In its simplest form, GLM may be used to determine whether the independent variables significantly effect the data. In extended form, GLM can be used to test for significant interactions between variables, e.g. does the effect of indomethacin on release depend on the vessel type. In all tests performed, statistical significance was set at p less than 0.05. Values of p greater than 0.05 were not included and the interaction considered not significant. P values less than 0.1 were noted as close to significance.

2.4 Isometric tension measurements

2.4.1 Dissection of vessels

Equine and bovine pulmonary arteries were removed from the parenchyma of lung tissue of recently killed horses and cattle. First, dissecting a thin strip (1 cm) off the edge of the lungs from the major lobes allowed exsanguination. The caudal third of each lung was then removed. The major arteries were rinsed with cold gassed (95% O_2 / 5% CO_2 at 4°C) Kreb's solution and the caudal third of the lobe was put into cold Kreb's solution and taken back to the laboratory. The arteries were traced from a large pulmonary artery to branched arterioles with an outer diameter of 1.5 - 4.0 mm. These were removed taking care not to damage the outer connective tissue

layer of the vessels and were put into cold gassed Kreb's solution. The vessels were either used immediately or kept in a refrigerated (4°C) air tight container until the next day. Before use, the vessels were trimmed of parenchyma and excess connective tissue.

2.4.2 Tissue Bath Studies

Individual arterial rings were cut to a width of 2 mm by laying the dissected vessel across a perspex holder with 5 scalpel blades spaced 2 mm apart and gently rolling a plastic test tube across the blood vessel. This guaranteed the uniform length of vessels. For each experiment (4 rings/animal), two rings had the endothelium left intact and two rings had the endothelium removed by gently rubbing the inner surface of the rings with a roughened match stick. The rings were placed on purpose-made triangular steel wire holders, taking care not to damage the endothelium. The rings were suspended from Grass FT03 force transducers by silk thread tied to the triangular holders. The suspended blood vessels were anchored in 10 ml organ baths (Figure 2.13) and bathed in Kreb's solution of the following composition (mM): NaCl 118.0, KCl 4.57, $CaCl_2$ 2.52, KH_2PO_4 1.19, $MgSO_4$ 1.19, $NaHCO_3$ 25.0 and glucose 11.1. Kreb's solution (37° ± 1°C) was gassed with 95% O_2 / 5% CO_2 continuously. Isometric tension was recorded with Grass FT03 force-displacement transducers (Grass Instrument Co, Quincy, MA, USA) linked to a Grass Model 7E Polygraph. The polygraph was calibrated with a set of weights (1,2, 3 and 4 g) before each experiment. Force produced by vessels was measured with reference to this calibration. At the end of each experiment the arterial rings were weighed. These values were used to express the tension produced by each vessel in terms of grams tension/gram wet weight (g tension/g wet wt). This is a standard procedure used to normalise the tension produced to the mass of the vessel (Schott et al 1993; Tsuchida et al 1994). Experiments were initially carried out to define the optimal resting tension required to achieve the maximum agonist response in both equine and bovine vessels. The maximum response to 10^{-6} M PE was observed with a resting tension of 3 to 4 g wt. In order to achieve a stable tension baseline,

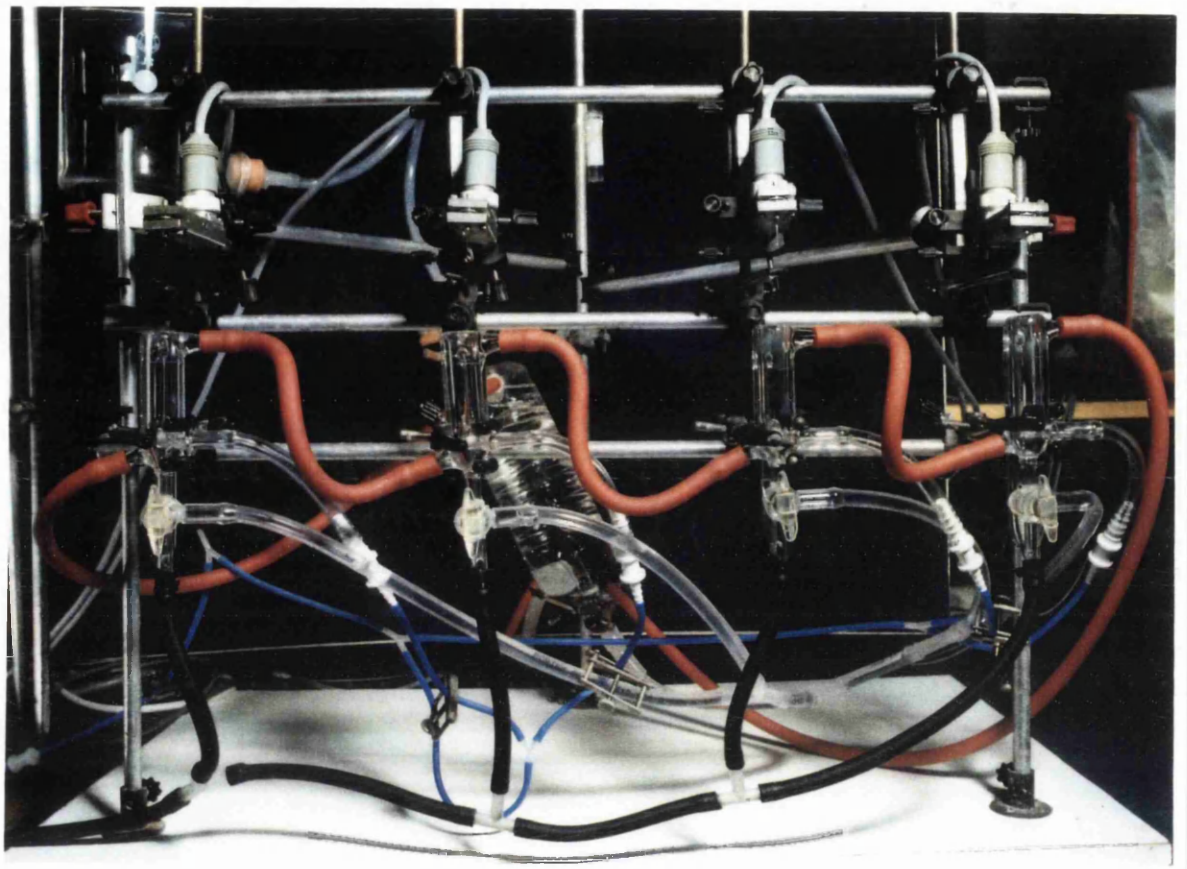


Figure 2.13: Photograph of tissue bath experimental setup. Four water-jacketed pyrex tissue baths were arranged below 4 Grass tension transducers. Individual rings of blood vessel were held within each tissue bath and bubbled continuously with 5% CO_2/95% O_2 (or hypoxic gas).

the vessels' diameters were increased incrementally over 1- 2 hours until this resting tension was achieved.

2.4.2.1 Studies of phenylephrine and 5-hydroxytryptamine

Cumulative dose response curves were constructed in equine and bovine vessels (contracted in a dose-dependent manner) with PE (10^{-10} M to 3 x 10^{-5} M) and 5-HT (10^{-10} M to 3 x 10^{-5} M). The response to 5-HT in equine pulmonary arteries was poor. Consequently, PE was used to induce tone in these vessels to study the effects of Bk, ACh and hypoxia. In bovine vessels, 5-HT elicited a stronger response than in equine vessels, therefore 5-HT was used in the equivalent bovine experiments. The concentration of PE and 5-HT that generated 50% of the maximal contraction (EC_{50}) and slope of the relationship were calculated for each experiment

2.4.2.2 Studies of endothelin response

To determine the type of ET receptor on the equine pulmonary arteries, the ET_A antagonist BQ 123 and the ET_B antagonist BQ 788 (Alexis Corp, Nottingham, UK) were used. A dose response curve for ET-1 was constructed by adding ET-1 cumulatively (10^{-10} M to 3 x 10^{-7} M) to equine pulmonary artery rings. The vessels were washed and force allowed to reach baseline. Using previously established concentrations, BQ 123 (3 x 10^{-6} M) or BQ 788 (10^{-7} M) were added to the tissue baths and incubated for 20 minutes (Douglas et al 1993; Ishikawa et al 1994). ET-1 was added as described above.

2.4.2.3 Studies of bradykinin and acetylcholine

The functional integrity of the endothelium of equine and bovine arterial rings was assessed by inducing vasodilation (relaxation) to Bk and ACh. This was achieved by contracting the vessel to a plateau by using the EC_{50} value of each agonist, and then adding 10^{-11} M - 10^{-6} M Bk or 10^{-9} M - 10^{-5} M ACh in a dose-dependent manner to relax the vessels. An investigation into the effects of indomethacin (10^{-5} M) and L-NAME (10^{-4} M) on the relaxation of Bk was carried out by adding either indomethacin or L-NAME or both to the tissue bath for 20 minutes before adding Bk. Then Bk was added in a dose-dependent manner as described above.

2.4.2.4 Hypoxic pulmonary vasoconstrictor response

In bovine pulmonary arterial rings, a concentration of agonist that gave approximately 50% of maximum force was added and allowed to reach a plateau. The vessels were gassed with 3 hypoxic gases, to test the effect on the vasoactivity of the vessels. Gas 1 consisted of 5% O_2 / 5% CO_2 / balance N_2; gas 2 consisted of 2% O_2 / 5% CO_2 / balance N_2 and gas 3 consisted of 5% CO_2 / balance N_2. Gas 1, 2 or 3 was added to the tissue baths through the same tubes instead of the normoxic gas until a plateau effect was achieved (approximately 20 min). The PO_2 of the solution in the baths was monitored with an oxygen electrode attached to an oxygen meter (Strathkelvin Instruments). Krebs bubbled with 95% O_2 gas had a PO_2 of > 200 mm Hg; with 5% O_2 gas, the PO_2 was 62 $\pm$ 6 mm Hg; with 2% O_2 gas the PO_2 was 25 $\pm$ 3 mm Hg and with 0% O_2 the PO_2 was 0.9 $\pm$ 1 mm Hg (n = 13). After the hypoxic period, the gas was switched back to the normoxic gas 95% O_2/5% CO_2 The tissues were then washed and force allowed to return to baseline.

After establishing this control response, tissues were then exposed to 10^{-5} M indomethacin, 10^{-4} M L-NAME or both for 20 minutes, then the gas was switched to the anoxic gas 95% N_2/5% CO_2 until the vessels reached maximal contraction.

2.4.3 Calculations and statistics

The data (g tension/g wet weight) were converted to percentage of maximum values by dividing each value by the maximum contraction and multiplying by 100. The EC_{50} and slope of the linear portion of the dose response curve, for each ring, were calculated by plotting the concentration of agonist against the percentage of maximum effect and fitting a logistic sigmoidal curve to the data (using the program Origin 3.54, Microcal Software, California). The sigmoidal logistic curve relating isometric tension (T) to concentration of agonist (D) is shown in Equation 8:

$$T = (T_{max} - T_{min})/(1+([D]/EC_{50})^{n}) + T_{min} \qquad \text{Equation 8}$$

where T_{max} and T_{min} are the maximum and minimum tension levels fixed at 100 and 0 respectively. EC_{50} is the concentration of agonist that produced 50% maximum force, and n is an exponent that determines the slope of the relationship. Two

methods of analysis of cumulative dose response curves are possible: (i) mean tensions can be calculated at each drug concentration and a single sigmoidal curve fitted to the mean data or (ii) results from individual vessels can be fitted with a sigmoidal relationship and the mean EC_{50} and slope (n) calculated from these numerous curves. Theoretical work based on the classical pharmacological model of drug-receptor interaction described in Tallarida & Jacob (1979) suggests that the latter of these two methods is the more valid one. Therefore, the values of EC_{50} and slope (n) were recorded from each dose response curve. As a measure of the force production from each ring of blood vessel, the maximal force produced by the agonist was noted. Thus for each experiment, maximal force, EC_{50} and slope (n) were calculated. The mean ($\pm$ s.e.m.) of these values were tabulated and compared with values under a range of experimental conditions. ANOVA (General Linear Model) was used to determine the statistical significance of the differences (section 2.3.6.4).

The ability of ACh and Bk to cause relaxation was measured by expressing the relaxation as a percentage of the initial force level. The relationship between the drug and the percentage relaxation was fitted with a logistic sigmoidal relationship as described above. The EC_{50}, slope (n) and maximal relaxation were calculated for each experiment and the mean $\pm$ s.e.m. of these values were tabulated and assessed using ANOVA (GLM).

Measurements of isometric tension during hypoxia were quantified by expressing the tension in hypoxia relative to that seen in normoxia (expressing this value as a percentage). Equine and bovine vessel responses, in intact and rubbed vessels, in the presence of indomethacin and L-NAME, were compared using ANOVA. A significance level of $p < 0.05$ was used.

Chapter 3: Results

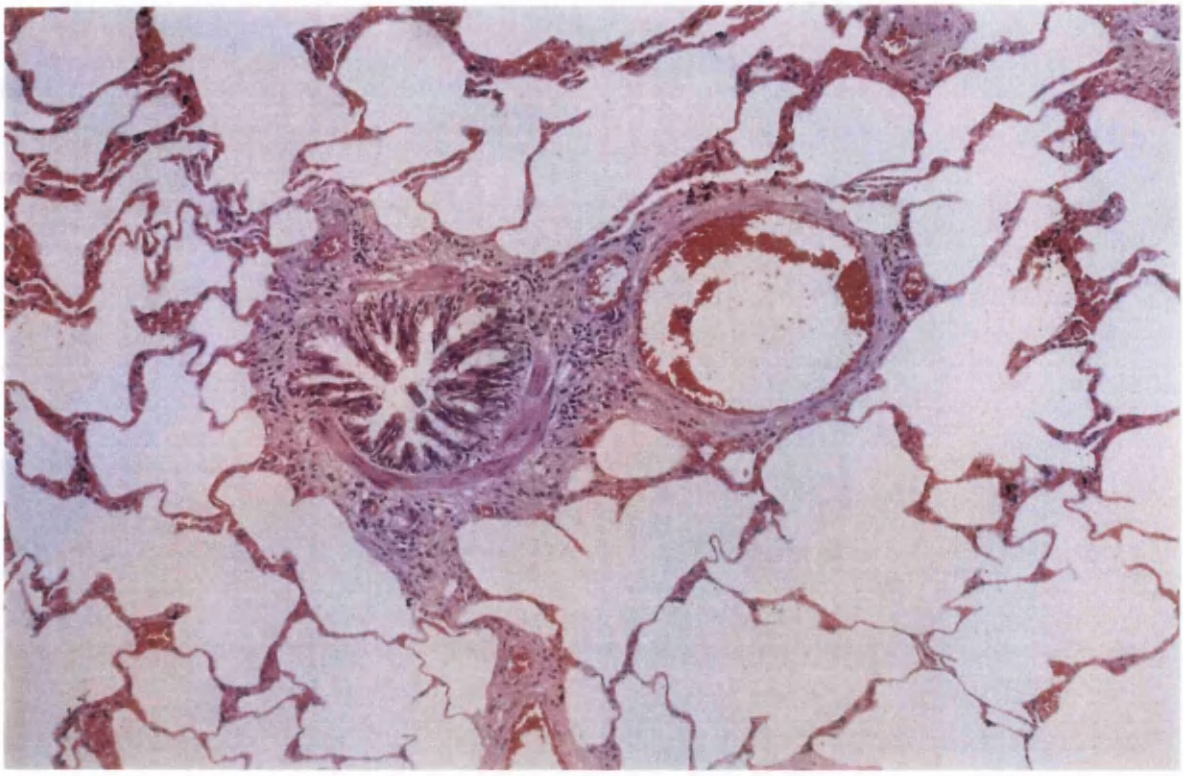
3.1 Pulmonary histology

On examination of freshly isolated lungs, the small arteries (2 - 3 mm o.d.) within the bovine lung appeared to be surrounded by more connective tissue compared to equine tissue from a comparable region of the lung. This feature made the dissection of bovine pulmonary vessels easier than equine. Upon isolation, the bovine vessel wall appeared to be thicker than equine pulmonary arteries of the same outer diameter. These observations were confirmed by examination of histological sections of bovine and equine lung. Figures 3.1A & B are photomicrographs of H & E stained equine and bovine lung parenchyma, with a small bronchus and accompanying artery centred in each. Although systematic studies were not performed, this figure supports the general impression that bovine lung parenchyma contains more connective tissue than equine.

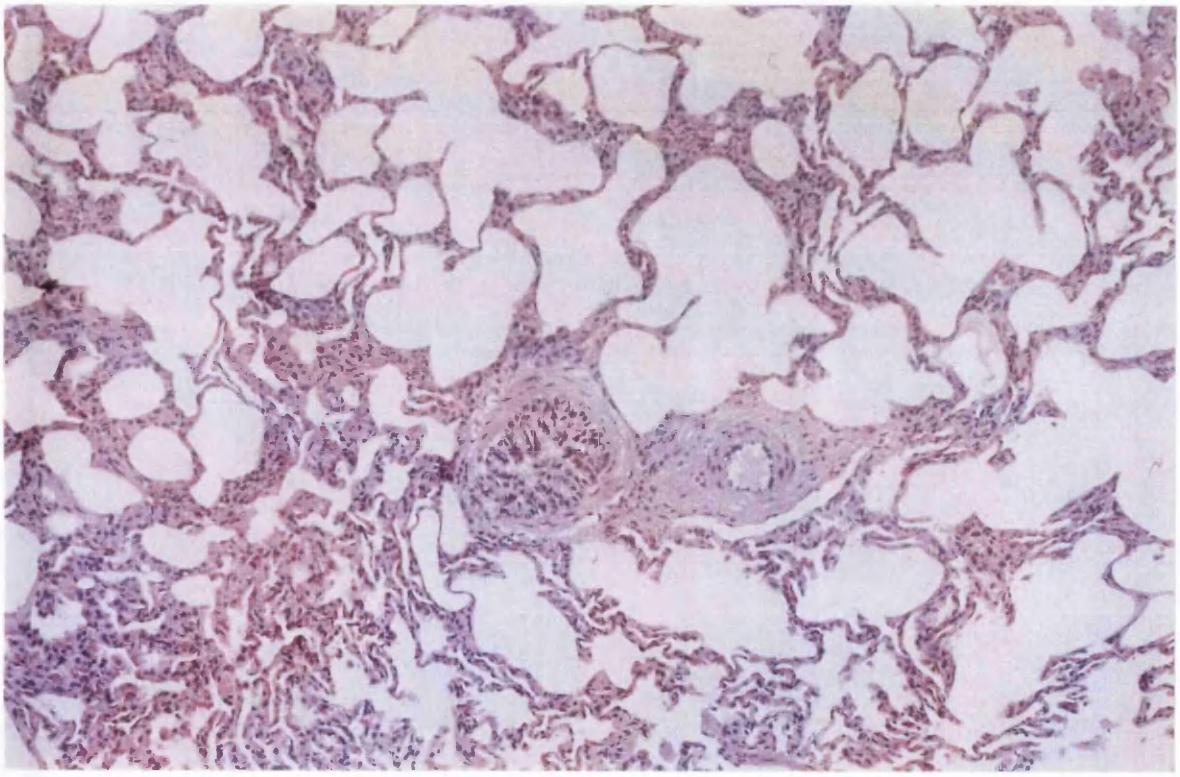
Figures 3.2A & B are cross sections of equine and bovine pulmonary arteries (of approximately the same outer diameter), stained with H & E. The inner endothelial cell layer can be seen at the top of the photographs. Bovine vessels appear to have proportionally more smooth muscle in the tunica media than equine vessels, although quantitative measurements have not been made.

Figure 3.3 shows equine (A) and bovine (B) pulmonary arteries in cross section, stained with Verhoeff's stain, which stains elastin black and collagen red and muscle cells yellow. Both species show a diffuse distribution of elastin in the tunica media. In the bovine artery, the internal elastic lamina between the tunica intima and tunica media is clearly seen at the top of the photograph.

The next set of photomicrographs (Figures 3.4 and 3.5) are cross sections of equine and bovine pulmonary arteries respectively, stained with von Willebrand factor (Factor VIII), specific for endothelial cells (section 2.2.2.2). The first photographs in each set (A) are pulmonary arteries with the intact endothelium and the second in

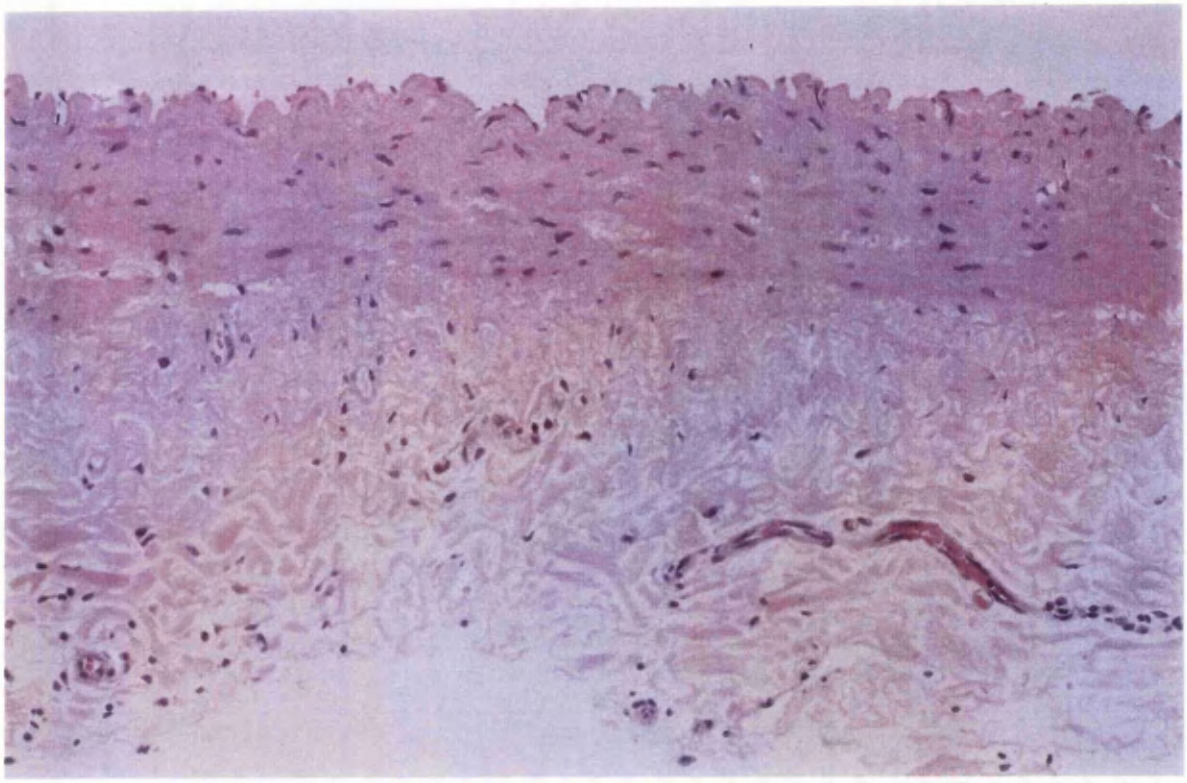


A

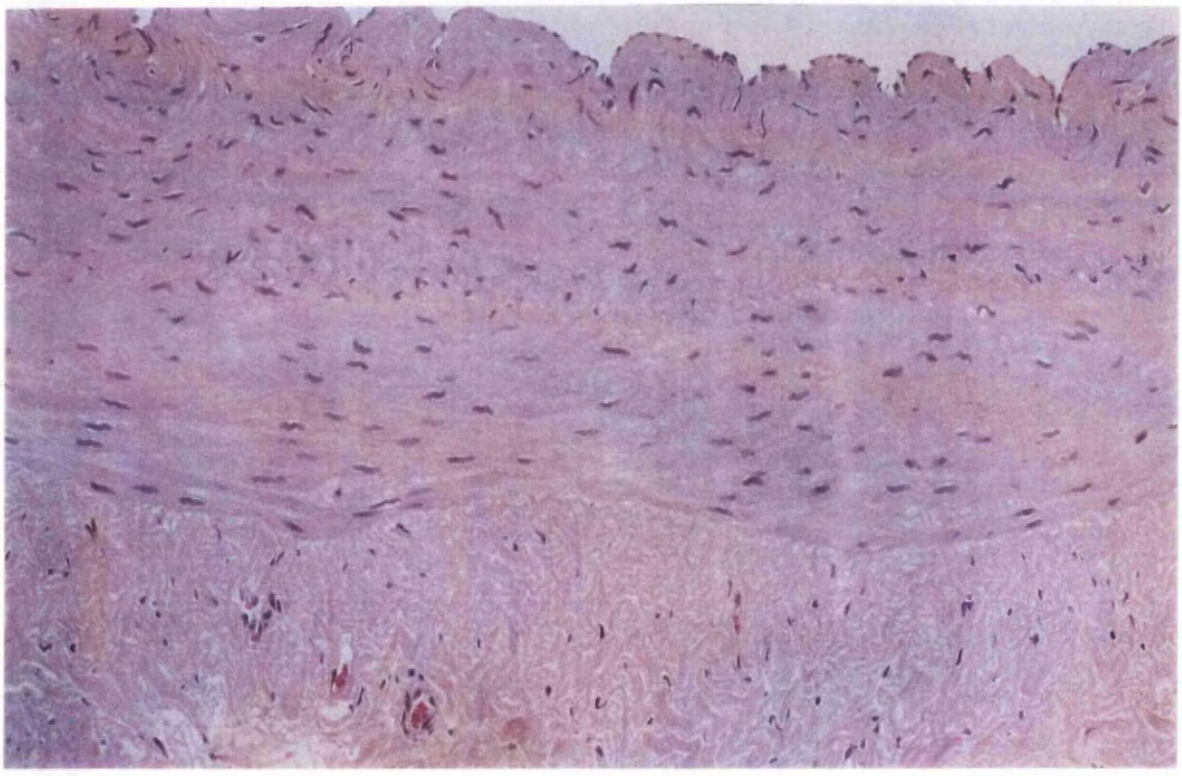


B

Figure 3.1: H & E staining of pulmonary parenchyma. **Panel A:** Equine lung. **Panel B:** Bovine lung. A small bronchi (left) and artery (right) are seen in the centre of each photograph, magnification 40 X. This staining method distinguishes cytoplasm and connective tissue (pink) from nuclei (purple) and red blood cells in the blood vessels (red).



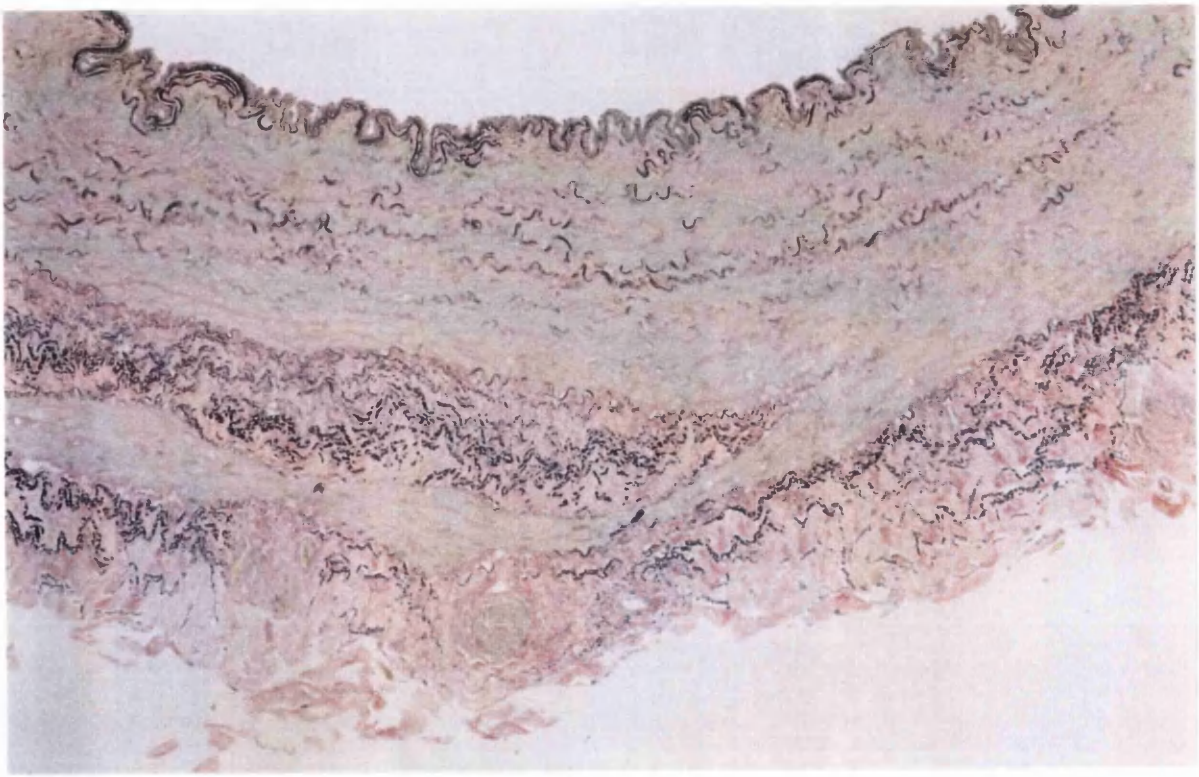
A



B

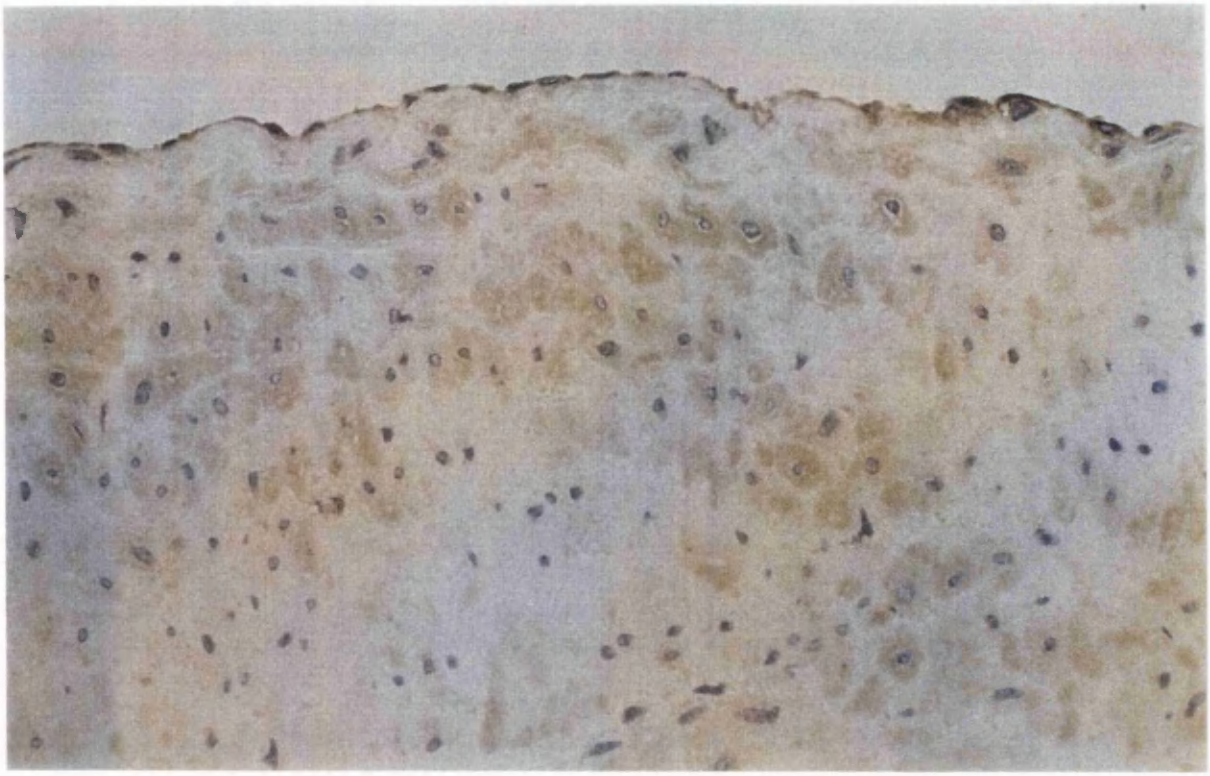
Figure 3.2: Cross sections of pulmonary vessels, lumen at top, H & E stain, magnification 70 X. **Panel A:** Equine pulmonary artery. **Panel B:** Bovine pulmonary artery.

A



B

Figure 3.3: Cross sections of pulmonary arteries stained using Verhoeff's haematoxylin for elastic fibres, vessel lumen at top. **Panel A:** Equine **Panel B:** Bovine. Elastic fibres and nuclei are stained black, collagen is counterstained red and remaining smooth muscle tissue yellow. Magnification 50 X.



A

B

Figure 3.4: Equine pulmonary arteries in cross section stained for von Willebrand factor (Factor VIII). **Panel A:** Equine pulmonary artery with endothelium left intact. **Panel B:** Rubbed equine pulmonary artery from the same animal. Positive staining gives an intense brown colour evident on the luminal surface (top) in panel A and not in panel B. Magnification 128 X

A

B

Figure 3.5: Bovine pulmonary arteries in cross section stained for von Willebrand factor (Factor VIII). **Panel A:** Bovine pulmonary artery with endothelium left intact. **Panel B:** Rubbed bovine pulmonary artery from the same animal. Positive staining gives an intense brown colour evident on the luminal surface (top) in panel A and not in panel B. Magnification 128 X

each set (B) have been rubbed in an attempt to remove the endothelial cell layer (section 2.4.2). Factor VIII stains endothelial layers dark brown, which can be seen as a single layer at the top of Figures 3.4A and 3.5A. Figures 3.4B and 3.5B do not have significant staining of this layer (top of photograph) confirming the absence of an endothelial layer with minimal damage to the underlying tissues.

Figure 3.6 shows a longitudinal section of equine pulmonary artery, with H & E staining, before and after enzymatic digestion with collagenase to remove the single layer of endothelial cells for culture (section 2.2.1). Figure 3.6A shows the single layer of endothelial cells at the top of the photomicrograph and Figure 3.6B shows the same vessel after digestion with collagenase. The endothelial layer is no longer evident.

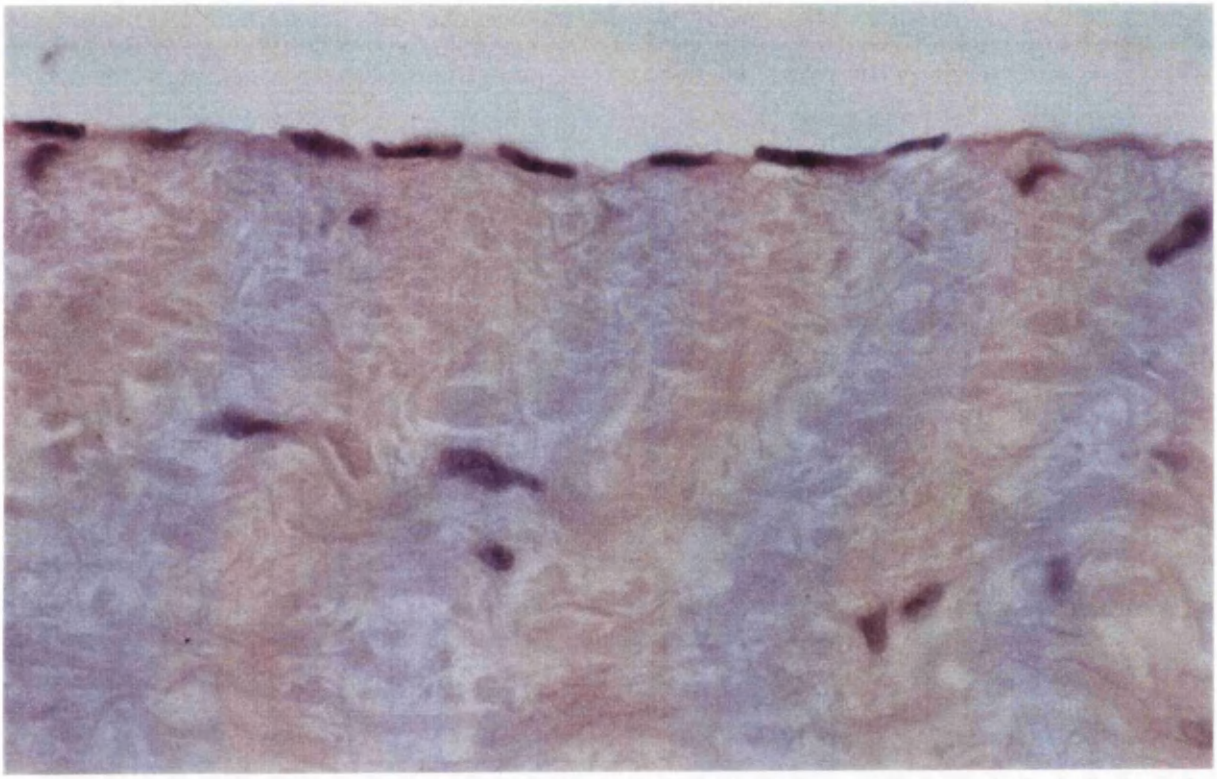
3.2 Endothelial cell culture and characterisation

3.2.1 Primary isolation of vascular endothelial cells

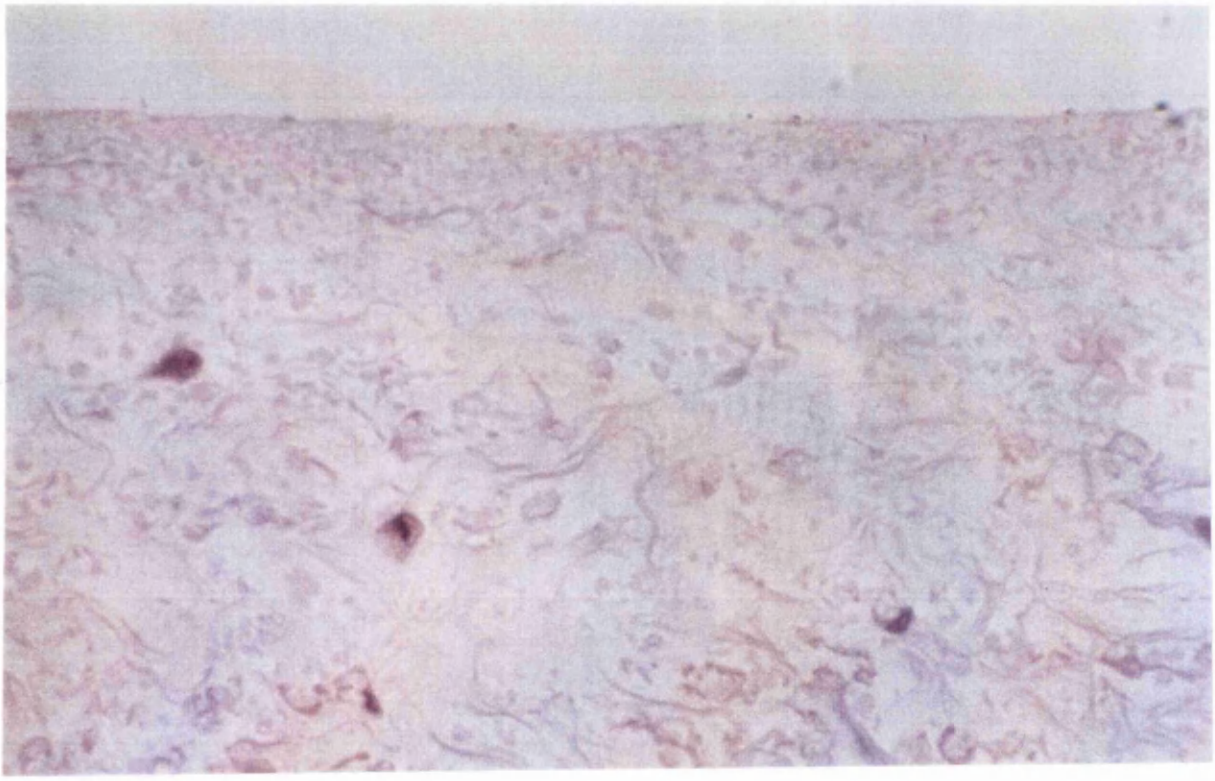
Equine endothelial cells grew to confluence in 6 days on average (range 5 - 8 days), whereas bovine cells achieved confluence over a shorter period of 4 - 6 days. Equine and bovine cells were homogeneous, closely packed and polygonal in shape (approximate diameter 8 μm), with a large centrally located nucleus. The cobblestone appearance of the cultured endothelial cells shown in Figure 3.7 (equine) and Figure 3.8 (bovine) are characteristic of endothelial cells in culture (Freshney 1994). Note, Figure 3.8 shows bovine endothelial cells growing from an aggregate of cells, as described in section 2.2.1.

3.2.2 Immunofluorescence studies

As described in section 2.2.2.2., staining with von Willebrand factor (Factor VIII) is a recognised method of identifying endothelial cell phenotype. Figure 3.9A is a photomicrograph of cells stained for von Willebrand factor. The obvious fluorescence from within the inner surface of the cell membrane contrasts with absence of any detail of the control photomicrograph (Figure 3.9B).



A



B

Figure 3.6: H & E staining of equine pulmonary artery longitudinal strips, inner surface at top of photographs. **Panel A:** Prior to enzymatic digestion with 0.1 % collagenase. **Panel B:** After treatment with collagenase for 45 minutes. Magnification 400 X

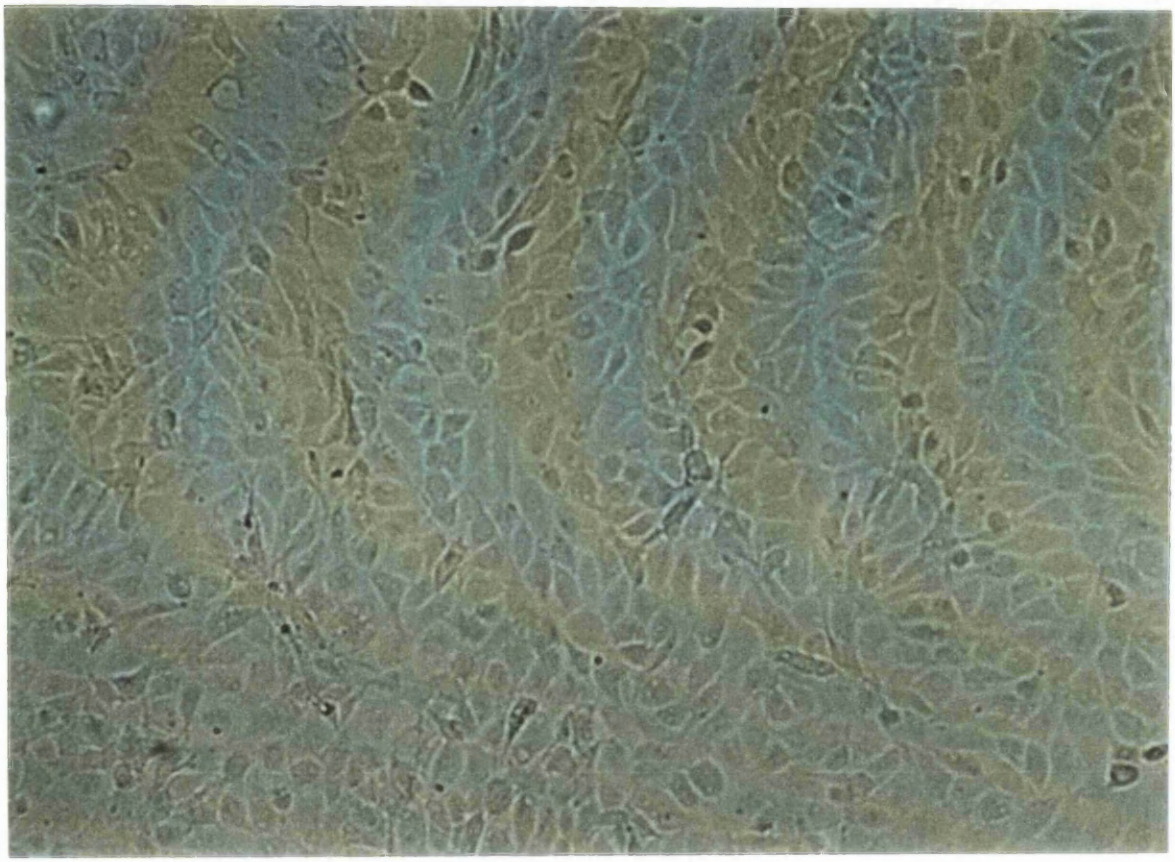


Figure 3.7: Light microscopy photograph of equine pulmonary artery endothelial cells grown to confluence in culture. Viewed with phase contrast optics, day 8, magnification 500 X.

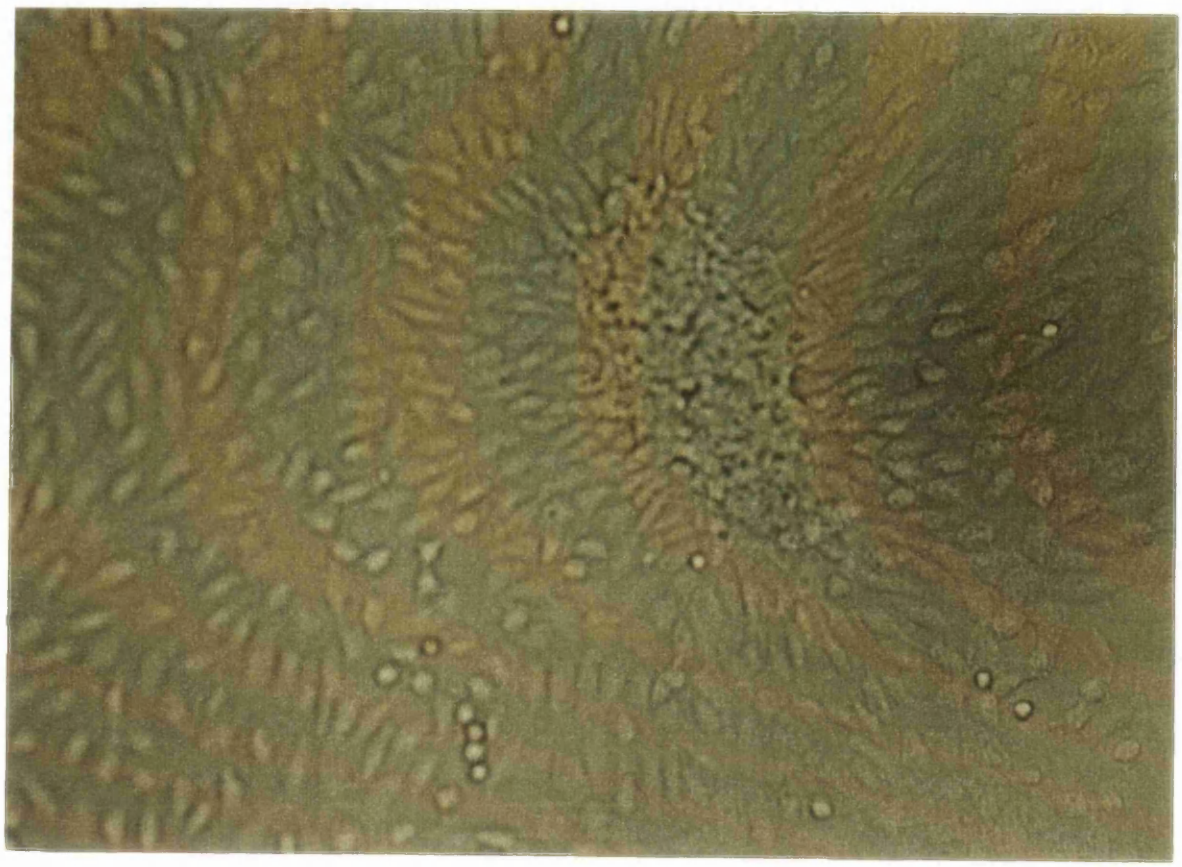
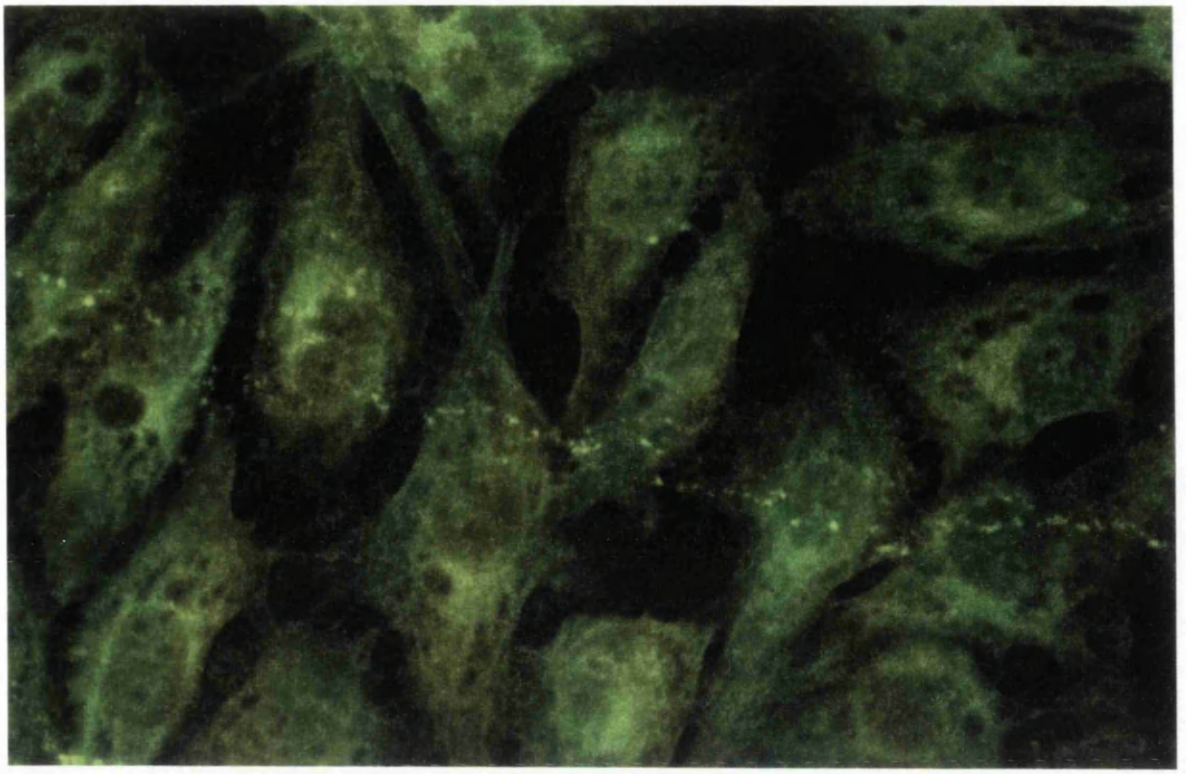
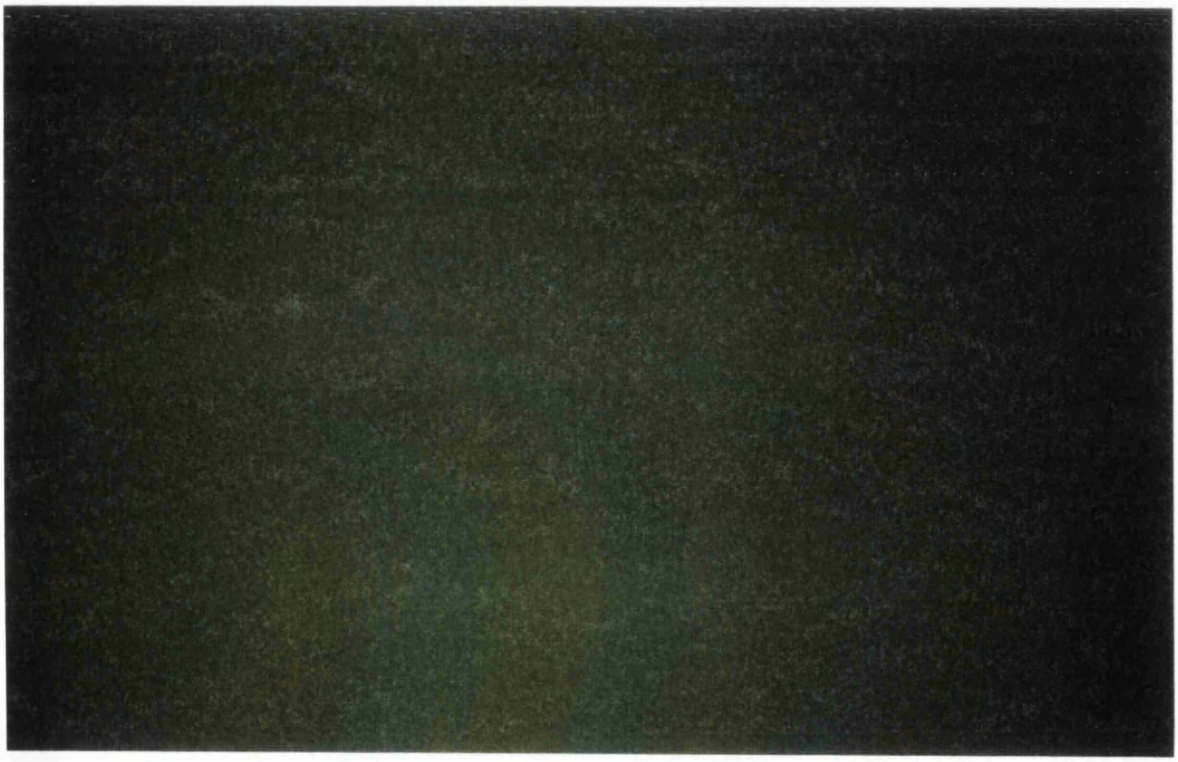


Figure 3.8: Light microscopy photograph of bovine pulmonary artery endothelial cells grown to confluence in culture. Day 4. Viewed with phase contrast optics with a yellow filter. Magnification 500 X.



A



B

Figure 3.9: Panel A: Confluent equine pulmonary artery endothelial cells. Treated with a primary antibody for von Willebrand factor and a secondary fluorescent antibody tag. Image obtained with UV illumination, magnification 2000 X. **Panel B**: Equine endothelial cells stained using the same procedure illustrated above, except with the omission of primary antibody Magnification 2000 X.

3.2.3 NADPH-diaphorase staining

Figure 3.10 illustrates cultured endothelial cells stained for NO synthase using NADPH-diaphorase. As shown in Figure 3.10A, the staining of bovine endothelial cells was heterogeneous in distribution with the highest staining density present in the peri-nuclear area and on the cytoplasmic cell membrane. In the equine endothelial cells, the staining was positive, but less well defined (Figure 3.10B).

3.2.4 Measurement of intracellular calcium concentration

Figure 3.11 illustrates the response of cultured endothelial cells to Bk. During the period indicated by the bar, the cells were superfused with 1μM Bk. As indicated by the fluorescence ratio, Bk caused a transient rise of intracellular [Ca^{2+}] which returned to pre-stimulation levels. Similar responses were seen in cells from two other equine pulmonary arteries. The transient response to Bk is similar to that observed in endothelial cell preparations from other species (section 1.3.1.1). These results support the endothelial phenotype of the cells cultured from equine pulmonary arteries.

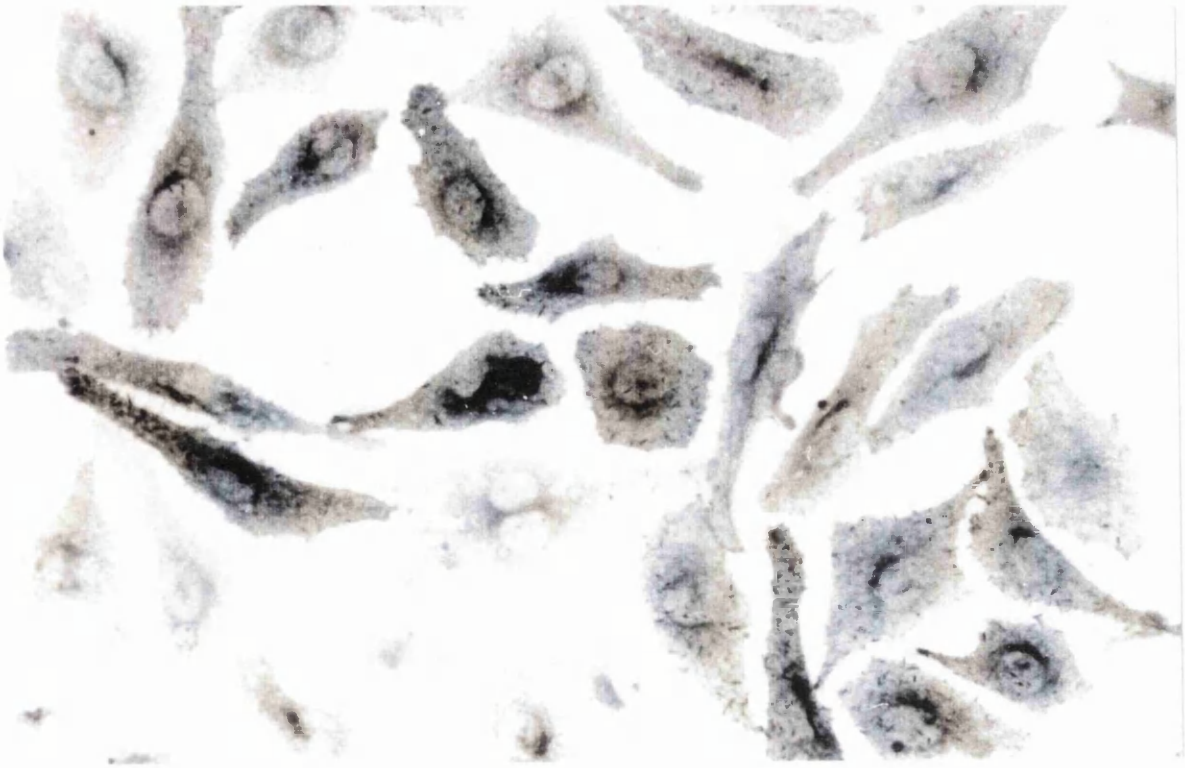
3.3 Results from endothelial cell assays

3.3.1 Endothelin production by cultured endothelial cells

3.3.1.1 Endothelin production in normoxia and hypoxia

As described in section 2.3.1.1, ET release was measured with the radioimmunoassay, over a period of 4 hours, using endothelial cells from equine pulmonary artery, equine aorta and bovine pulmonary artery under normoxic and hypoxic conditions. The results are summarised in Table 3.1 and plotted in Figure 3.12.

As can be seen from Table 3.1 and Figure 3.12, ET concentration ([ET]) progressively increased over the 4 h period, with an approximately linear time course. The values of ET shown are not thought to be assay specific, since the few measurements made using the ELISA-based system (section 2.3.1.2) gave



A

B

Figure 3.10: Panel A: NADPH-diaphorase staining in cultured bovine pulmonary artery endothelial cells. Cells were fixed and stained 15 minutes after treatment with 10^{-9} M Bk. Magnification 1000 X. **Panel B**: NADPH-diaphorase staining in cultured equine pulmonary artery endothelial cells. Cells were fixed and stained 15 minutes after treatment with 10^{-9} M Bk. Magnification 670 X.

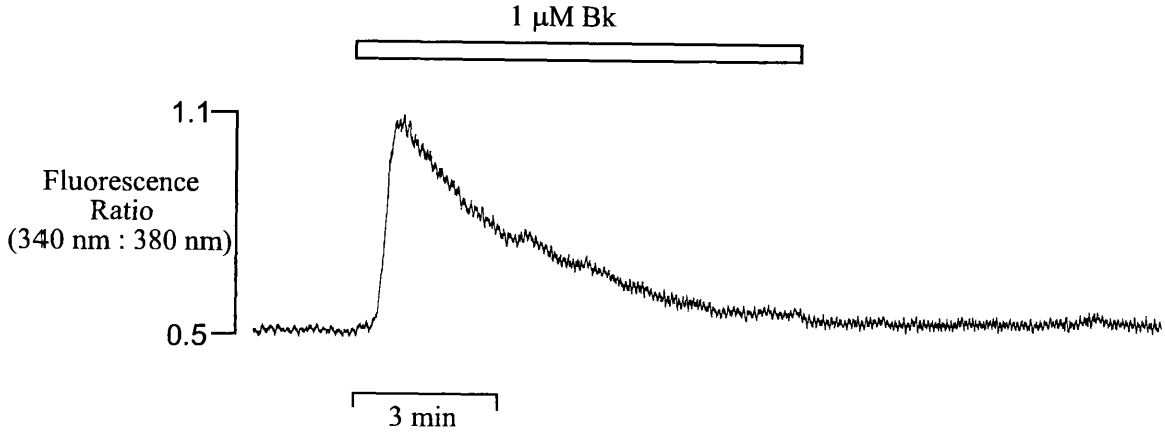


Figure 3.11: Transient release of intracellular Ca^{2+} in cultured equine pulmonary artery endothelial cells, stimulated by 1 μM bradykinin (Bk) over 10 minutes. Time is plotted against fluorescence ratio of 340 nm : 380 nm.

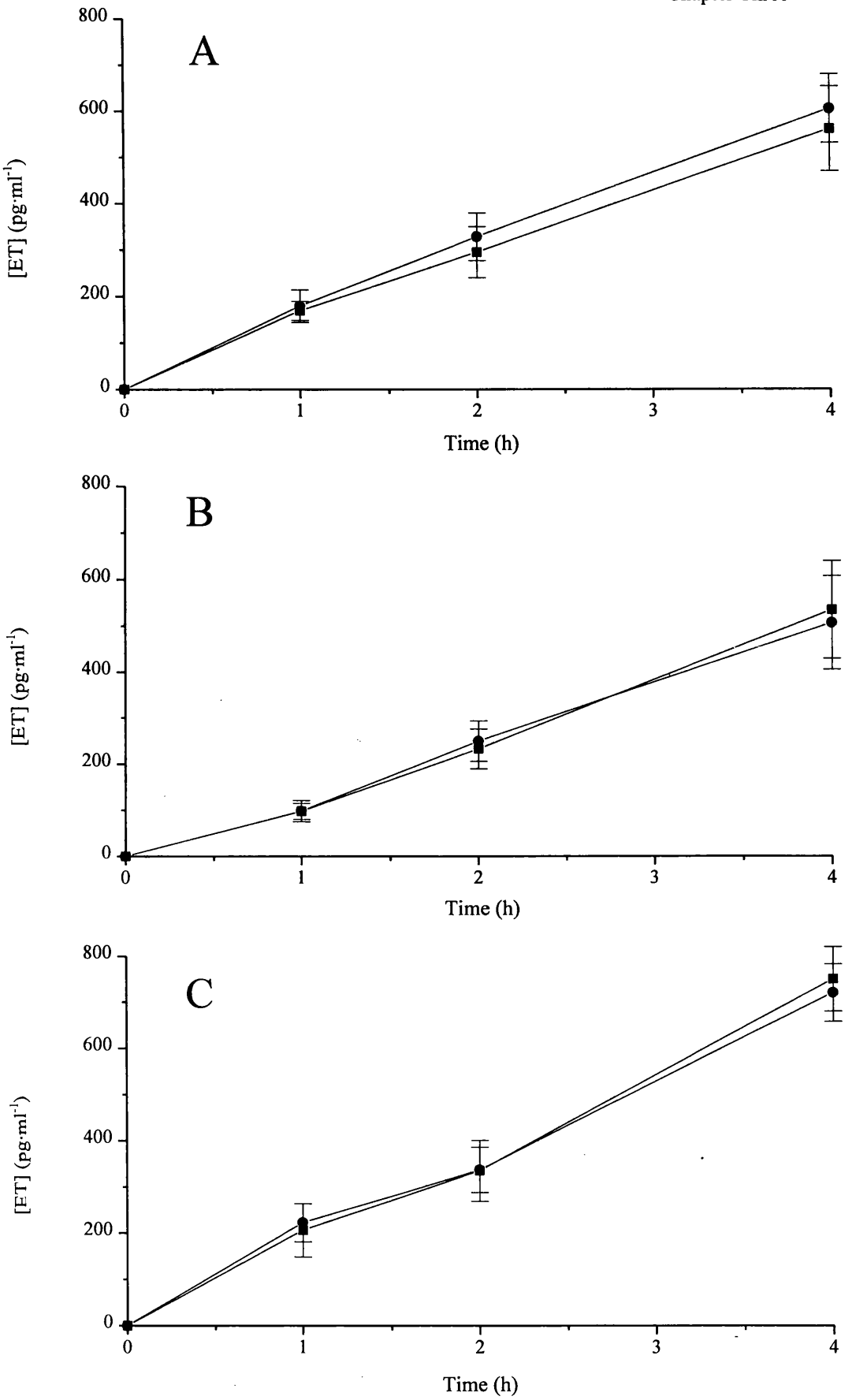


Figure 3.12: Panel A: Endothelin [ET] production (mean ± s.e.m.) in equine pulmonary artery cells. **Panel B:** [ET] production in equine aorta cells. **Panel C:** [ET] production in bovine pulmonary artery cells. Results are 1, 2 and 4 h incubation. (■), values obtained in air / 5% CO_2 incubation; (●), values obtained in 95% N_2 / 5% CO_2.

comparable values (results not shown). As described in section 2.3.6, analysis of variance was applied to all data points. No significant difference in ET production in normoxic and hypoxic conditions was evident in all 3 vessel types. However, vessel type was a significant factor ($p = 0.002$). Bovine pulmonary artery endothelial cells produced significantly more ET than equine pulmonary artery ($p = 0.011$) or aorta endothelial cells ($p < 0.001$). Equine pulmonary artery and aorta endothelial cells produced similar quantities of ET.

		Equine PA	Equine Aorta	Bovine PA
Oxygen	Time (h)	[ET] ($pg \cdot ml^{-1}$)	[ET] ($pg \cdot ml^{-1}$)	[ET] ($pg \cdot ml^{-1}$)
Normoxia		(n = 16)	(n = 11)	(n = 11)
	0	0	0	0
	1	169 ± 20	98 ± 17	206 ± 58
	2	295 ± 55	233 ± 43	334 ± 66
	4	560 ± 92	534 ± 106	750 ± 70
Hypoxia		(n = 16)	(n = 11)	(n = 11)
	0	0	0	0
	1	179 ± 35	99 ± 23	222 ± 41
	2	328 ± 51	250 ± 44	336 ± 49
	4	604 ± 74	506 ± 101	720 ± 63

Table 3.1: Endothelin (ET) production (mean ± s.e.m.) in $pg \cdot ml^{-1}$ by endothelial cells from equine pulmonary artery (PA), equine aorta, and bovine pulmonary artery (PA) at 1, 2 and 4 h, under normoxic and hypoxic conditions.

3.3.1.2 Endothelin production in the presence of indomethacin and L-NAME

Indomethacin (a cyclo-oxygenase inhibitor) and L-NAME (inhibitor of NO production) were individually found to effect the contractility of isolated pulmonary vessels, particularly in response to Bk or hypoxia (section 3.4.1.5). To investigate the possibility that these agents act by altering ET production, endothelial cells were incubated with 10^{-5} M indomethacin and 10^{-4} M L-NAME separately, under normoxic and hypoxic conditions. The results are shown in Table 3.2 and Figure 3.13.

			Equine PA	Equine Aorta	Bovine PA
Oxygen	Drug	Time (h)	[ET] ($pg \cdot ml^{-1}$)	[ET] ($pg \cdot ml^{-1}$)	[ET] ($pg \cdot ml^{-1}$)
Normoxia	Control		(n = 9)	(n = 9)	(n = 7)
		0	0	0	0
		1	180 ± 27	87 ± 17	252 ± 73
		2	362 ± 73	227 ± 35	428 ± 84
		4	692 ± 115	550 ± 119	790 ± 97
	Indomethacin		(n = 5)	(n = 5)	(n = 5)
		0	0	0	0
		1	115 ± 9	72 ± 18	294 ± 100
		2	257 ± 63	155 ± 44	356 ± 94
		4	606 ± 93	434 ± 54	679 ± 152
	L-NAME		(n = 4)	(n = 4)	(n = 2)
		0	0	0	0
		1	255 ± 21	109 ± 45	153 ± 80
		2	447 ± 128	213 ± 56	386 ± 90
		4	668 ± 176	528 ± 133	933 ± 24
Hypoxia	Control		(n = 9)	(n = 9)	(n = 7)
		0	0	0	0
		1	195 ± 44	74 ± 12	235 ± 54
		2	380 ± 70	235 ± 44	396 ± 60
		4	701 ± 93	517 ± 107	730 ± 97
	Indomethacin		(n = 5)	(n = 5)	(n = 5)
		0	0	0	0
		1	90 ± 19	42 ± 7	338 ± 104
		2	249 ± 21	159 ± 31	370 ± 75
		4	604 ± 86	412 ± 68	644 ± 142
	L-NAME		(n = 4)	(n = 4)	(n = 2)
		0	0	0	0
		1	273 ± 74	127 ± 30	189 ± 141
		2	518 ± 150	291 ± 65	685 ± 79
		4	687 ± 183	483 ± 127	925 ± 15

Table 3.2: Endothelin (ET) production (mean ± s.e.m.) in $pg \cdot ml^{-1}$ from equine pulmonary artery (PA), equine aorta, and bovine pulmonary artery (PA) endothelial cells at 1, 2 and 4 h ± the addition of 10^{-5} M indomethacin or 10^{-4}M L-NAME, under normoxic and hypoxic conditions.

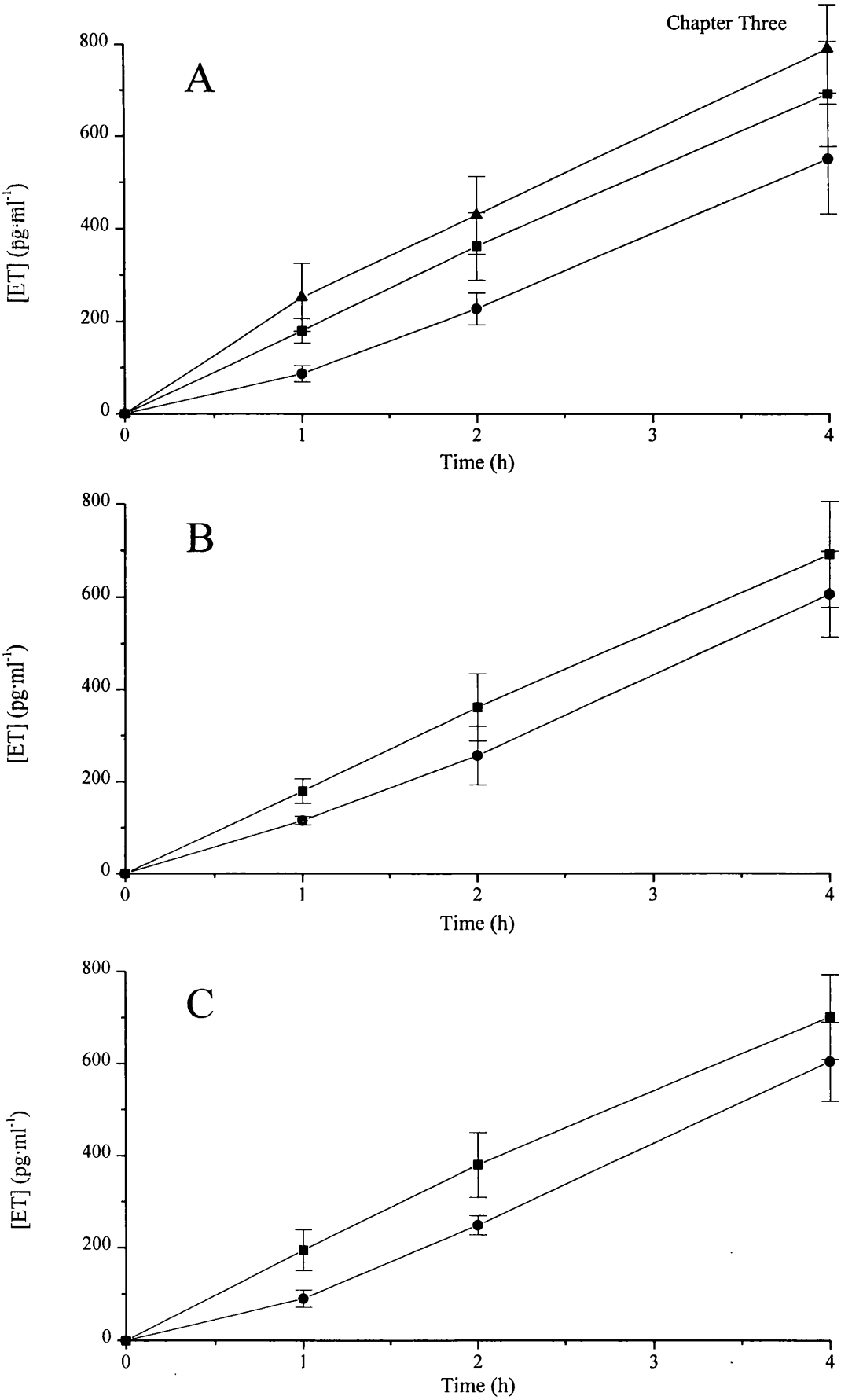


Figure 3.13: Panel A: Endothelin [ET] production (mean ± s.e.m.) at 1, 2 and 4 h in equine pulmonary artery cells (■), equine aorta cells, (●), and bovine pulmonary artery cells (▲). **Panel B:** Endothelin production in equine pulmonary artery cells at 1, 2 and 4 h (■) and with 10^{-5} M indomethacin added (●). **Panel C:** Endothelin production in equine pulmonary artery cells at 1, 2 and 4 h, (■), values obtained in 95% N_2 / 5% CO_2 incubation; and (●), with 10^{-5} M indomethacin and 95% N_2 / 5% CO_2.

Table 3.2 and Figure 3.13 show an approximately linear increase in ET production over the 4 h period. Statistical analysis confirmed the previous results that there was no significant difference in ET production under normoxic and hypoxic conditions in all 3 vessel types. However, unlike the previous set of experiments, bovine pulmonary artery and equine pulmonary artery cells produced significantly more ET than equine aorta endothelial cells (Figure 3.13A). The presence of L-NAME had no significant effect, but the presence of indomethacin did significantly alter ET production ($p = 0.015$). Further analysis revealed that indomethacin significantly reduced ET production in equine pulmonary artery cells ($p = 0.042$), but indomethacin failed to produce an effect in the other two vessel types. Modelling an interaction between the effect of indomethacin and the presence of oxygen failed to improve the statistical significance of the result in all three vessel types. Figure 3.13B is a graph of ET release from equine pulmonary artery cells in the presence and absence of 10^{-5} M indomethacin illustrating the reduction in ET production. A similar reduction in ET production is shown in Figure 3.13C under hypoxic conditions.

3.3.1.3 Endothelin production in the presence of halothane

In a preliminary set of experiments, ET production was measured in the presence of 2% halothane (section 2.2.5.3). As shown in Table 3.3, only one set of experiments was performed under normoxic conditions, yet there appeared to be no marked differences compared to the control data, shown in Tables 3.1 and 3.2, suggesting that this concentration of halothane had minimal effects on ET production by cultured equine endothelial cells.

Time (h)	[ET] ($pg \cdot ml^{-1}$) Control (n = 1)	[ET] ($pg \cdot ml^{-1}$) Halothane (n = 1)
1	161.7	227.9
2	339.6	618.2
4	754.8	936.4

Table 3.3: Endothelin (ET) production from equine pulmonary artery endothelial cells, at 1, 2 and 4 h, under normoxic conditions in the presence of 2 % halothane.

3.3.2 Prostacyclin production by cultured endothelial cells

Production of PGI_2 (detected as its stable metabolite, 6-keto-$PGF_{1\alpha}$) was measured in cultured endothelial cells from equine and bovine pulmonary artery, using radioimmunoassay as described in section 2.3.2.1. Measurements were made in the presence of 10^{-5} M indomethacin and 10^{-4} M L-NAME under normoxic and hypoxic conditions. The results are shown in Table 3.4.

The characteristics of $PGF_{1\alpha}$ production differed markedly between equine and bovine pulmonary artery endothelial cells. The [$PGF_{1\alpha}$] gradually increased over the 4 h time period in equine pulmonary artery cells, indicating continuous release of PGI_2. However, measurements from bovine pulmonary artery cells indicated a quite different pattern of release. By the first hour, bovine endothelial cells had produced a significantly higher concentration of $PGF_{1\alpha}$ than equine cells. This was followed by a gradual decrease over the subsequent 2 and 4 h periods (Figure 3.14A). This trend was seen in each of the 4 bovine experiments that make up the mean data. The difference in $PGF_{1\alpha}$ release between blood vessel types was statistically significant ($p < 0.001$). When the results from both vessel types are grouped, the presence of L-NAME was not a significant factor, while indomethacin significantly reduced $PGF_{1\alpha}$ production ($p = 0.016$). The data associated with each vessel type was then analysed separately. The data from equine pulmonary artery endothelial cells revealed that indomethacin significantly reduced $PGF_{1\alpha}$ production under normoxic and hypoxic conditions ($p = 0.016$, Figure 3.14B and 3.14C). But neither L-NAME nor hypoxia alone significantly altered $PGF_{1\alpha}$ production.

When the results from bovine pulmonary artery endothelial cells were considered, the low number of experiments allowed only a limited set of comparisons. Statistical analysis revealed that none of the experimental conditions affected $PGF_{1\alpha}$ production. When the effect of hypoxia was considered in isolation, the hypothesis that hypoxia increased $PGF_{1\alpha}$ production just failed to reach statistical significance ($p = 0.079$), despite the marked difference in $PGF_{1\alpha}$ production observed (Figure 3.15) in some experiments.

			Equine PA	Bovine PA
Oxygen Status	Drug	Time (h)	$[PGF_{1\alpha}]$ ($ng \cdot ml^{-1}$)	$[PGF_{1\alpha}]$ ($ng \cdot ml^{-1}$)
Normoxia	Control		(n = 7)	(n = 4)
		0	0	0
		1	8.4 ± 3.1	16.0 ± 4.6
		2	10.7 ± 3.4	15.3 ± 4.9
		4	19.8 ± 8.7	11.4 ± 4.3
	Indomethacin		(n = 4)	(n = 1)
		0	0	0
		1	5.3 ± 1.0	15.7
		2	8.3 ± 3.5	16.0
		4	7.6 ± 1.9	16.3
	L-NAME		(n = 5)	(n = 2)
		0	0	0
		1	6.5 ± 3.2	10.9 ± 0.5
		2	8.3 ± 1.9	23.8 ± 12.1
		4	12.1 ± 3.8	44.9 ± 26.1
Hypoxia	Control		(n = 7)	(n = 4)
		0	0	0
		1	11.2 ± 2.6	32.4 ± 7.5
		2	12.7 ± 2.9	52.5 ± 30.3
		4	30.5 ± 11.2	80.3 ± 58.4
	Indomethacin		(n = 4)	(n = 1)
		0	0	0
		1	7.3 ± 1.2	24.8
		2	9.0 ± 2.5	16.7
		4	6.0 ± 0.4	21.1
	L-NAME		(n = 5)	(n = 2)
		0	0	0
		1	9.4 ± 3.7	20.5 ± 4.2
		2	15.2 ± 6.8	20.7 ± 7.3
		4	13.3 ± 5.1	29.4 ± 14.3

Table 3.4: Prostacyclin $[PGF_{1\alpha}]$ production (mean ± s.e.m.) in $ng \cdot ml^{-1}$ from equine and bovine pulmonary artery (PA) endothelial cells with and without 10^{-5} M indomethacin and 10^{-4} M L-NAME under normoxic and hypoxic conditions at 1, 2 and 4 h.

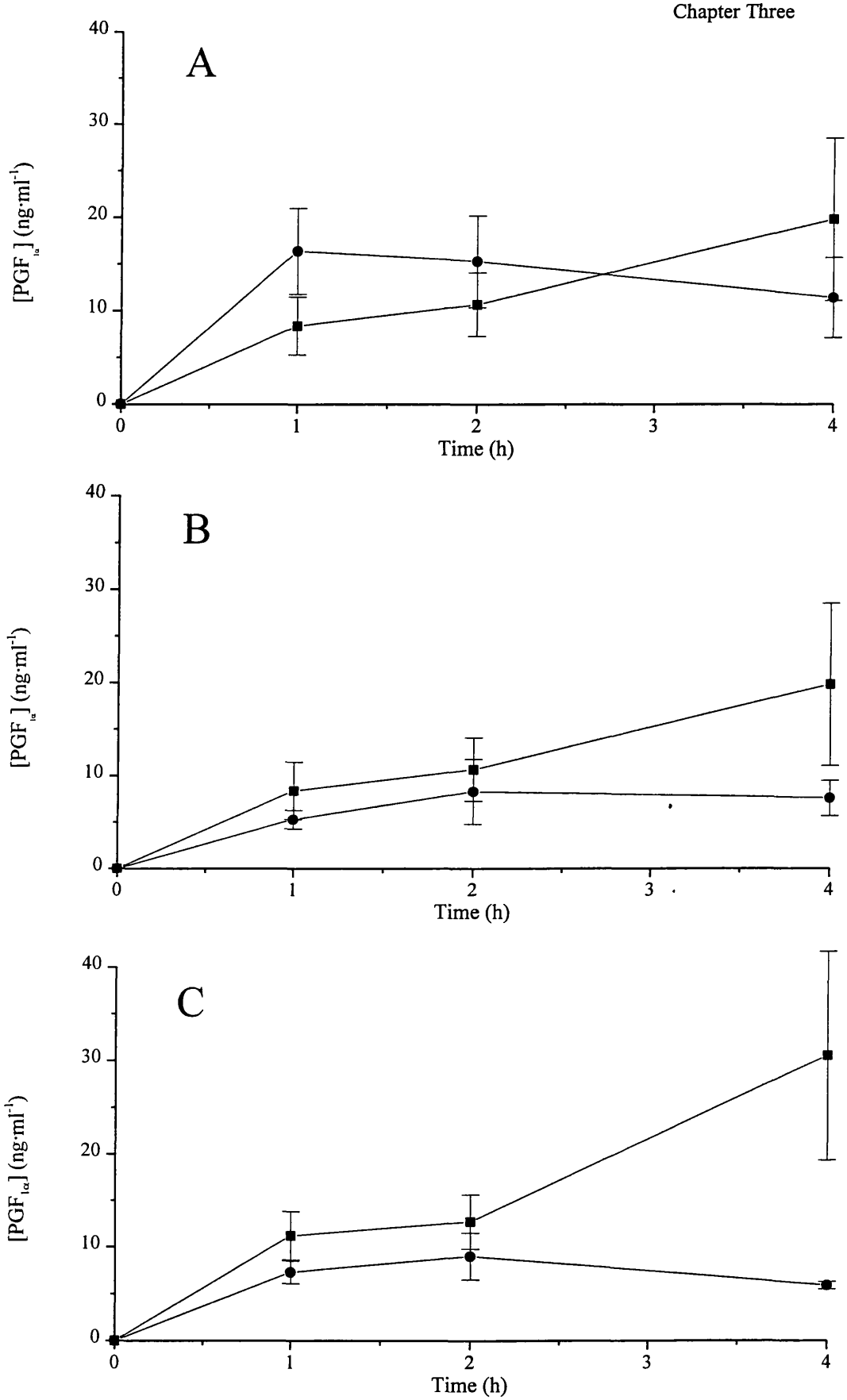


Figure 3.14: Panel A: Prostacyclin [$PGF_{1\alpha}$] production (mean ± s.e.m.) at 1, 2 and 4 h in equine pulmonary artery cells (■) and bovine pulmonary artery cells (●). **Panel B:** $PGF_{1\alpha}$ production in equine pulmonary artery cells at 1, 2 and 4 h (■) and with 10^{-5} M indomethacin added (●). **Panel C:** $PGF_{1\alpha}$ production in equine pulmonary artery cells at 1, 2 and 4 h with 95% N_2 / 5% CO_2 (■) and 10^{-5} M indomethacin with 95% N_2 / 5% CO_2 (●).

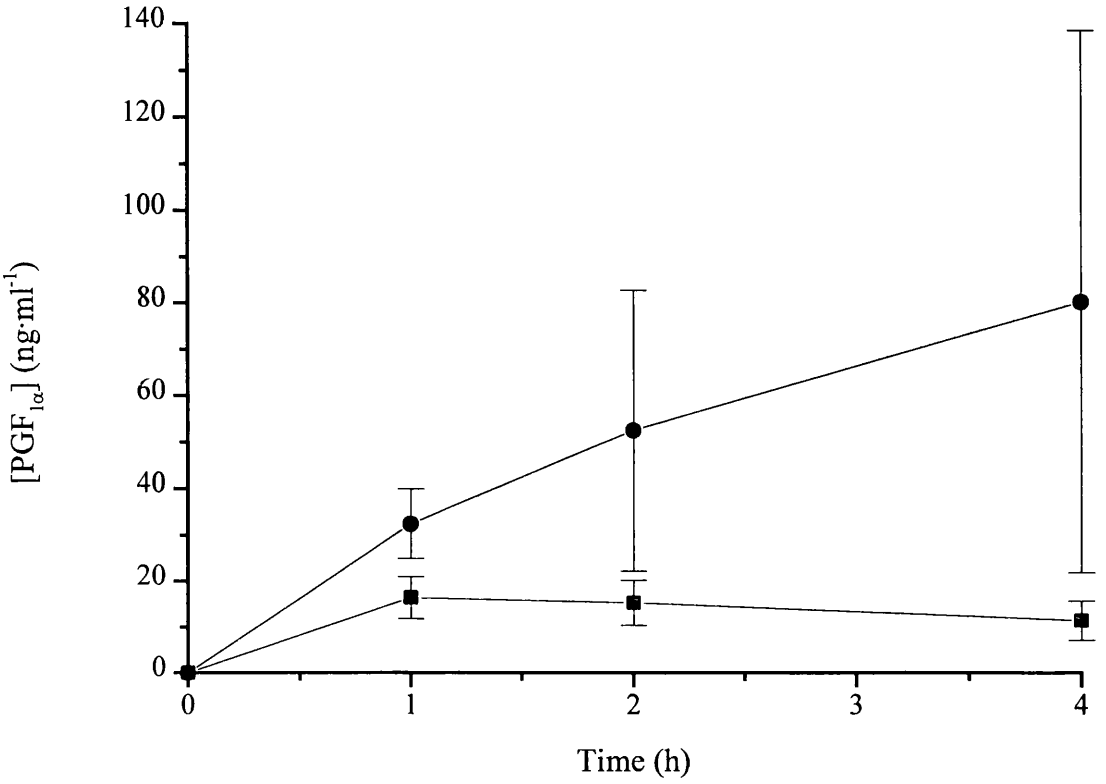


Figure 3.15: Prostacyclin [$PGF_{1\alpha}$] production (mean ± s.e.m.) in bovine pulmonary artery cells at 1, 2 and 4 h in (■), air / 5% CO_2 and (●), 95% N_2 / 5% CO_2.

3.3.3 Arachidonic acid stimulated thromboxane production by cultured endothelial cells

As described in section 1.3.1.4, endothelial cells release the vasoactive agent TxA_2. Initial experiments measuring the stable metabolite TxB_2 production were carried out on cultured endothelial cells from bovine pulmonary artery and aorta. Addition of 50 and 200 $\mu g \cdot ml^{-1}$ arachidonic acid stimulated TxA_2 (measured as TxB_2) production (Table 3.5). Only a limited number of these measurements were made and measurements were not made under hypoxic conditions nor with equine pulmonary artery endothelial cells. However, these data indicate that the cultured bovine endothelial cells used in these studies responded in a similar manner to previously published studies.

Drug	Bovine PA TxB_2 production ($pg \cdot ml^{-1}$) (n = 2)	Bovine Aorta TxB_2 production ($pg \cdot ml^{-1}$) (n =2)
Control	20	393
50 $\mu g \cdot ml^{-1}$ arachidonic acid	26175	20405
200 $\mu g \cdot ml^{-1}$ arachidonic acid	27010	11257

Table 3.5: Measurement of thromboxane B_2 (TxB_2) production ($pg \cdot ml^{-1}$) from cultured bovine pulmonary artery (PA) and bovine aorta cells, sample after 1 h.

3.3.4 Nitric oxide levels in cultured endothelial cells

Using the Griess-Isolvay reagent system, all the experimental samples were below the detection limit for nitrite (10 μM). Employing a modified Griess assay, lowered the detection level of nitrite to 0.3 μM, in agreement with the detection limit published for the method (section 2.3.4.2). Despite the extra sensitivity of this method, no significant nitrite production could be measured from cultured endothelial cells. One reason for this may be the high background absorbance of the culture medium. As with most culture mediums, the SFM contained significant

concentrations of a colorimetric pH indicator, which limits the use of these assay systems for the detection of low levels of nitrite.

3.3.5 Total protein content of cultured endothelial cells

Two types of protein assay systems (Lowry and Coomassie methods) were used to measure the total protein content of cultured endothelial cells. The measurement of total protein is a technique routinely used to quantify the total cellular mass of cultured cells. The protein content can be used to standardise other measures of endothelial cell activity, e.g. ET production. In this study, total protein content was measured by harvesting all the endothelial cells from one culture plate (20 wells), into an Eppendorf tube. In Figure 3.16, the estimates of total protein are plotted in the form of a histogram to demonstrate the range of values obtained by 3 separate assays using the Coomassie method and 1 assay using the Lowry method. There was a large spread of values both within each assay and between assays. Furthermore, the average total protein levels measured using the Lowry technique appeared lower than those measured using the Coomassie system. The large variation between assays suggests that either the cellular content in each tray varied widely or that the method was an unreliable measure of total protein content in these cultured cells. To distinguish between these two possibilities, the relationship between ET production under control conditions and total protein content of the cells was examined (Figure 3.17). The lack of correlation between these two values suggests the estimate of total protein content was unreliable and could not be used to standardise endothelial factor production. The cause of the low reliability of this method is unknown. However, one possibility is the variable retrieval of the cells from the culture plate.

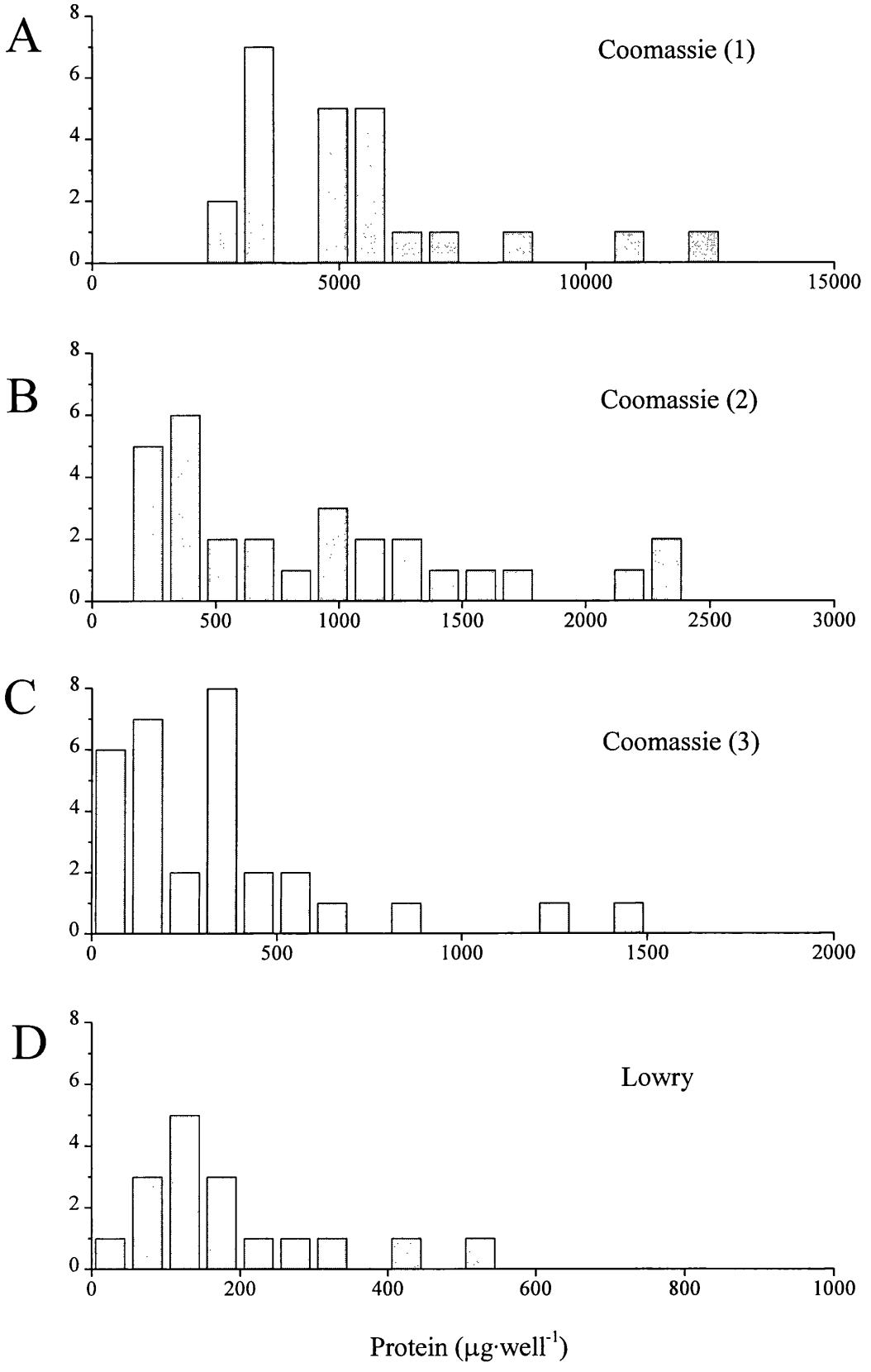


Figure 3.16: Histograms of protein concentration using Coomassie (Panels A, B, C) and Lowry (Panel D) total protein assays. The Y axis of each histogram represents the number of assays, the X axis reflects protein content (μg) in each well.

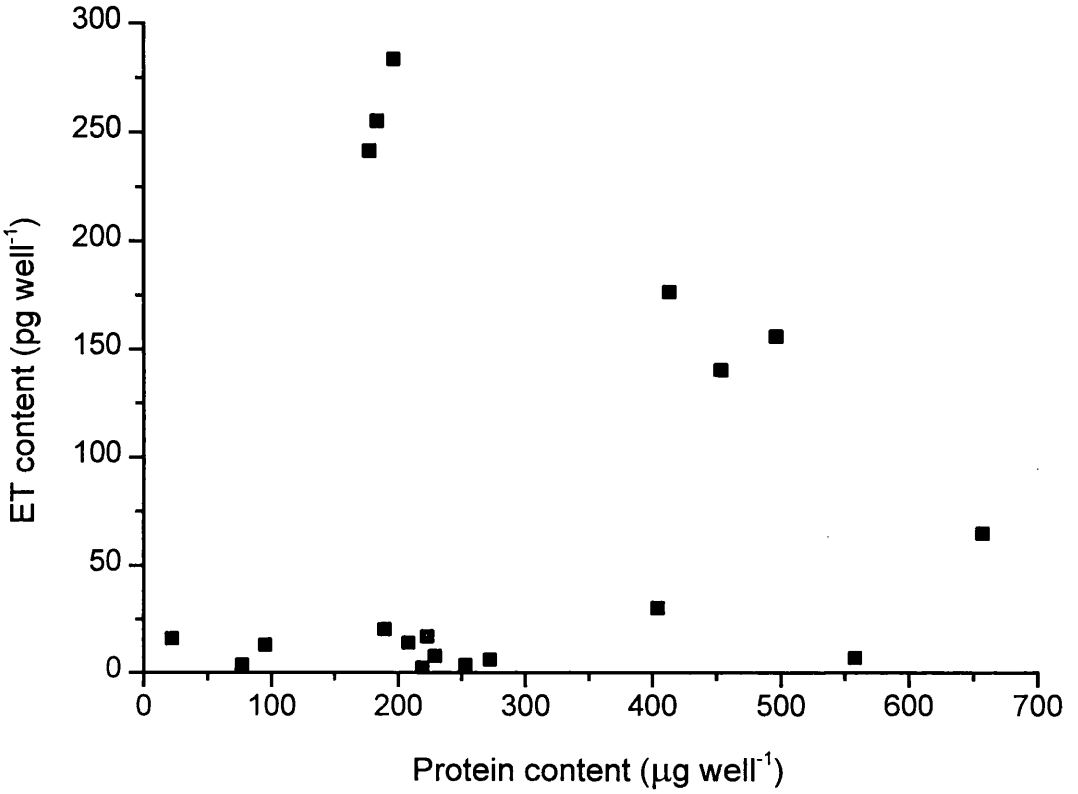


Figure 3.17: Relationship between total protein content measured with the Coomassie method and endothelin [ET] concentration at 1 h in cultured equine endothelial cells grown to confluence.

3.4 Isometric tension measurements

3.4.1 Pharmacological characterisation of equine and bovine pulmonary artery

This section details the responses of isolated equine and bovine pulmonary arteries to a range of drugs. Phenylephrine, 5-HT and ET-1 are used to increase tone, while Bk and ACh were used to study endothelium-dependent relaxation.

3.4.1.1 Contractile response to phenylephrine

As described in section 2.4.2.1, isometric tension measurements were made from rings of bovine and equine pulmonary artery while PE concentration was increased in a cumulative fashion (10^{-10} M to 3 x 10^{-5} M). An example of the tension measurements are shown in Figures 3.18A & B. Increasing concentrations of PE caused a step-wise increase in isometric tension. Measurements were made from vessels within 24 hours post mortem (day 1), 48 hours post mortem (day 2) in equine and bovine vessels. Rubbed equine and bovine vessels, in which the endothelium was presumed to be removed, were also examined. As described in section 2.4.3, measurements of tension and PE concentration for each vessel were plotted and fitted to a sigmoidal logistic relationship (Figure 3.19). Each curve had an associated estimate of EC_{50} and slope. Maximum force production was measured as the highest tension levels achieved at the higher range of agonist concentrations. Table 3.6 shows the average EC_{50}, slope and maximal contraction to PE in both equine and bovine pulmonary arteries.

Species	Day	Endothelium	Number of animals	EC_{50} (M)	Slope	Maximum contraction (g /g wet wt)
Equine	1	intact	5	$2.33 \pm 0.93 \times 10^{-6}$	1.23 ± 0.13	187 ± 22
	2	intact	26	$1.72 \pm 0.40 \times 10^{-6}$	1.46 ± 0.10	322 ± 21
	2	rubbed	23	$2.69 \pm 0.85 \times 10^{-6}$	1.49 ± 0.16	265 ± 25
Bovine	2	intact	10	$9.51 \pm 5.36 \times 10^{-5}$	0.98 ± 0.08	641 ± 92
	2	rubbed	6	$1.22 \pm 0.46 \times 10^{-5}$	0.72 ± 0.11	498 ± 80

Table 3.6: EC_{50}, slope and maximum contraction (mean $\pm$ s.e.m.) of the response of equine and bovine pulmonary arterial rings to PE, with the endothelium intact or rubbed.

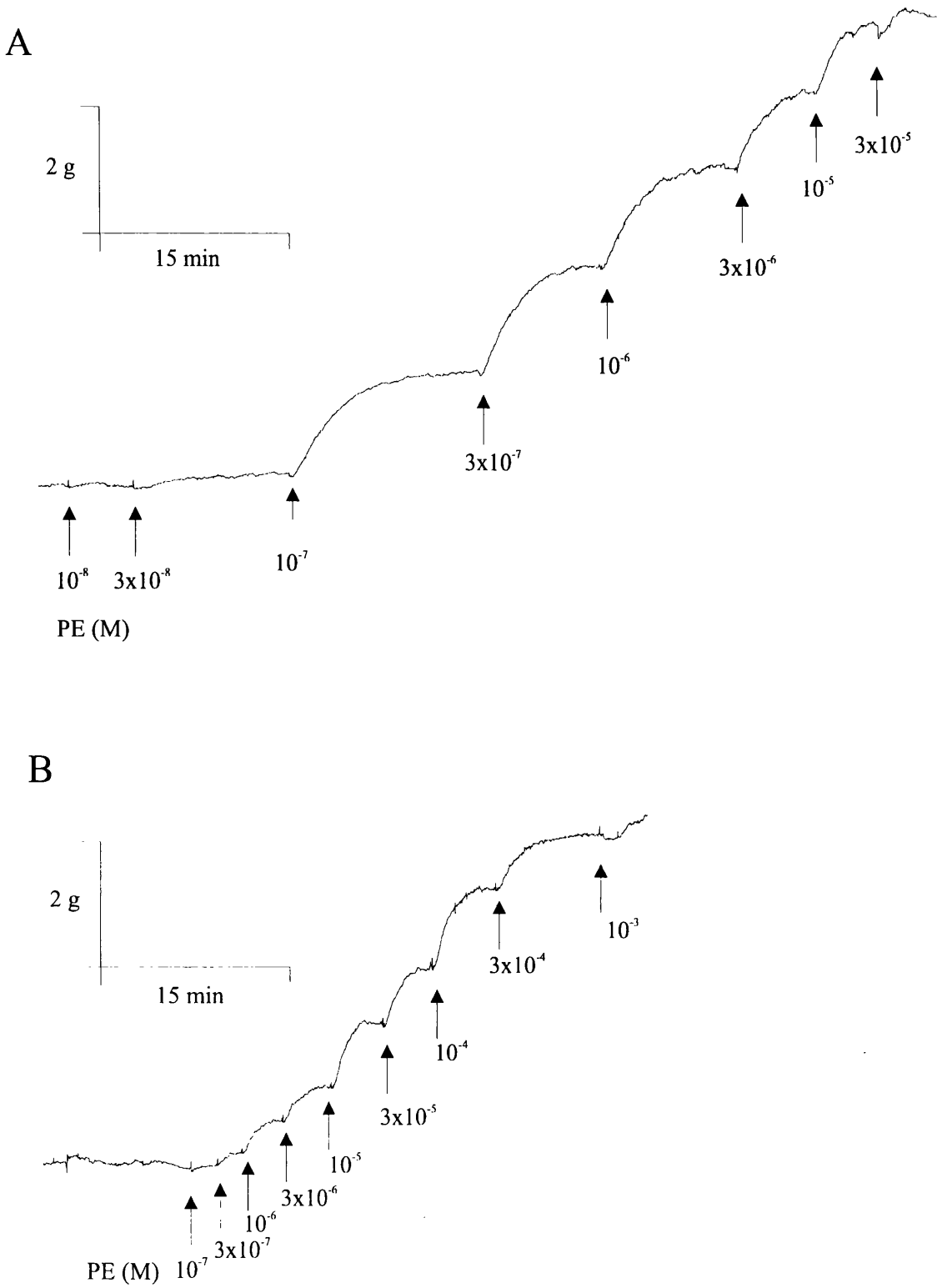


Figure 3.18: Typical isometric tension recordings from pulmonary artery rings in response to phenylephrine (PE). **Panel A:** Incremental doses of PE (10^{-8} M to 3 x 10^{-5} M) were added at the points indicated by the arrows to equine pulmonary artery ring. **Panel B:** Similar trace of incremental doses of PE (10^{-7} M to 10^{-3} M) added to a bovine pulmonary artery ring.

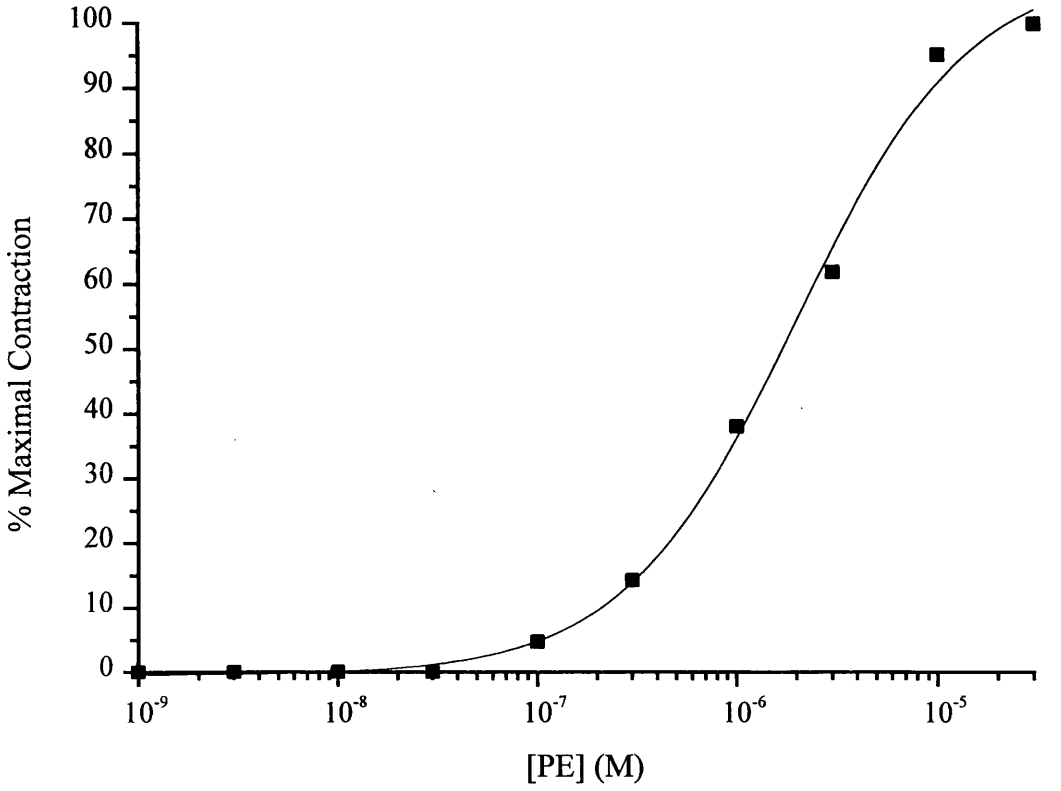


Figure 3.19: Typical relationship between maximal contraction and PE concentration in equine pulmonary artery. Solid line is the best fit sigmoidal relationship between the percentage maximal contraction (% Maximal Contraction) and [PE] using the equation:

$$\% \text{ Maximal Contraction} = (A_{max} - A_{min})/(1+([PE]/EC_{50})^{n}) + A_{min}$$

Where A_{max} = 108.85 ± 4.45 %; A_{min} = -0.47 ± 1.39 %; EC_{50} = 1.97 ± 0.25 x 10^{-6} M and n = 1.00 ± 0.10.

Analysis of the full data set revealed that the EC_{50}, slope and maximal contraction depended significantly on the vessel type ($p = 0.002$, $p < 0.001$, $p < 0.001$ respectively). Maximal contraction to PE was significantly greater in equine pulmonary artery rings on day 2 than day 1 ($p = 0.012$), bovine vessels produce significantly more tension than equine vessels ($p < 0.001$); and the removal of the endothelium significantly reduced the maximal tension in both vessel types ($p = 0.048$). This latter observation suggests that removal of the endothelium may be associated with damage to the underlying smooth muscle. Alternatively, endothelial derived factors may enhance force production by PE. This is discussed in more detail in section 4.3.1.1.

3.4.1.2 Contractile response to 5-hydroxytryptamine

The addition of increasing concentrations of 5-HT caused stepwise increases in tension in both bovine and equine pulmonary arteries as illustrated in Figure 3.20. The response of equine pulmonary artery rings to 5-HT was extremely variable compared to bovine pulmonary artery. In particular, maximum contraction varied from 78 g/g wet wt to 2232 g/g wet wt across the equine vessels studied. EC_{50} values were not significantly different, but the slope was significantly larger ($p < 0.001$) and the maximal contraction significantly lower ($p < 0.001$) in equine vessels compared to bovine vessels (Table 3.7).

Species	Day	Endothelium	Number of animals	EC_{50} (M)	Slope	Maximum contraction (g /g wet wt)
Equine	2	intact	4	$4.79 \pm 2.17 \times 10^{-7}$	2.94 ± 0.57	687 ± 329
	2	rubbed	4	$2.26 \pm 0.99 \times 10^{-7}$	2.50 ± 0.41	695 ± 351
Bovine	1	intact	3	$2.24 \pm 1.03 \times 10^{-6}$	0.62 ± 0.05	1431 ± 139
	2	intact	20	$6.18 \pm 1.64 \times 10^{-7}$	0.69 ± 0.03	1740 ± 66
	2	rubbed	13	$2.72 \pm 0.76 \times 10^{-7}$	0.66 ± 0.04	1632 ± 102
	3	intact	4	$6.75 \pm 1.18 \times 10^{-7}$	1.17 ± 0.15	1328 ± 136

Table 3.7: Mean ($\pm$ s.e.m.) EC_{50}, slope and maximal contraction to 5-HT in equine and bovine pulmonary arterial rings, with the endothelium intact or rubbed.

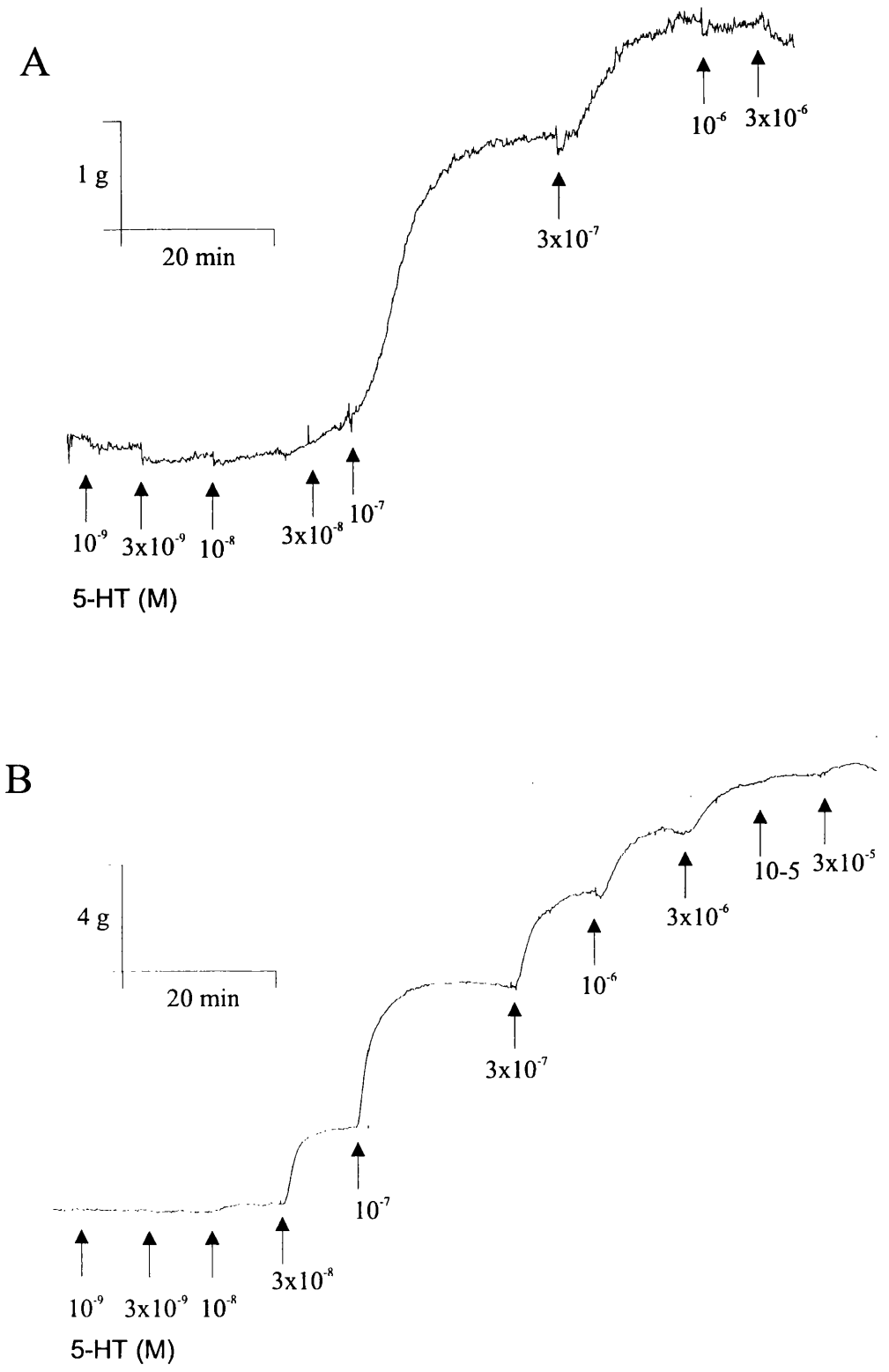


Figure 3.20: Typical isometric tension recording from rings of pulmonary artery in response to 5-HT. **Panel A:** Incremental doses of 5-HT (10^{-9} M to 3 x 10^{-6} M) were added at the points indicated by the arrows to an equine pulmonary artery ring. **Panel B:** Similar trace of incremental doses of 5-HT (10^{-9} M to 3 x 10^{-5} M) added to a bovine pulmonary artery ring.

When considering the bovine data alone, the day that the experiment was performed was the only significant factor affecting EC_{50} ($p = 0.001$). In particular, the EC_{50} for day 1 was significantly greater than for day 2 and day 3 ($p = 0.003$). In contrast, the slopes for day 1 and day 2 results were significantly less that those for day 3 ($p < 0.001$). Finally, maximal contraction on day 3 was significantly less than day 2 and day 1 ($p = 0.007$). The removal of endothelium did not significantly effect the maximal contraction or slope, but the reduction of the EC_{50} value in bovine vessels approached significance ($p = 0.064$). In equine pulmonary artery rings, the EC_{50}, slope or maximal contraction were unaffected by the removal of the endothelium.

3.4.1.3 Characterisation of endothelin-1 response

As shown in Figure 3.21A, addition of ET-1 caused an increase in force of contraction, but with a much slower time course than that seen in response to PE or 5-HT (Figures 3.18 and 3.20, respectively). In Figure 3.21B, the vessel had been incubated for approximately 20 minutes with the ET_A receptor antagonist BQ 123 (3×10^{-6} M) Addition of ET-1 failed to produce a significant tension response until the concentration reached 10^{-7} M, suggesting effective antagonism of ET-1. Using the ET_B receptor antagonist BQ 788 (10^{-7} M), the response of the blood vessel to ET-1 was similar to the control response (Figure 3.21C).

The steady state tension responses of individual vessels were plotted and fitted to a sigmoidal logistic relationship. The mean ($\pm$ s.e.m.) of the EC_{50}, slope and maximal contraction obtained from these measurements are shown in Table 3.8.

Drug	Number of animals	EC_{50} (M)	Slope	Maximal contraction (g/g wet wt)
ET-1 only	5	$2.70 \pm 0.43 \times 10^{-9}$	3.19 ± 0.78	282.5 ± 39.4
ET-1 + ET_A antagonist	5	$8.16 \pm 3.18 \times 10^{-8}$	5.05 ± 1.94	201.7 ± 63.8
ET-1 + ET_B antagonist	3	$2.95 \pm 1.12 \times 10^{-9}$	1.94 ± 0.92	352.5 ± 170.2

Table 3.8: Effects of ET-1 and the ET_A receptor antagonist (BQ 123) and ET_B receptor antagonist (BQ 788) on EC_{50}, slope and maximal contraction in equine pulmonary arterial rings, day 2, intact vessels.

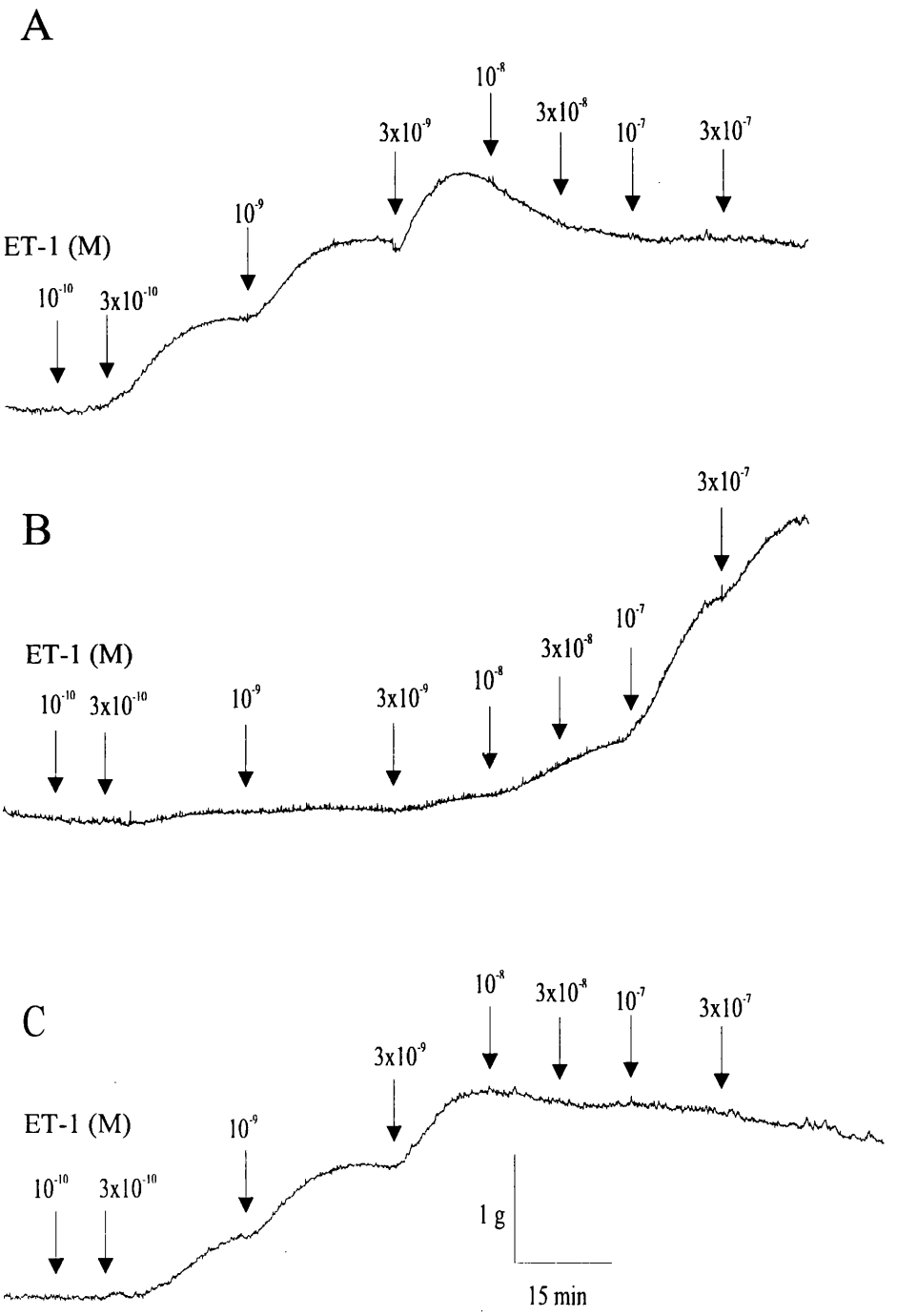


Figure 3.21: Typical isometric tension recording from rings of equine pulmonary artery in response to endothelin-1 (ET-1). **Panel A:** Incremental doses of ET-1 were added at the points indicated by the arrows **Panel B:** Recordings from a different vessel in the presence of BQ123. **Panel C:** Recordings from a different vessel in the presence of BQ788.

Analysis of variance indicated that the presence of BQ 123 significantly increased the EC_{50} for ET-1 ($p < 0.001$), but did not affect either the slope or the maximal contraction. Incubation with BQ 788 did not alter any of the 3 parameters. These results suggest that the ET receptor in small equine pulmonary arteries is exclusively the ET_A type.

3.4.1.4 Relaxation to bradykinin

As described in section 2.4.2.3, the integrity of the endothelium was assessed by measuring the ability of Bk (10^{-11} M to 10^{-6} M) to relax precontracted equine and bovine vessels. In each experiment, measurements were made from 4 vessels simultaneously. Normally, two of these vessels were rubbed to remove the endothelial layer (section 2.4.2), and two vessels were left intact. The effectiveness of this treatment was evident from the extent to which the vessel relaxed on addition of increasing concentrations of Bk (Figure 3.22). Addition of 10^{-9} M Bk to intact equine vessels previously contracted with 10^{-6} M PE caused a rapid and profound relaxation of the tension. Further increases in Bk concentration caused a small additional relaxation (Figure 3.22A). However, only a small fall in force was observed in rubbed vessels, indicating that the majority of the endothelium had been removed (Figure 3.22B). In a limited number of rubbed vessels, only an intermediate level of relaxation was achieved at high concentrations of Bk, suggesting that there was incomplete removal of the endothelium. The sensitivity of the vessel to Bk was assessed by plotting the relative relaxation against the Bk concentration and using a sigmoidal logistic relationship to fit the results and provide a value of EC_{50} and slope (Figure 3.23). The maximum degree of relaxation was expressed as a percentage of the precontracted level. The results are shown in Table 3.9.

Bovine pulmonary vessels were significantly less sensitive to Bk than equine vessels ($p < 0.001$), but neither the slope of the relationship nor the extent of maximal relaxation were different between species. In both vessel types, the process of rubbing the intima significantly ($p < 0.001$) reduced the maximal relaxation caused by Bk from approximately 90% to approximately 25%.

Species	Endothelium	Number of animals	EC_{50} (M)	Slope	Maximum relaxation (%)
Equine	intact	22	$7.88 \pm 4.00 \times 10^{-10}$	2.51 ± 0.44	87.2 ± 3.4
	rubbed	19	$3.29 \pm 1.92 \times 10^{-8}$	1.81 ± 0.39	24.7 ± 3.7
Bovine	intact	10	$1.43 \pm 0.41 \times 10^{-8}$	1.20 ± 0.16	88.7 ± 3.4
	rubbed	9	$6.70 \pm 2.79 \times 10^{-9}$	1.03 ± 0.19	27.8 ± 8.8

Table 3.9: EC_{50}, slope and maximum relaxation of bradykinin-induced relaxation (mean $\pm$ s.e.m.), after agonist precontraction, in equine and bovine pulmonary arterial rings (day 2).

Although Bk had a small relaxation effect on rubbed vessels, the EC_{50} and slope of this effect was not significantly different from vessels with an intact endothelium.

3.4.1.5 Relaxation to bradykinin in the presence of indomethacin and L-NAME

Indomethacin and L-NAME inhibit the endothelial production of PGI_2 and NO respectively. The action of these drugs and the ability of Bk to relax isolated vessels was studied in both intact and rubbed equine vessels precontracted to approximately 50% of maximal tension with PE. After an initial challenge with progressively increasing concentrations of Bk, both PE and Bk were washed off and the preparation was incubated for approximately 20 min in either indomethacin or L-NAME. PE was then reapplied before Bk was added and the extent of relaxation was monitored in the continued presence of the inhibitor. In a subset of experiments, the effect of both inhibitors was studied. Typical records illustrating the effect of indomethacin and L-NAME on Bk relaxation are shown in Figures 3.24 & 3.25.

The relationship between the Bk concentration and the relative steady state relaxation was plotted and fitted with a sigmoidal logistic relationship. The maximum degree of relaxation was expressed as a percentage of the precontracted level. The results are shown in Table 3.10. Neither L-NAME or indomethacin significantly affected the EC_{50} or slope of the Bk-induced relaxation.

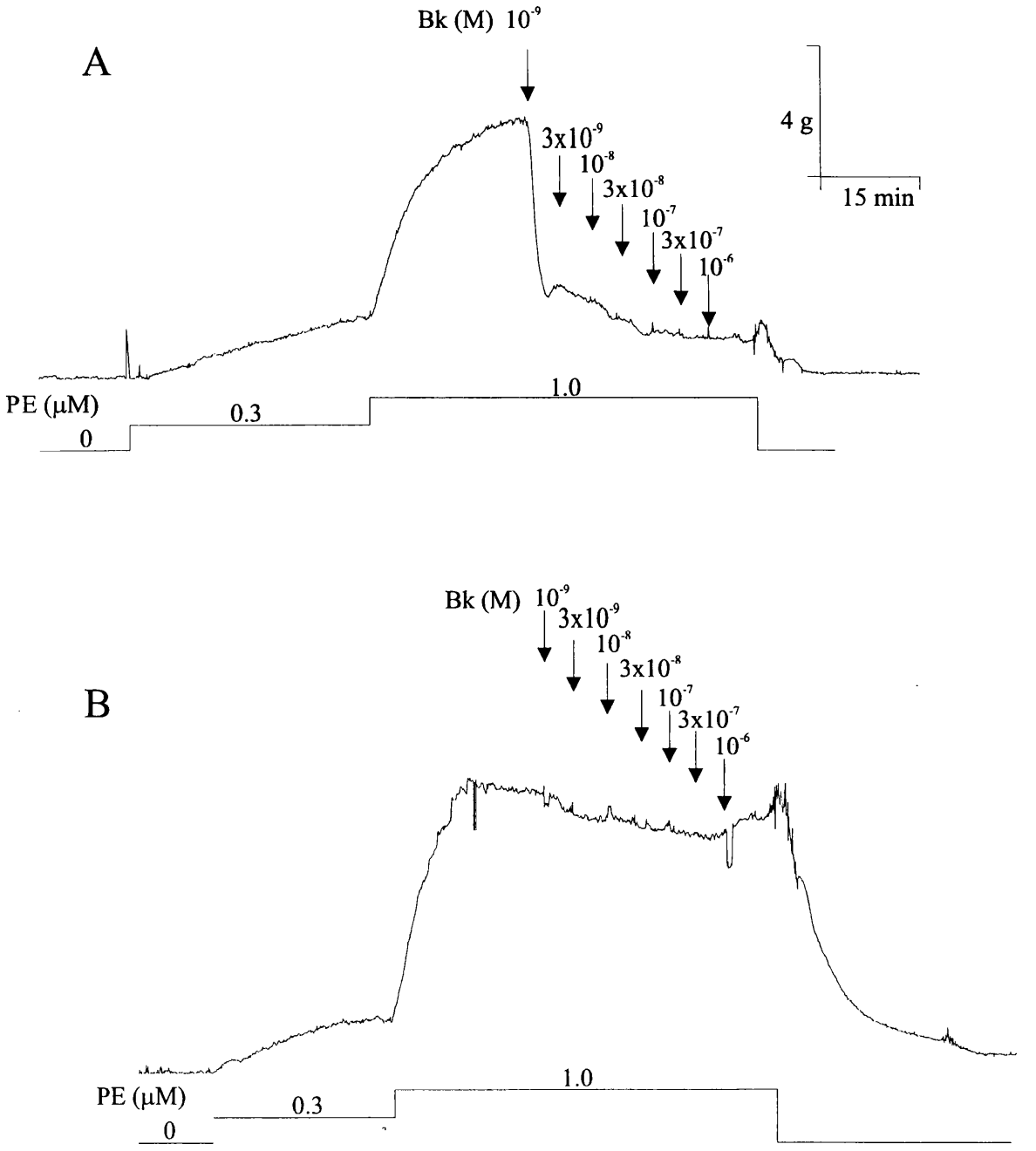


Figure 3.22: Typical isometric tension recording from rings of equine pulmonary artery in response to bradykinin (Bk). Vessels were precontracted with 1 μM phenylephrine (PE) as indicated below the trace. **Panel A:** Intact equine pulmonary artery, exposed to increasing concentrations of Bk (10^{-9} M to 10^{-6} M) as indicated by the downward pointing arrows. **Panel B:** Rubbed equine pulmonary artery, exposed to PE and Bk as above. Calibration bar in panel A also applies to panel B.

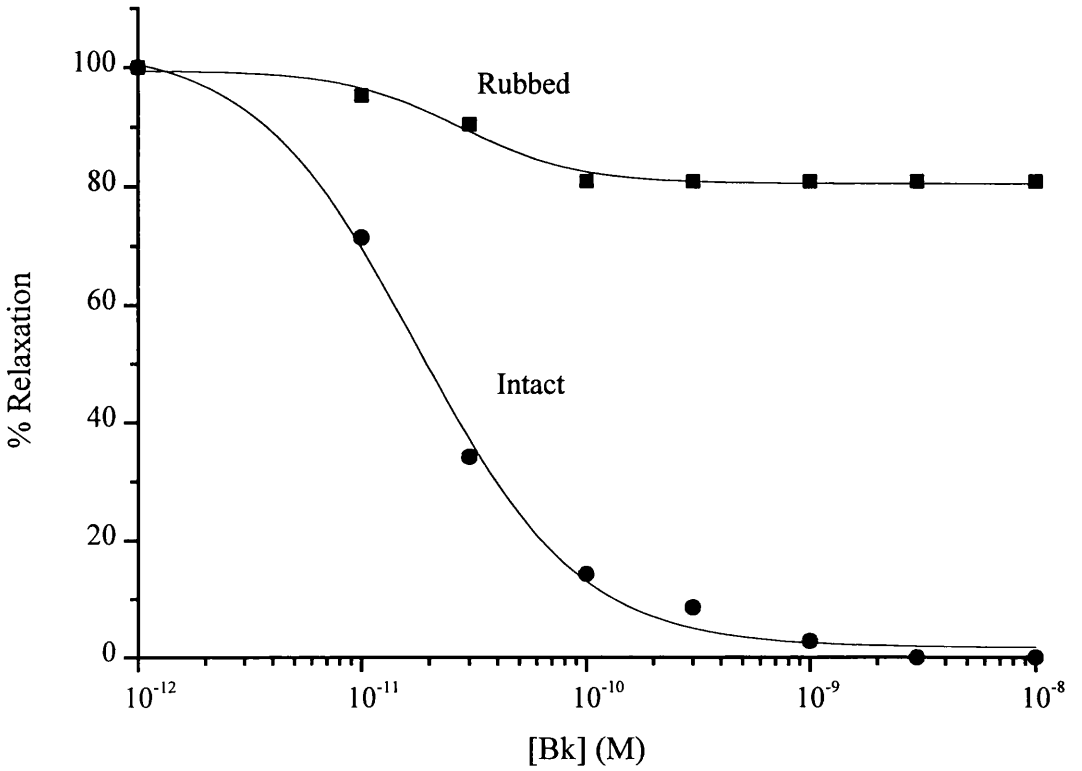


Figure 3.23: Typical response of equine pulmonary artery (precontracted with PE) to Bk in rubbed and intact vessels. Solid line is the best fit sigmoidal relationship between the percentage maximal relaxation (% Maximal Relaxation) and [Bk] using the equation:

$$\% \text{ Maximal Relaxation} = (A_{min} - A_{max})/(1+([Bk]/EC_{50})^{n}) + A_{max}$$

In the rubbed vessel: $A_{max} = 99.47 \pm 1.28$ %; $A_{min} = 80.52 \pm 0.66$ %; $EC_{50} = 2.71 \pm 0.49 \times 10^{-11}$ M and $n = 1.66 \pm 0.44$. In the intact vessel: $A_{max} = 103.64 \pm 3.76$ %; $A_{min} = 1.71 \pm 1.64$ %; $EC_{50} = 1.78 \pm 0.18 \times 10^{-11}$ M and $n = 1.18 \pm 0.13$.

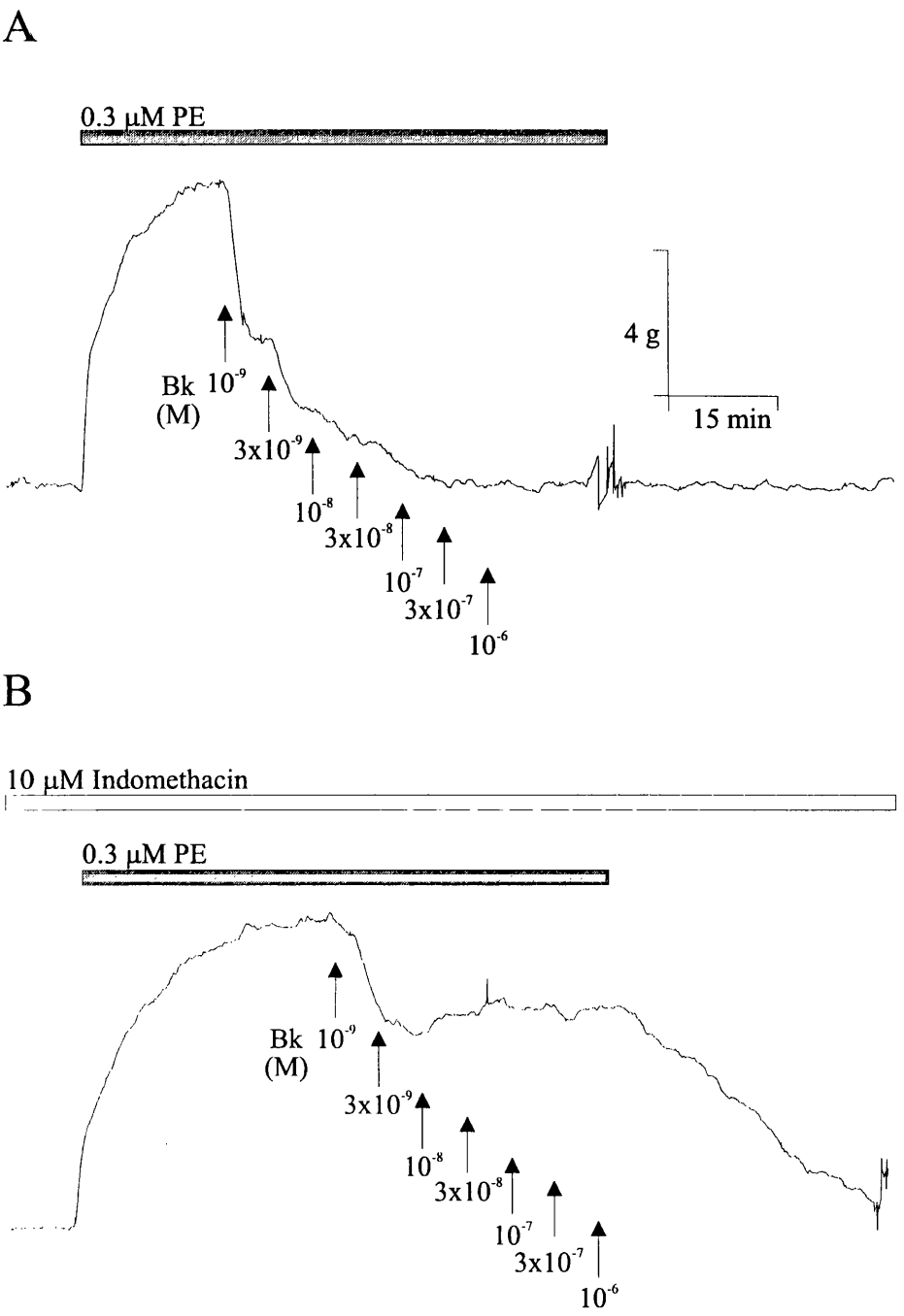


Figure 3.24: Typical isometric tension recording from rings of equine pulmonary artery in response to bradykinin (Bk) (precontracted with phenylephrine, PE) under control conditions (Panel A) and in the presence of 10 μM indomethacin (Panel B). Application of PE is indicated by bars above the trace; the concentrations of Bk (M) are indicated by the upward arrows at the points indicated. Calibration bar in panel A also applies to panel B.

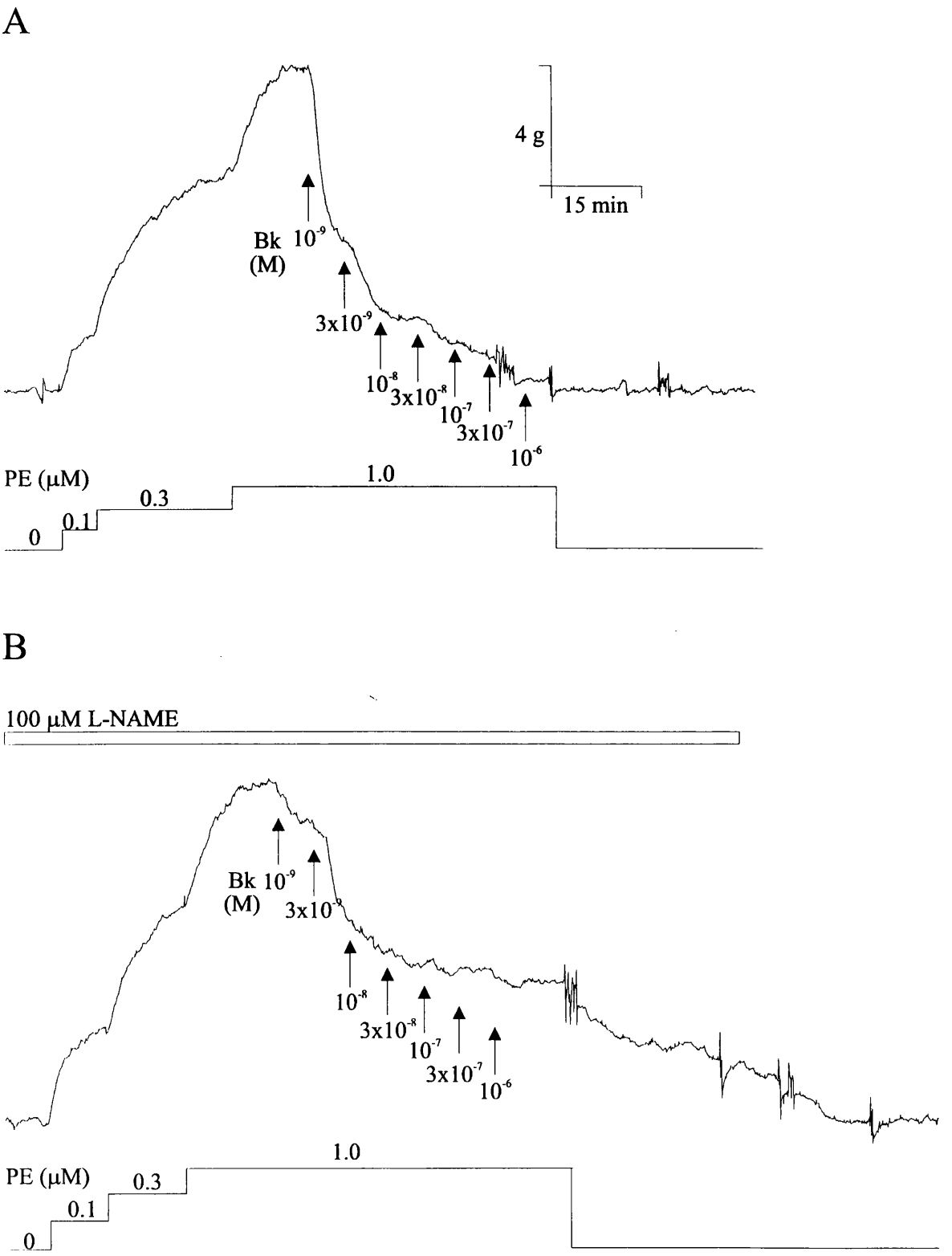


Figure 3.25: Typical isometric tension recording from rings of equine pulmonary artery in response to bradykinin (Bk) (precontracted with phenylephrine, PE) under control conditions (Panel A) and in the presence of 100 μM L-NAME (Panel B). The cumulative application of PE occurred as indicated below the trace. The concentrations of Bk (M) are indicated by the upward arrows at the points indicated. Calibration bars in panel A also apply to panel B.

Endothelium	Drug	Number of animals	EC_{50} (M)	Slope	Maximum relaxation (%)
Intact	Control	8	$8.12 \pm 2.14 \times 10^{-11}$	1.92 ± 0.562	95.4 ± 2.0
	L-NAME	6	$3.01 \pm 1.05 \times 10^{-10}$	1.17 ± 0.15	68.4 ± 6.4
	Indomethacin	3	$8.39 \pm 5.53 \times 10^{-10}$	1.81 ± 0.43	72.3 ± 18.6
	Both	5	$5.26 \pm 1.12 \times 10^{-10}$	7.66 ± 2.53	31.7 ± 7.3
Rubbed	Control	8	$4.39 \pm 2.15 \times 10^{-9}$	2.96 ± 1.05	27.8 ± 2.7
	L-NAME	6	$3.50 \pm 2.56 \times 10^{-10}$	0.55 ± 0.27	24.5 ± 9.1
	Indomethacin	3	$8.56 \pm 4.59 \times 10^{-11}$	9.08 ± 5.24	-10.0 ± 3.6
	Both	5	$4.79 \pm 3.12 \times 10^{-8}$	0.79 ± 0.22	1.8 ± 1.7

Table 3.10: Relaxation of intact and rubbed equine pulmonary arterial rings to bradykinin (Bk) in the presence of 10^{-4} L-NAME and 10^{-5} indomethacin, day 2. Results are expressed as the mean $\pm$ s.e.m. of the EC_{50}, slope and percentage maximal relaxation.

As in Table 3.9, rubbed vessels showed only a small fall in force on addition of Bk. The large range of EC_{50} values measured in rubbed vessels is related to the poor relaxation seen under these conditions leading to large errors in the estimate of these parameters. Maximal relaxation was found to be significantly reduced by indomethacin ($p = 0.042$) or L-NAME ($p = 0.003$) in intact vessels, but in rubbed vessels only indomethacin significantly effected relaxation ($p < 0.001$). As indicated in the table, addition of BK to rubbed vessels, pretreated with indomethacin, caused a small increase in tension. When both L-NAME and indomethacin were present, the combined effects resulted in a profound inhibition of relaxation in intact and rubbed vessels ($p < 0.001$).

3.4.1.6 Relaxation to acetylcholine

In a separate study, the ability of ACh to induce endothelium dependant relaxation was studied in rings from equine and bovine pulmonary arteries. The protocol and analysis were similar to those used to study Bk. The mean ($\pm$ s.e.m.) of the individual EC50's and slopes are tabulated along with the maximum relaxation observed at a saturating dose of ACh (Table 3.11).

	Equine (n = 3)	Bovine (n = 8)	Unpaired, 2-tailed, Student's t test
EC_{50}	$3.92 \pm 0.95 \times 10^{-8}$	$7.83 \pm 2.43 \times 10^{-8}$	p = 0.37 ns
Slope	1.52 ± 0.53	1.33 ± 0.39	p = 0.88 ns
Maximal relaxation (%)	45.9 ± 7.3	75.2 ± 6.2	p = 0.03

Table 3.11: Relaxation to ACh (10^{-9} M to 10^{-5} M) in equine and bovine pulmonary arterial rings. EC_{50}, slope percentage maximal relaxation are expressed as mean $\pm$ s.e.m.

These results, compared using a Student's t test, suggest that ACh has a similar EC_{50} and slope in both equine and bovine pulmonary vessels. However, ACh produced significantly greater relaxation in bovine vessels.

3.4.2 Effects of hypoxia on equine and bovine pulmonary vessels

In this section, the responses of bovine and equine vessels to hypoxia are examined. In both types of vessels, the hypoxic gases were introduced after the vessels had developed significant levels of force using PE in equine vessels and 5-HT in bovine vessels.

3.4.2.1 Isometric tension responses to 0%, 2% and 5% O_2 in bovine pulmonary artery rings

Figure 3.26A illustrates the response of isolated bovine vessels to hypoxic gases. After the initial development of a sustained contraction in the presence of 0.1 μM 5-HT, the gas bubbling the vessel was changed from 95% O_2 / 5% CO_2 to 5% O_2 / 5% CO_2 / 90% N_2. This caused a slow relaxation to a lower steady state level, which reversed when the gases were returned to normal. In contrast to this response, on switching the gas bubbling the vessel to 0% O_2 / 5% CO_2 / 95% N_2 a transient relaxation was observed before a sustained contraction developed which was significantly higher than the level recorded under normoxic condition (Figure 3.26B). Similar procedures were carried out using 2 % O_2 mixtures: the mean $\pm$ s.e.m. of the responses of vessels to all 3 oxygen levels are shown in Table 3.12. The tension responses are expressed as a percentage relative to the contraction in PE

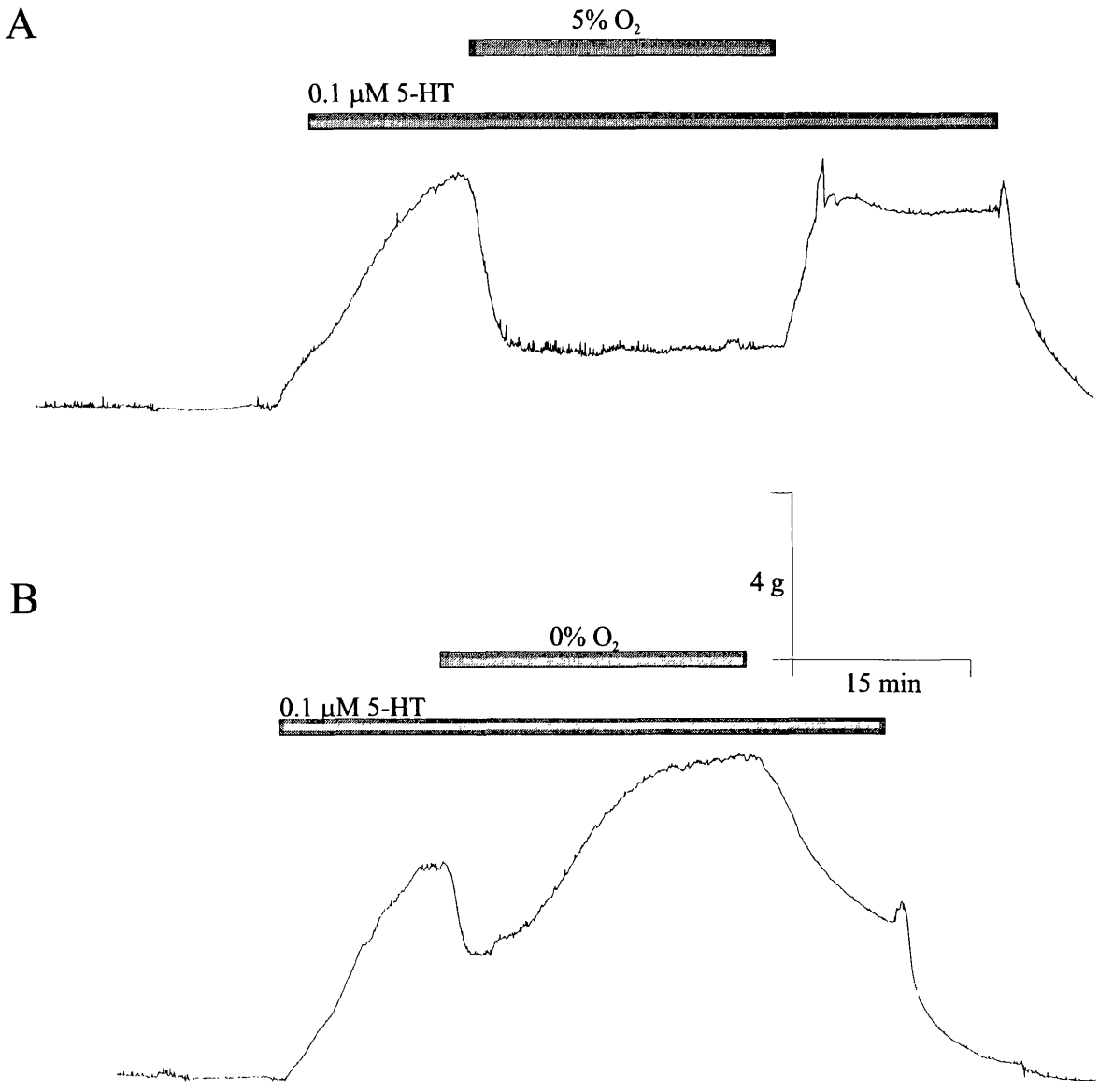


Figure 3.26 Typical isometric tension measurements from an bovine pulmonary artery ring in response to hypoxia. In both vessels, steady state tone was achieved by addition of 0.1μM phenylephrine (PE) for the period indicated **Panel A** shows the response to switching the gas bubbling the preparation to 5% O_2 / 5% CO_2 / 90% N_2 for the period indicated by the bar above the trace. Indicated. **Panel B** shows the response to switching the gas bubbling the preparation to 0% O_2 / 5% CO_2 / 95% N_2 for the period indicated by the bar above the trace.

in 95 % O_2. Results from vessels that did not relax significantly to Bk were not included.

% Oxygen	Number of animals	Tension (% of precontracted levels)
0	11	237.92 ± 31.95
2	8	- 32.78 ± 6.19
5	4	- 48.26 ± 7.78

Table 3.12: Relative tension (mean ± s.e.m.) produced in intact bovine pulmonary arterial rings, day 2. These results indicate that 5% and 2% O_2 caused relaxation, while 0% O_2 caused contraction. The relaxation in 5% appeared to be greater than that seen in 2% O_2, but this difference was not significant (p = 0.08, Student's t test).

3.4.2.2 Isometric tension responses to 0% O_2 in equine and bovine pulmonary arteries

In this section, the response of equine and bovine pulmonary blood vessels to nominally 0% O_2 is studied in the presence and absence of a functional endothelium. In the absence of agonist induced force, 0 % O_2 did not alter the level of resting tension in both equine and bovine vessels (results not shown). However, significant contractile responses were observed after the vessel had developed tone in the presence of an agonist. Figure 3.27 illustrates the responses from 2 equine pulmonary arteries: Panel A, an intact vessel, Panel B, a rubbed vessel. In the intact vessel, increasing doses of Bk almost completely reversed the contraction to PE, confirming a functional endothelium. When this vessel was exposed to 0% O_2 there was a transient relaxation followed by sustained contraction similar to that seen in bovine pulmonary arteries (not shown). When a similar protocol was repeated on a rubbed vessel, Bk was much less effective in producing relaxation but the vessels still contracted to 0% O_2. These results would indicate that the presence of a functional endothelium is not essential to the hypoxic response. In Table 3.13, the mean contractile response (± s.e.m.) to hypoxia is expressed relative to the tension level in 95% O_2.

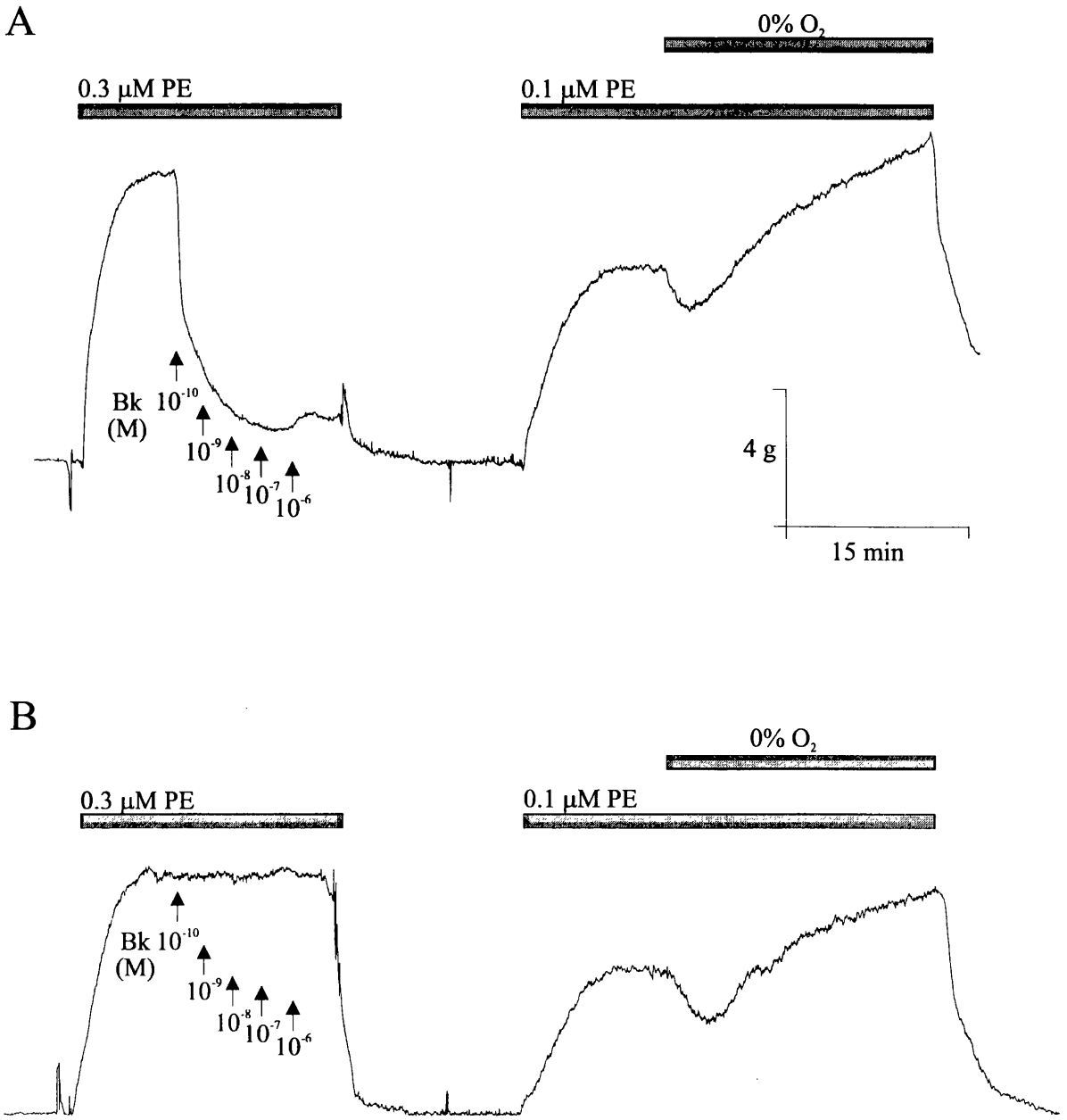


Figure 3.27 Typical isometric tension measurements from isolated equine pulmonary arteries exposed to hypoxia. Both vessels were contracted with 0.3μM phenylephrine (PE) as indicated above the trace and then were exposed to increasing concentrations of bradykinin (Bk) as indicated by the arrows. Next the PE and Bk were withdrawn and the vessels were contracted with 0.1μM PE, before exposure to hypoxic gases (0% O_2 / 5% CO_2 / 95% N_2). **Panel A**: Record from an intact vessel; **Panel B:** Record from a rubbed vessel.

Species	Endothelium	Number of animals	Tension (% of precontracted levels)
Equine	intact	13	196.5 ± 19.8
	rubbed	13	127.9 ± 16.3
Bovine	intact	17	289.9 ± 32.1
	rubbed	14	165.0 ± 18.0

Table 3.13: Relative tension (mean ± s.e.m.) produced (of precontracted levels) in equine and bovine pulmonary arterial rings, day 2, in intact and rubbed vessels exposed to 95% N_2/5% CO_2 for approximately 20 min.

Analysis of the above results revealed that bovine blood vessels contracted to significantly higher levels in 0% O_2 than equine vessels ($p = 0.007$). In both vessel types, the rubbed vessels contracted significantly less than the intact vessels (equine $p = 0.011$, bovine $p = 0.002$). The absence of a functional endothelium appeared to attenuate the contractile response to 0% O_2 by approximately 30%. An alternative method of analysing these data is to correlate the degree of relaxation induced by Bk to the relative amplitude of the hypoxic contracture. This relationship is shown in Figure 3.28. It is clear that rubbed vessels relaxed to a lesser extent than intact vessels. However, within each group there was no clear relationship between the degree of Bk induced relaxation and the size of the hypoxic contraction. Figure 3.28B, is a plot of the same measurements from bovine pulmonary vessels. As with equine vessels, intact vessels showed a greater relaxation to Bk and a larger hypoxic contraction, but within each group there was no clear correlation between the two parameters.

3.4.2.3 Hypoxia-induced contraction in the presence of indomethacin and L-NAME.

In this set of experiments, the function of the endothelium was selectively inhibited by pre-incubation of the vessel with either L-NAME (10^{-4} M) or indomethacin (10^{-5} M). These agents were shown previously shown to significantly attenuate the response of pulmonary vessels to Bk (section 3.4.1.5). Typical isometric tension measurements illustrating the effects of L-NAME and indomethacin on hypoxic

vascular contraction are shown in Figure 3.29. In these examples, indomethacin inhibited the hypoxia-induced contraction (Figure 3.29A), while L-NAME blocked the hypoxic response incompletely (Figure 3.29B). The average results from a number of experiments are given in Table 3.14.

Statistical analysis revealed that only indomethacin significantly reduced the response to hypoxia in equine pulmonary arteries ($p = 0.028$), the effect of L-NAME was similar, but the low number of observations prevented the difference from being significant ($p = 0.1$). In bovine pulmonary arteries, L-NAME significantly reduced the contractile response to hypoxia ($p = 0.026$), but the response in the presence of indomethacin was not significantly different from control. As discussed in section 4.3.2.2, these results indicate that although not essential for the contractile response to hypoxia, a functional endothelium significantly enhances the sensitivity of the vessels to hypoxia.

Species	Drug	Number of animals	Tension (% of precontracted levels)
Equine	Control	10	178.7 ± 28.8
	L-NAME	4	103.7 ± 9.2
	Indomethacin	6	100.6 ± 7.9
Bovine	Control	9	292.9 ± 44.7
	L-NAME	4	148.9 ± 21.3
	Indomethacin	5	223.4 ± 21.9

Table 3.14: Relative isometric tension (mean ± s.e.m.) produced in intact equine and bovine pulmonary arterial rings exposed to 95% N_2 / 5% CO_2 in the presence of 10^{-4} M L-NAME and 10^{-5} M indomethacin.

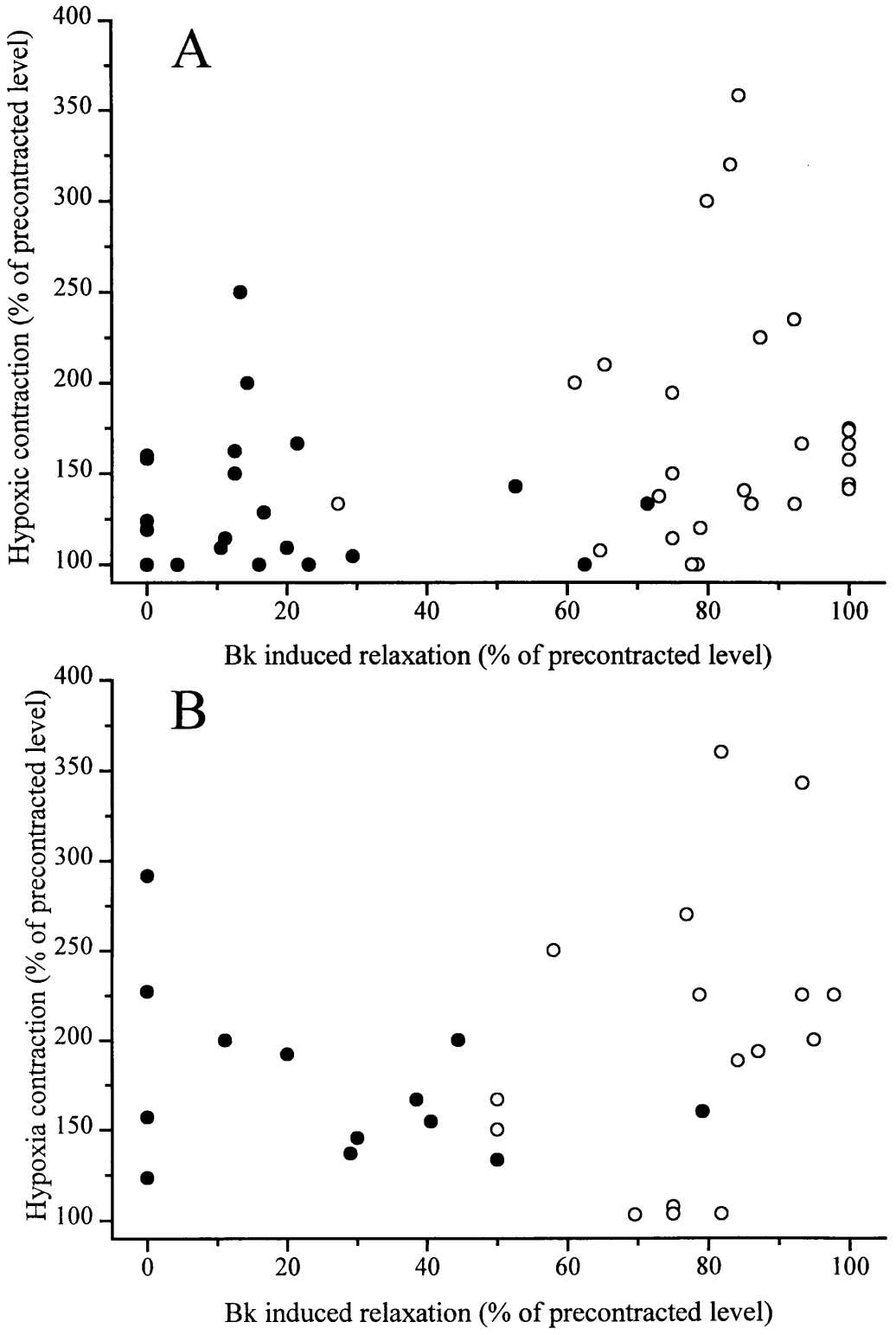


Figure 3.28: Correlation of the maximum degree of relaxation in Bk with the relative amplitude of the hypoxic contraction in rubbed (●) and intact vessels (○) from equine (Panel A) and bovine (Panel B) pulmonary artery rings.

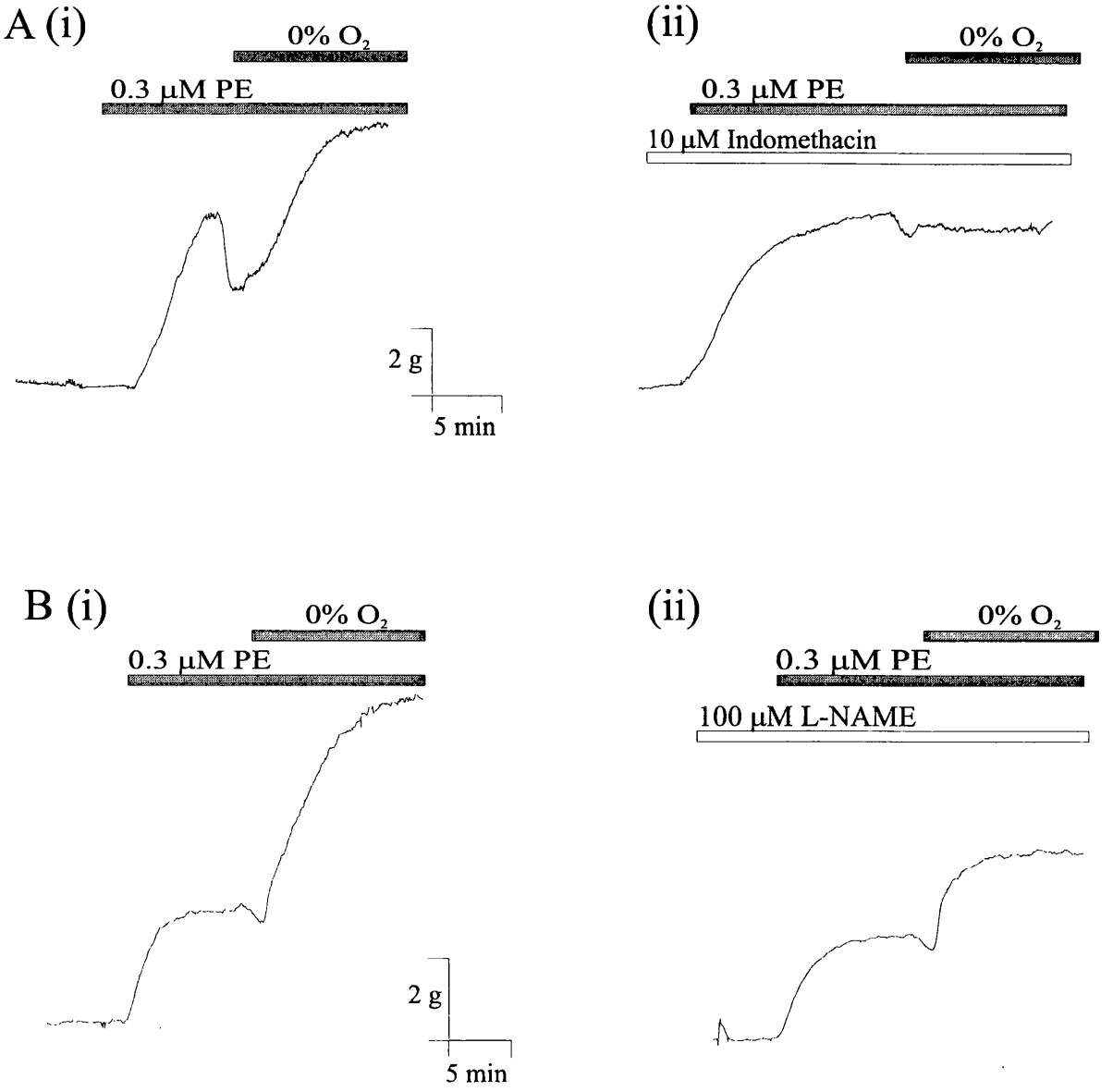


Figure 3.29: Typical isometric tension measurements from intact equine pulmonary arteries precontracted with 0.3 μM phenylephrine (PE) and exposed to hypoxic gas mixtures (0% O_2 / 5% CO_2 / 95% N_2) as indicated by the bars above the traces. **Panel A(i):** Control. **Panel A(ii):** After 20 min incubation with 10 μM indomethacin. **Panel B(i):** Control. **Panel B(ii):** After 20 min incubation with 100 μM L-NAME.

Chapter 4: Discussion

4.1 Special features of equine lung morphology and function

As discussed in Chapter 1, two related features which differentiate equine from bovine lung are (i) the degree of lobulation and (ii) the extent of collateral ventilatory pathways. The lungs of cattle, sheep and pigs have well defined lobules separated by extensive connective tissue and no collateral ventilation. In contrast, equine lung has a poorly defined lobular structure with little connective tissue and extensive collateral ventilatory pathways (McLaughlin et al 1961; Lekeux 1993). The preliminary histological studies presented in section 3.1 confirm the larger amounts of connective tissue associated with bovine lung parenchyma. In addition, equine blood vessels appeared to have a thinner wall with less smooth muscle when compared to equivalent vessels in bovine lung. A more systematic anatomical study by McLaughlin (1961) also found significantly thinner blood vessel walls in equine compared to bovine pulmonary systems. The functional consequences of this difference were implied by Tucker (1975) in a study of a number of species. He suggested a positive correlation between the thickness of the tunica media and the strength of the *in vivo* HPV response. It is interesting to note that species with no collateral ventilatory pathways (pigs, sheep and cattle) are thought to have a strong HPV response, while animals with extensive collateral pathways have only a weak HPV response (dog, horse and human). One explanation for this association would be that both the HPV response and collateral ventilation serve to normalise ventilation and perfusion within the lung, i.e. that a strong HPV response is required when there is insufficient collateral ventilation and vice versa.

Another anatomical feature peculiar to the horse that appears to have detrimental consequences on lung function is the pronounced dorsal-sternal angle of the diaphragm (Figure 1.3). This shape, which contrasts to the more upright diaphragm in cattle (Dyce et al 1987), can give rise to the significant compression of the dorsal lobes of the lung during recumbency (particularly dorsal) due to the pressure of the abdominal contents. Compression of part of the lung will reduce the volume

available for respiration, leading to an imbalance of ventilation and perfusion and lower arterial PO_2. Conscious horses can be trained to remain in lateral recumbency for approximately 30 min; during this time only mild arterial hypoxaemia develops (Hall 1979). It would be difficult to make equivalent measurements on conscious animals in dorsal recumbency, but it would be anticipated that the arterial hypoxaemia would be greater in this situation due to the bilateral compression of the dorsal lobes of the lung. General anaesthesia increases the mild arterial hypoxaemia observed during lateral recumbency to the point that it may be a serious complication during surgery (Gillespie et al 1969). The cause of this is unknown, although several reasons have been suggested (i) anaesthesia-induced cardiovascular depression, in particular reduced cardiac output (ii) reduced ventilatory capacity due to compression of lung regions by the abdominal contents and, (iii) a weak HPV response in equine pulmonary blood vessels which is further weakened by the anaesthetic agent. It is difficult to assess the contribution of (iii) on the basis of the current literature. The purpose of the experimental work of this thesis was to study isolated equine pulmonary blood vessels in response to a range of vasoconstrictor and vasodilator agents. These results are compared with those from the equivalent bovine vessel. In addition, equine and bovine pulmonary vascular endothelial cell function was examined using tissue culture techniques.

4.2 Equine endothelial cells in culture

4.2.1 Development of a method for endothelial cell culture

Methods for isolation and culture of equine endothelial cells from the pulmonary artery, aorta and the microvasculature of the omentum have been described by other workers, but have not been widely used in this species. The method of culturing cells used in this study differs from other workers and offers a simple, reliable and reproducible technique. During the period of this study, the technique has been reproduced successfully over 50 times in horses and over 100 times in cattle. Both Bottoms et al (1985) and Bochsler et al (1989) used at least twice the concentration of collagenase used in this study (0.1 %). At this lower concentration the

endothelial layer was removed from the aorta and pulmonary blood vessels, but minimal damage to the smooth muscle layer was induced, thereby preventing contamination with overgrowth of smooth muscle cells. It is clear from the data reported here (Figure 3.6) that complete removal of the endothelium was achieved with a lower concentration of collagenase than previously described in bovine and porcine endothelial cell culture (Booyse et al 1975; Ryan et al 1978; Chung-Welch et al 1988; Nakamura et al 1990). Moreover, centrifugation of cells at a lower g force (as described here) would be anticipated to cause less damage to the cells. Amphotericin B, an antifungal agent, was used in both studies by Bottoms et al (1985) and Lamar et al (1986), however this drug is toxic in some cells (Darling & Morgan 1994). The inclusion of penicillin and streptomycin without amphotericin in the culture medium was sufficient to prevent contamination in this study.

It is possible that the phenotypic expression, in particular cell growth and organisation may not be the same as within the tunica intima of intact vessels. Cultured cells may differ from *in vivo* tissue because they lack the interaction of receptor types on the cell surface with specific sites in the extracellular matrix (Freshney 1994). Therefore the choice of matrix may be important. The type of culture substrate varied between the studies, Lamar et al (1986) used 1% gelatin-coated and positive surface charged petri dishes, whereas, Bochsler et al (1989) plated the cells on to flasks coated with fibronectin. The growth of human umbilical endothelial cells on a range of matrices was investigated by Sirois et al (1993). On the basis of the growth pattern, they concluded that the presence of growth factors within the culture medium was more influential than the nature of the matrix. The data presented in this thesis indicated that irradiated polystyrene and DMEM medium provides a reproducible substrate for growing endothelial cells. Further studies are required to determine the most natural medium for the growth cultured endothelial cells.

The cell preparation differed in previously reported studies. Bottoms et al (1985) used dispersed cells, whereas Turek et al (1987) and Bochsler et al (1989) used passaged cells. Changes occur in passaged cells or cell lines after six to nine

passages, cytoplasmic vacuolation occurs after ten passages, and endothelial cells are no longer reactive to antisera to human factor VIII associated proteins (Ryan et al 1978). In passaged cells, the ploidy changes from diploid to heteroploid and contact inhibition in the monolayer is lost (Freshney 1994). Therefore transformation (a permanent alteration in the cell phenotype) occurs by an irreversible genetic change which occurs in all passaged cells. In this study, which was designed to investigate physiological responses of equine endothelial cells to stimuli, only primary cultures were used to ensure the phenotype of the cells under investigation. As long as there are adequate sources of material, this is the preferred method of cell isolation.

Clearly, endothelial cells grown as a monolayer in culture dishes are not exposed to the same conditions as they would be *in vivo.* In particular, shear stress is thought to modulate the release of vasoactive compounds from endothelial cells, although some controversy exists over the nature of these effects (Yoshizumi et al 1989; Malek & Izumo 1992). However, physical factors such as shear stress vary according to the physiological states of the animal and are difficult to study *in vitro.* Nevertheless, primary cultures of endothelial cells offer a unique opportunity to study these cells in isolation in a species where *in vivo* studies are frequently not possible.

4.2.2 Phenotypic characterisation of cultured endothelial cells

The simplest method of characterisation is visual inspection, endothelial cells are easily distinguished from fibroblasts or smooth muscle cells on morphological grounds. Both fibroblasts and smooth muscle cells appear as long, slender, spindle-shaped cells, with distinct cell borders, growing in parallel arrays with whorling and multiple overlapping layers. As described in section 3.2.1, the cells produced with the current method are approximately 8 μm diameter, have a 'cobblestone' appearance and centrally located nucleus; all characteristics of endothelial cells (Freshney 1994).

Two methods of assessing endothelial cell character by detecting specific plasma membrane proteins were attempted. The use of immunofluorescence staining techniques failed to identify the presence of the glycoprotein CD31, despite published evidence of the presence of this glycoprotein in human vascular endothelial cells (Parums et al 1990; Hewett & Murray 1993). The reasons for the failure to detect CD31 are unknown, the monoclonal antibody may not be appropriate despite the manufacturers claim that it reacted well with horse and elephant CD31. An alternative explanation is that the procedures for staining were sub-optimum, even though a range of primary and secondary antibody dilutions were used. To resolve these difficulties, attempts should be made to detect CD31 with a tissue used by previous workers, e.g. human systemic microvessel endothelial cells (Hewett & Murray 1993).

In a separate series of experiments, an FITC-conjugated antibody for the endothelial membrane protein von Willebrand factor was used. This technique successfully identified von Willebrand factor on equine endothelial cells (Figure 3.9). Similar staining patterns were observed in human (Hoyer et al 1973; Jaffe et al 1973) and bovine endothelial cells (Booyse et al 1975).

Using the NADPH diaphorase staining technique, active nitric oxide synthase (NOS) was identified in equine and bovine endothelial monolayers, suggesting that the cells retain their ability to synthesise NO. Loesch et al (1995) have shown that the highest NADPH-diaphorase activity is found in the perinuclear cytoplasm of rabbit aortic endothelial cells while similar staining patterns have been revealed in neuronal cells (Morris et al 1997). The results shown in Figure 3.10 confirm this finding in equine and bovine pulmonary endothelial cells. This method does not distinguish between the two isoforms of NOS, namely iNOS and cNOS (section 1.3.1.1). Prior to the assay, the cells were incubated with Bk (1 nM) which would be expected to activate cNOS activity, but parallel incubations were not performed in the absence of Bk for equine endothelial cells to determine the unstimulated NOS activity. In separate experiments on bovine cultured endothelial cells, unstimulated and stimulated cells were compared using this technique (Morris et al 1997). The

non-stimulated cells showed prominent NADPH diaphorase staining indicating a significant basal NOS activity. The activity in the presence of 1nM Bk was approximately 60% higher than control in bovine cells. These measurements would suggest that basal NOS activity was high, yet still could be increased further by Bk, suggesting that both cNOS and iNOS contribute to NADPH diaphorase staining. However, the relative contributions of iNOS and cNOS cannot be easily distinguished with this method, however one possible way to assess cNOS activity is to compare the control NADPH diaphorase stain with that in the presence of a calmodulin antagonist (e.g. KN 62) a manoeuvre that should block cNOS activation.

The measurement of intracellular [Ca^{2+}] using Fura-2 fluorescence indicated that equine cultured endothelial cells responded to Bk with a response that was similar to previously published records from endothelial cells isolated from cattle (Busse et al 1993; Loeb et al 1993) and pigs (Busse et al 1991). These data in particular support the endothelial cell character of these cells since cultured fibroblasts and smooth muscle cells respond poorly to similar levels of Bk (Calixto & Medeiros 1992).

4.2.3 Measurements of the release of endothelial derived agents

In order to characterise the mediators which may control equine pulmonary vascular tone, assay systems were developed to measure the release of a range of vasoactive agents from endothelial cells, since no such data are available in this species. The four agents studied were NO, PGI_2, TxA_2 and ET. Short term regulation of vessel diameter is thought to be mediated by the release of vasodilators NO and PGI_2, countered by the release of the vasoconstrictor TxA_2. ET is thought to be involved in the longer term (hours to days) control of vascular tone (see review by Pearson & Vanhoutte 1993).

4.2.3.1 Assay systems for the measurement of nitric oxide release

Nitric oxide is difficult to measure because it is an unstable molecule that has a half-life of 5 - 30 sec, breaking down into nitrate and nitrite (Moncada et al 1991). Several methods for detecting nitrite have been reported. The Griess-Ilosvay reagent method by Green et al (1982) has a limit of detection of 10 μM nitrite.

Standard concentrations of nitrite were measured to this level, but nitrite was not detected in the culture medium at any time. A second, more sensitive method was investigated, the modified Griess reagent had a detection limit of 0.6 μM nitrite, yet this method did not detect NO production from the endothelial cells in culture. Therefore more sensitive methods of detection need to be investigated. Currently there appear to be three alternatives: (i) high-performance liquid chromatography with electrochemical detection will detect concentrations as low as 0.1 μM (Kaku et al 1994). While this method has been used to detect NO release from macrophages, it has yet to be applied to endothelial cells. (ii) A modified chemiluminescence system which can detect NO (1 nM limit) has been used to detect NO release from isolated blood vessels (Hoshino et al 1994). However, this measurements system is complex and involves the use of an expensive thermal energy analyser system (Sung et al 1992). (iii) A bioassay system, normally rabbit aortic strips, which will relax when perfused with solutions containing NO. Usually the endothelial cells are grown on microcarrier beads to enable the perfusate to be readily directed onto the aortic strips and this system can detect NO release from cultured endothelial cells (e.g. de Nucci et al 1988). This system will readily detect NO released from a column containing 10 - 20 x 10^6 cells, approximately 10 times the maximum number that would constitute a confluent monolayer within a microwell of the type used in this study.

4.2.3.2 Thromboxane release from bovine endothelial cells

A significant effort was invested in developing and optimising a sensitive thromboxane assay with the intention of measuring thromboxane release from equine and bovine endothelial cells under normoxic and hypoxic conditions. After determining the optimum conditions for the assay, i.e. antibody and charcoal concentrations, incubation time and temperature, initial experiments on bovine cells established that this technique could be used to measure basal thromboxane release, and verified the stimulatory effects of arachidonic acid on thromboxane release (Table 3.5). However, this technique was not applied to equine and bovine cells under experimental conditions.

4.2.3.3 Endothelin release from equine and bovine cultured endothelial cells

Two ET assay systems (radioimmunoassay and ELISA) were used to measure ET release from endothelial cells. The typical calibration curve for the radioimmunoassay system (Figure 2.2) was obtained by following the instructions given by the manufacturer (Peninsula Laboratories) but the slope of the curve was steeper than expected. Normally the maximum sensitivity from an assay system is achieved with slope value close to unity, yet in these assays, the slope was routinely 2.0 - 2.5. This would suggest that the recommended concentration of antibody was higher than optimal (Chard 1990). Despite this limitation, the assay system was able to provide a reproducible estimate of the ET concentration in the incubation medium. This assay system cannot differentiate ET-1, ET-2 or ET-3 i.e. it measures the total ET concentration. The few estimates made using the ELISA method, which specifically assayed ET-1, gave similar estimates of ET concentration to those obtained using the radioimmunoassay system. This suggests that ET-1 was the main ET released from bovine and equine vascular endothelial cells. Endothelial cells from equine and bovine pulmonary artery and equine aorta synthesised and released ET at a relatively constant rate over a 4 h period. However, data on the amount of ET release were controversial. In the group of experiments summarised in Table 3.1, bovine pulmonary artery endothelial cells appeared to produce significantly more ET than cells from equine pulmonary artery and aorta. Subsequently, equine pulmonary artery endothelial cells appeared to produced significantly more ET than the earlier set of experiments (Table 3.2). In this latter set of assays, bovine and equine pulmonary endothelial cells produced equivalent amounts while equine aorta endothelial cells produced significantly less than the other cell types. The reason for the differences between experiments is not known. It is unlikely to simply reflect differences in cell numbers, since each set of experiments were an average of large numbers of assays. Differences observed within each assay are unlikely to represent differences due to species or vessel type, thus the results indicate that the three endothelial cell types produced comparable quantities of ET over the 4 h period.

4.2.3.4 The effect of hypoxia, indomethacin and L-NAME on endothelin production

Incubation under hypoxic conditions for up to 4 h did not significantly affect ET production in any of the three types of cultured endothelial cell. As indicated in section 2.2.5.2, without active bubbling of the solution, PO_2 of the incubation fluid decreased slowly, reaching only 20 mm Hg after the first hour. Significant hypoxia (less than 10 mm Hg) existed after 2 h. Thus differences in ET production would only be evident after 2 h. Examining the ET concentration at 4 h revealed no significant difference as a result of hypoxic incubation in all three vessel types. Published studies on bovine pulmonary artery endothelial cells showed no change in ET release after 24 h of hypoxia (Hassoun et al 1992). However, ET release from bovine coronary artery endothelial cells was unaffected at 4 h but was significantly higher than control values after 24 h of hypoxia (Hieda & Gomez-Sanchez 1990). These results from systemic and pulmonary cells may suggest that ET release is not involved in the acute hypoxic response, although, hypoxia may have an effect on ET production on long term exposure to hypoxia, as suggested by MacLean et al (1994) from work on isolated rat pulmonary vessels.

L-NAME (0.1 mM) did not significantly affect ET production in any cell type either under normoxic or hypoxic conditions. This result runs counter to published results which suggest that endothelial derived NO inhibits ET production. L-NAME (3 h exposure at 0.1 mM) increased ET production in human umbilical vein endothelial cells (Cao et al 1994) and porcine aorta endothelial cells (Boulanger & Luscher 1990), an effect that is thought to be mediated by cGMP and G-kinase modulation of endothelin converting enzyme (Pearson & Vanhoutte 1993), although the exact cellular mechanism is not understood. The values of ET release measured in this study from bovine pulmonary endothelial cells after 4 h incubation with L-NAME appeared higher than control values in both normoxia and hypoxia (Table 3.2). However, the low sample number associated with these results meant that the difference did not achieve statistical significance. No comparable effect of L-NAME was observed in equine endothelial cells despite the larger sample numbers

suggesting that NO does not have an inhibitory effect on ET production in equine pulmonary artery or aorta endothelial cells. Further experiments, particularly on bovine endothelial cells, are required to establish if this is a real difference in endothelial cell function between the two species.

Indomethacin (a non-selective cyclooxygenase inhibitor) appeared to reduce ET production significantly in equine pulmonary artery endothelial cells under normoxic and hypoxic conditions (Figure 3.13 B & C). However, indomethacin had no effect on bovine pulmonary artery and equine aorta endothelial cells. A number of agents have been found to reduce ET production e.g. agents that activate protein kinase C (Emori et al 1989) or raise cGMP (Saijonmaa et al 1990), or raise cAMP (Magnusson et al 1994) or increase intracellular [Ca^{2+}] (Yanagisawa et al 1988). None of these second messengers can be directly linked to the known effects of indomethacin, and measurements of cAMP, cGMP and intracellular [Ca^{2+}] would be good starting points to investigate this effect of indomethacin further.

4.2.3.5 Effects of halothane on release of endothelin

In one experiment, ET release was monitored in the presence of 2% halothane. This procedure appeared not to affect ET release from equine endothelial cells, but this experiment should be repeated to obtain conclusive results. The basis for these experiments was the numerous published studies showing that halothane interfered with the ability of Bk and ACh to release NO, PGI_2 and TxA_2 (Blaise et al 1994; Loeb et al 1994; Boyle & Maher 1995; Zuo et al 1996) and the observations *in vivo* that a ventilation perfusion disturbance increases in halothane anaesthesia in horses (Gillespie 1969). However, there is no published work establishing the effects of halothane on ET production from endothelial cells. As previously mentioned, ET production is influenced by several intracellular factors, in particular intracellular [Ca^{2+}] (Yanagisawa et al 1988). Halothane depresses intracellular [Ca^{2+}] in cultured bovine endothelial cells (Loeb et al 1994; Simoneau et al 1996) and may alter other intracellular second messenger systems and through this mechanism affect ET release, however exposure to halothane for longer than 1 h may be required before ET release is affected.

One criticism that applies to this and other experiments is the lack of a suitable time control for these experiments. It is unclear how stable ET is during a 4 h incubation at 37°C. One possibility is that the slow rise in ET concentration observed in these experiments represents a balance between a higher rate of production by the endothelial cells and a fixed rate of degradation of ET in the medium bathing the cells. In hindsight, one means of controlling for this would have been to allocate one or more wells without cells a standard amount of ET in the incubation medium. The rate of decline of ET purely due to non-biological degradation would be obvious as a decrease in the standard concentration over the 4 h period.

4.2.3.6 Prostacyclin release from equine and bovine cultured endothelial cells

Two forms of PGI_2 assay were investigated. Both were based on the measurement of the labelled form of stable metabolite $PGF_{1\alpha}$. The radioimmunoassay based system was sensitive to $PGF_{1\alpha}$ concentrations as low as 140 $pg \cdot ml^{-1}$. The commercial kit from Amersham gave close to an ideal calibration curve (Figure 2.4): the slope of the curve was close to unity and B_{max} and B_{min} were insignificantly different from 100% and 0% respectively. The samples were measured using this assay. Regrettably, before all experimental samples could be completed, the assay was discontinued. An alternative enzyme-immunoassay was investigated, but the calibration curves were poor, with high slope values and reduced maximal binding. Furthermore, there was a large inter-assay variation in the EC_{50} value. For this reason this assay system was not used to measure the remaining experimental samples.

4.2.3.7 Comparison of $PGF_{1\alpha}$ release from equine and bovine pulmonary endothelial cells

There were marked differences in the time course of the release of $PGF_{1\alpha}$ between equine and bovine pulmonary artery endothelial cells (Figure 3.14A). Equine cells released $PGF_{1\alpha}$ at an approximately constant rate over the 4 h incubation period. However, the high concentration of $PGF_{1\alpha}$ after 1 h incubation indicated an initially higher rate of release of $PGF_{1\alpha}$ from bovine cells compared to equine cells. The

subsequent fall in $PGF_{1\alpha}$ concentration suggests that production fell to very low levels in the following 3 h and may have ceased altogether. Degradation of the vasoactive agent in the incubation medium may significantly attenuate the apparent rate of production of the vasoactive agent. While this may explain the decline in the $PGF_{1\alpha}$ concentration observed between 1 h to 4 h in bovine cells, the difference between equine and bovine cells is a significant finding. Alternative explanations for the fall in $PGF_{1\alpha}$ concentration are that: bovine cells actively absorb $PGF_{1\alpha}$ during the course of the experiment; or a slow acting negative feedback mechanism exists where an increase in extracellular concentration of $PGF_{1\alpha}$ depresses basal release of $PGF_{1\alpha}$ from endothelial cells. However, this phenomenon has not been noted in previous studies.

4.2.3.8 The effects of hypoxia, indomethacin and L-NAME on PGI_2 production

Hypoxia did not appear to affect the rate of $PGF_{1\alpha}$ production in equine pulmonary artery endothelial cells. As expected, the cyclooxygenase inhibitor indomethacin markedly reduced the rate of $PGF_{1\alpha}$ production by equine endothelial cells as was evident from the reduced $PGF_{1\alpha}$ levels measured in the incubation medium (Figure 4.14 A&B), but L-NAME had no effect. The results from bovine endothelial cells were unclear due to the low sample numbers and the large spread of results, so a comparison of the effects of indomethacin and L-NAME was not possible. Hypoxia appeared to increase $PGF_{1\alpha}$ release in bovine cells, yet due to the large variation between animals, the difference was not statistically significant ($p=0.08$). The literature concerning the effects of hypoxia on PGI_2 release is varied, results on systemic and pulmonary endothelial cells will be considered separately. Work by Busse et al (1984) on canine femoral and coronary arteries indicated that hypoxia (PO_2 20 mm Hg) caused a marked increase in PGI_2 release (measured as $PGF_{2\alpha}$) which mediated hypoxic vasodilation. Richards et al (1991) measured PGI_2 release from porcine aortic endothelial cells and found that a 30 min exposure to hypoxia (PO_2 22 mm Hg) had no effect on PGI_2 release. On the other hand, Madden (1986) showed a marked decrease in PGI_2 production (from 2.7 to 0.054 $ng \cdot ml^{-1}$) in bovine pulmonary artery endothelial cells exposed to nominally 0% O_2 for 4 h. In

contrast, Martin et al (1992) showed a 30% increase in PGI_2 from bovine pulmonary endothelial cells after 3 h hypoxic (4% O_2) incubation. The basis for these differing results may be the range of PO_2 values used. Only Madden et al (1986) used a PO_2 below 10 mm Hg O_2, although this study also noted a 50% reduction in PGI_2 release when the PO_2 was reduced from 160 mm Hg to 75 mm Hg. Another reason may be the different vascular sites from which the endothelial cells were derived. Yet Madden (1986) and Martin et al (1992) both used bovine pulmonary artery endothelial cells. Therefore there appears to be no consensus in the literature as to the effects of hypoxia on PGI_2 release from vascular endothelial cells. Only the study by Busse et al (1984) directly related the increased PGI_2 release to hypoxia induced relaxation of the blood vessels. As described in chapter 1, the synthesis of PGI_2 involves oxidation of arachidonic acid catalysed by the enzyme cyclooxygenase (Rang & Dale 1991). Because this reaction requires molecular oxygen (O_2), it should be inhibited by anoxia. However, the affinity of the enzyme for oxygen is unknown, and therefore the minimum PO_2 required for the reaction to proceed is not clear. In summary, $PGF_{1\alpha}$ production by equine pulmonary artery endothelial cells was unaffected by hypoxia, while there was some indication that hypoxia increased $PGF_{1\alpha}$ production in bovine pulmonary endothelial cells. It is difficult to find confirmation of these results in the literature since the published results seem contradictory.

4.2.3.9 Total protein determination

As is clear for Table 3.1, 3.2 and 3.3, measurements of both ET and $PGF_{1\alpha}$ under a standard set of condition showed high inter-experimental variation. One cause of this variation is a range in the cell density of the endothelial cells from one batch of cells to the next. A commonly used method for compensating for this variation is to express the concentration of vasoactive compound relative to the total protein content of the batch of cultured cells. Attempts were made to measure total protein content using both Coomassie and Lowry methods. These methods gave large inter- and intra-method variation (Figure 3.16). The lack of correlation between the concentration of an endothelial derived agent and the total protein concentration was

demonstrated in Figure 3.17. In this graph, the ET-1 concentration measured after 1 h incubation was plotted against the total protein concentration measured by the Coomassie method. The large scatter of the results suggests that total protein cannot be used to normalise the ET-1 measurements. The reason for this large variation is unknown, one possible cause may be inconsistent retrieval of the cells from the culture plates.

4.3 Isometric tension measurements on isolated vessels

4.3.1 Pharmacological characterisation of equine and bovine pulmonary artery

4.3.1.1 Contractile response to phenylephrine

Equine pulmonary arteries (2 - 3 mm o.d.) contracted in response to PE with an EC_{50} of approximately 2 μM in intact and rubbed vessels, 1 or 2 days post mortem. The maximum force produced 2 days post mortem was higher than that produced on day 1, and the processes of rubbing the endothelial layer reduced the maximum force produced in both equine and bovine vessels by approximately 20% (Table 3.6). Intact bovine vessels were less sensitive to PE (EC_{50} approximately 10 μM) than equine vessels, yet rubbed bovine vessels had a much higher sensitivity to PE than intact vessels, achieving a value that was comparable to equine vessels. Both intact and rubbed bovine vessels produced approximately twice as much maximum force as equine vessels to PE. This disparity cannot necessarily be attributed to the thicker media of bovine vessels since these tensions were corrected for the weight of the preparation. Instead, this disparity may reflect either intrinsic differences in the contractile properties of the smooth muscle from these two species or differences in the adrenoreceptor population. The published sensitivities of a range of systemic and pulmonary vessels to PE suggest a range of values ranging from 0.3 - 3.0 μM (reviewed by McGrath 1985). Work on rat pulmonary vessels indicate an EC_{50} of approximately 0.5 μM (MacLean et al 1993), which is significantly lower than the measurements from both equine and bovine vessels reported in this study. Since PE is considered to be a specific agonist of α_1-adrenoreceptors (McGrath et al 1989),

the contractile response observed is thought to be via the activation of these receptor subtypes. Yet, systemic and pulmonary vessels also possess α_2-adrenoreceptors on both the endothelial cell layer and the smooth muscle (McGrath et al 1989). Activation of endothelial α_2-adrenoreceptors causes an endothelium dependent relaxation via the production of NO. Stimulation of the α_2-adrenoreceptors on vascular smooth muscle causes contraction possibly by lowering intracellular cAMP levels (McGrath et al 1989). Despite these conflicting effects, activation of α_2-adrenoreceptors in intact rabbit pulmonary arteries (using a synthetic agonist UK14304) causes contraction with an EC_{50} of 2.5 μM. Addition of L-NAME enhanced the force produced by UK14304, indicating that a powerful endothelium derived inhibitory effect existed in addition to the smooth muscle based vasoconstricting effects (MacLean et al 1993). When considering the results presented in this study, the possibility that PE may be exerting its effects via α_2-adrenoreceptors cannot be rejected since evidence exists that PE may have a significant affinity for α_2-adrenoreceptors in canine coronary arteries (Guimaraes et al 1987). Thus, the lower sensitivity to PE in both bovine and equine pulmonary vessels suggests that either these vessels (i) have α_1-adrenoceptors with unusually low affinities for this agonist, or (ii) possess predominately α_2-adrenoceptors. However, unlike the studies by MacLean et al (1993), when the influence of the endothelium was removed, maximal force production was reduced in both equine and bovine vessels. This would suggest that PE was not activating a significant population of α_2-adrenoreceptors in the endothelium. At present, the only method of distinguishing α_1- and α_2-adrenoreceptors mediated responses would be to conduct a series of experiments with PE, α_1-adrenoreceptors antagonists (e.g. prazosin), α_2-adrenoreceptor agonists (e.g. UK14304) and α_2-adrenoreceptors antagonists (e.g. rauwolscine). Only by considering the response to all of these agents can the adrenoreceptors be characterised (McGrath 1985).

4.3.1.2 Contractile response to 5-hydroxytryptamine

As discussed in section 1.4.1.4, work on human pulmonary artery by MacLean (1996), suggests that the contractile response is due to activation of 5-HT_1 receptors.

The contractile response observed in other species studied which appear to use 5-HT_2 receptors (Drummond & Wadsworth 1994; Ellis et al 1995). The sensitivity to 5-HT of these two receptor subtypes are not obviously different: both appear to result in an EC_{50} of approximately 0.3 μM, which approximates to the values measured in both equine and bovine pulmonary arteries in this study (Table 3.7). As with the adrenoreceptor subtypes, the use of specific agonists (sumatriptan for 5-HT_1) and antagonists (ketanserin for 5-HT_2, and methiothepin for 5-HT_1 & 5-HT_2) can be used to functionally classify the response. Removal of the endothelium appeared to cause an increase in the sensitivity of bovine pulmonary artery to 5-HT ($p = 0.064$), but had no obvious effect on maximal tension. A similar effect was observed in equine vessels, but the difference was not close to statistical significance due to large variation in the EC_{50} under control conditions. Maximum force was unaffected by removal of the endothelium, but both intact and rubbed vessels showed a large variation in maximum force, much larger than the variation seen in bovine vessels, or in equine vessels in response to PE. The cause of this variation is unknown. MacLean et al (1994) observed a similar shift in sensitivity to 5-HT in rubbed bovine pulmonary arteries, and in vessels treated with L-NAME. In addition to this, these workers observed a marked increase in the maximum contractile response to 5-HT after L-NAME treatment. They attribute this effect to the known 5-HT stimulated release of NO from the endothelium, but the specific receptor responsible for this response is unknown (Graven et al 1993). One reason for the absence of an increase in maximal force in rubbed vessels may be the damage of underlying smooth muscle which would counteract the increase in force expected once NO release was inhibited. That the removal of the endothelium did not depress maximal force in response to 5-HT, but did in response to PE in this study, supports this explanation.

4.3.1.3 Relaxation to bradykinin

As discussed in section 1.4.1.6, Bk caused relaxation of vascular smooth muscle via the production of both PGI_2 and NO from the endothelium. The purpose of this part of the work was to characterise the effect of Bk in equine pulmonary vessels and

assess the relative contributions of PGI_2 and NO pathways in the response. As is evident from Table 3.8, Bk caused profound relaxation in both equine and bovine pulmonary vessels to approximately 10% of the original force level. However, there appeared to be a difference in the sensitivity of the response to Bk, equine vessels responded with an EC_{50} of approximately 0.8 nM, while an EC_{50} of approximately 14 nM was observed in bovine vessels. The EC_{50} and degree of relaxation in bovine vessels measured in this study were similar to that observed by other workers (Furchgott 1984; Busse et al 1993). After removal of the endothelium, Bk was still able to reduce force to approximately 75% of its original level in both vessel types and the removal of the endothelium appeared to affect the EC_{50} of the remaining relaxing effect of Bk. This may be an apparent shift, the remaining endothelium may be operating normally, but because of the small changes in force, accurate measurements from the chart record were difficult. However, an alternative explanation to be considered is that Bk may act on the remaining smooth muscle to cause a weak vasorelaxant effect with a different sensitivity to the endothelial based effect. Studies have shown Bk induced PGI_2 release in cultured smooth muscle cells (Levesque et al 1993). Yet, from studies on intact vessels, there appears to be no basis for this effect. In fact, at concentrations above 1 μM, Bk causes a contraction in vascular smooth muscle (Calixto & Medeiros 1992). In this study, high concentrations of Bk (1 μM) caused a small increase in force in both equine and bovine pulmonary vessels as illustrated in Figure 3.21B.

The use of L-NAME or indomethacin allows selective inhibition of either NO or PGI_2 release. The use of either inhibitor appeared to reduce the relaxant effect of Bk by approximately 30% (Table 3.9), and consistent with these individual effects, the combined effects of L-NAME and indomethacin reduced the ability of Bk to relax pulmonary vessels by approximately 60%. The presence of L-NAME and indomethacin produced an effect similar in magnitude to removal of the endothelium. The reason for the remaining relaxation is unknown, but it does suggest that NO and PGI_2 were the sole mediators of endothelial relaxation in

pulmonary vessels, and that both agents were approximately equipotent. The co-release of NO and PGI_2 induced by Bk from aortic endothelial cells was first measured by De Nucci et al (1988), and suggests a common mechanism for release. These authors propose that a Bk-induced rise of intracellular $[Ca^{2+}]$ may be the event which links the two intracellular second messenger systems.

4.3.1.4 Relaxation to acetylcholine

The responses of equine and bovine pulmonary vessels to ACh were compared. As shown in Table 3.10, similar sensitivities to ACh were evident between the two species, however equine vessels relaxed less than bovine vessels to maximal concentrations of ACh. The almost complete relaxation to ACh observed in bovine vessels is commonly seen in isolated arterial vessels from both systemic and pulmonary systems (Furchgott 1983) although a systematic studies across species has not been published. However, vessel type may be a factor since bovine pulmonary veins relax poorly to ACh in comparison to pulmonary arteries (Gruetter & Lemke 1986). In a study of isolated equine coronary arteries pre-contracted with PE, (Obi et al 1994) noted almost complete relaxation to ACh with a similar EC_{50} to that measured in this study. ACh can mediate relaxation via two distinct receptors, M_2 receptors activate the release of NO and PGI_2, while activation of M_1 receptors causes relaxation via the release of an unidentified factor that causes hyperpolarisation of the smooth muscle membrane (EDHF) (Pearson & Vanhoutte 1993). Normally, both receptors are present, however the smaller extent of relaxation observed in equine vessels may result from an altered muscarinic receptor distribution or number in equine pulmonary vessels.

4.3.1.5 Contractile response to endothelin

The response of equine pulmonary vessels to ET-1 was studied in a series of experiments using specific blockers of either ET_A receptors (BQ123) or ET_B receptors (BQ788). As observed in vessels from other species, the time course of the contractile response to ET-1 was slow and sustained. Measurements by Sakata et al (1989) suggested that ET-1 causes a transient rise of intracellular $[Ca^{2+}]$ but a

sustained contraction because of an increase in the Ca^{2+} sensitivity of the myofilaments. A number of studies on large pulmonary arterial vessels (2 - 3 mm i.d.) from a range of species including rat, dog, pig cow and human have shown that ET-1 caused an increase in force via activation of ET_A receptors (Watanabe et al 1991; Nakamichi et al 1992; Douglas et al 1993; Nakashima & Vanhoutte 1993; Buchan et al 1994). However, the contractile response in rabbit large pulmonary artery (Panek et al 1992) and rat small pulmonary artery (approximately 0.3mm i.d. MacLean et al 1994) appears to be mediated via an ET_B receptor. From the results shown in Table 3.11, it is clear that the selective ET_A receptor antagonist effectively shifted the EC_{50} for ET-1 from approximately 3 nM to 80 nM, but the ET_B receptor antagonist had no effect. Thus it appears that the ET_A receptor is the dominant receptor responsible for ET-1 mediated contractions in large (2 - 3 mm o.d.) equine pulmonary artery. While maximal force was unaffected by BQ123, the level of maximal force appeared very variable in the presence of BQ788 and generally greater than induced by ET-1 alone, but the difference in magnitude was not statistically significant. One possible explanation for this effect is the well documented effect of ET-1 on the endothelium. As reviewed by Sakurai (1992), ET-1 can activate ET_B receptors on the endothelium and stimulate the release of NO. Thus, the net effect of the addition of ET-1 appears to be a balance between endothelium mediated relaxation and smooth muscle mediated contraction (MacLean et al 1994; Riezebos et al 1994), in much the same way as previously described for adrenoreceptor and 5-HT receptor function. Therefore, the use of the ET_B receptor antagonist BQ788 in this study may have blocked the ET-1 induced release of NO from the endothelium and thus increased maximal force in some of the vessels studied. The variation in maximal force observed may be due to partially damaged endothelium in some of the vessels studied. Further studies using L-NAME and rubbed blood vessels are required to confirm presence of ET receptors on the endothelium of equine pulmonary arteries.

4.3.2 Response of equine and bovine vessels to hypoxia

4.3.2.1 Isometric tension responses to 0%, 2% and 5% O_2 in bovine pulmonary artery rings

As shown in Table 3.12, 5% O_2 and 2% O_2 caused a relaxation of force in isolated bovine pulmonary arteries while nominally 0% O_2 (approximately 0.9 mm Hg) caused a steady state contraction. 5% O_2 appeared to produce a more profound relaxation than 2%, but this effect did not quite achieve statistical significance ($p = 0.08$). A similar graded relaxation was observed in porcine pulmonary arteries by Kovitz et al (1993). In this publication, relaxation was observed in 10% , 4% and 0% O_2 in small and medium sized arteries (i.e. 1 - 2 mm and 3 - 7 mm i.d. respectively), while in large and proximal arteries (8 - 10 mm and 10 - 12 mm i.d. respectively), 0% O_2 caused a contraction. Hypoxia-stimulated increases in PGI_2 and NO release from the endothelium have been shown to be responsible for hypoxic vasodilation in systemic vessels (Busse et al 1984; Graser & Vanhoutte 1991) and appears to be responsible for approximately 50% of the relaxation observed. The remaining relaxation is due to a direct effect of hypoxia on the smooth muscle of the tunica media. As discussed in section 1.4.5, one of the main mechanisms responsible for hypoxia induced relaxation is the activation of K_{ATP} channels and the subsequent hyperpolarisation of the smooth muscle sarcolemma (Wiener et al 1995). Hypoxia is thought to disturb the metabolism of the smooth muscle, lowering [ATP] and activating these channels. It should be noted the graded relaxation observed in this study and by Kovitz et al (1993) is not a universal observation, 5% O_2 failed to produce any effects in isolated sheep large pulmonary artery (2 - 4 mm i.d.), and only a small relaxant effects on rings of small human pulmonary arteries (0.4 - 0.6 mm i.d.) (Demiryurek et al 1993).

4.3.2.2 Isometric tension responses to 0% O_2 in equine and bovine pulmonary arteries

The time course of the response of equine and bovine pulmonary vessels to 0% O_2 was similar (Figures 3.26B and 3.27). Initially, the tension (induced by PE or

5-HT) decreased, before rising slowly to above pre-contracted levels. The initial fall probably represented a relaxing effect of intermediate O_2 levels achieved as the PO_2 fell within the vessel wall. This time course contrasted to those published in the literature. Monophasic increases in tension were observed in sheep and human pulmonary arteries (Demiryurek et al 1993; Demiryurek et al 1994), but both these arteries failed to relax in 5% O_2 and therefore would not have relaxed to the intermediate PO_2 on exposure to anoxic solutions. Rat pulmonary arteries responded to hypoxia (< 2%) with a rapid phasic contraction followed by a weaker, more slowly developing contraction (Rodman et al 1989; Jin et al 1992; Leach et al 1994; Robertson et al 1995). As described in section 1.4.4, this phasic response appeared to be partially endothelium dependent. A transient contraction followed by a sustained phase was also observed in pig pulmonary arteries but only in large (> 8 mm i.d.) vessels. Thus equine pulmonary vessels appear to respond to hypoxia (< 2%) in a similar way to bovine, ovine and human vessels. However, comparisons with most studies are hampered by the differing conditions used by investigators.

Bovine pulmonary artery appeared to produce a stronger HPV response than equine vessels (Table 3.13). This comparison was made after correcting the tensions for the weight of the vessel (i.e. cross sectional area) and therefore cannot simply be explained by the thicker media of the bovine vessels. It should be remembered that bovine vessels were pre-contracted to approximately 50% of maximum force levels with 5-HT (0.1μM) and equine vessels with PE (0.3 μM). These different agonists may be the reason for the differing relative responses to hypoxia, since alternative sets of second messenger systems may be involved promoting force by activation of 5-HT receptors (sub-class 1 or 2) or α-adrenoreceptors (sub-class 1 or 2) (sections 1.3.2 and 4.3.1). As described previously, these agonists were used to ensure stable tension levels in equine and bovine vessels, but a more valid comparison of the response to hypoxia would be possible if similar levels of tension were generated by activation of the same receptor type (e.g. α_1-adrenoreceptors) in the two species. These experiments will have to await a complete pharmacological analysis of receptor expression in both bovine and equine vessels.

In both vessel types, removal of the endothelium reduced but did not abolish the response to hypoxia; in both cases the contraction was reduced to 30 - 35% of the pre-contracted value. With both intact and rubbed vessels, this value represented the mean of a range of values that reflected a range of endothelial function (as assessed by the maximal effect of Bk). This theory was tested by plotting the extent of the relaxation observed in Bk against the relative size of the hypoxic response. As shown in Figure 3.28, no correlation between endothelial cell function and hypoxic contraction was present in the intact or rubbed groups (of either species) yet the rubbed vessels clearly had a smaller response to Bk and a smaller hypoxic contraction. One explanation for this lack of correlation could be that the endothelial influence over the hypoxic response was not accurately assessed by the response to Bk. Alternatively, the smaller hypoxic response observed in rubbed vessels may be a result of damage to the underlying smooth muscle. However, the simplest view of damage is the loss of functional smooth muscle cells, which might be expected to scale down both the response to an agonist and the hypoxic response to a similar degree and therefore not affect the relative response. The hypoxia-induced contractions were entirely abolished by removal of the endothelium in pig pulmonary artery (Kovitz et al 1993) and rat pulmonary artery (Bennie et al 1991). But in other studies on rat vessels, removal of the endothelium merely attenuated the response (Rodman et al 1989; Robertson et al 1995; Zhang et al 1995) and similar attenuation was seen in dog (Graser & Vanhoutte 1991). The responses in human and ovine pulmonary artery appear to be completely endothelium dependant (Demiryurek et al 1991; Demiryurek et al 1993).

The endothelial dependence of the response to hypoxia was also examined using specific inhibitors of NO and PGI_2 release. As shown in Table 3.14, indomethacin and L-NAME both effectively abolished the hypoxic response in equine vessels, but only L-NAME was partially effective in bovine vessels. The effects of L-NAME on bovine vessels were compatible with the results from rubbed vessels. L-NAME reduced the amplitude of the response to hypoxia to approximately 30% of the control value i.e. a similar hypoxic contraction was measured in rubbed bovine

vessels. This suggests that the endothelial component to the response to hypoxia was mainly due to the reduced release of NO from the endothelium. The phasic response to hypoxia seen in several studies was similarly inhibited by L-NAME (Johns et al 1989; Graser & Vanhoutte 1991; Jin et al 1992; MacLean et al 1993), but to date, no studies have shown a sustained response that was thought to be mediated by reduced NO production. Some workers have suggested that part of the sustained response to hypoxia is mediated by an endothelium derived constricting factor which does not appear to be ET (Rubanyi & Vanhoutte 1985; Kovitz et al 1993; Demiryurek et al 1994). There was no evidence for this unknown vasoconstrictor substance in this study, since the contraction to hypoxia in the presence of L-NAME was comparable to the contraction seen in rubbed bovine vessels.

In contrast to the results in bovine vessels, indomethacin and L-NAME had a more profound effect on the hypoxic contraction in equine vessels when compared with rubbed vessels. In some vessels, the complete absence of a response to Bk was still accompanied by a significant response to hypoxia. Yet L-NAME or indomethacin appeared to abolish the response to hypoxia. This is an unusual effect, and does not seem to have been shown in published work on pulmonary vessels. There are several possible explanations of this result requiring further investigation to distinguish: (i) both L-NAME and indomethacin may have direct effects on the smooth muscle that inhibit the direct action of hypoxia. It has been shown by a number of studies that smooth muscle can generate NO and PGI_2 which may act locally to affect smooth muscle tone (reviewed in Pearson & Vanhoutte 1993). One way to distinguish this might be to determine the effects of L-NAME and indomethacin on the hypoxic response in vessels where the endothelium had been disrupted. (ii) The absence of a response to Bk may not represent the complete absence of a functioning endothelium. If sufficient endothelium remained to modulate a hypoxic contraction, then the complete inhibition of the hypoxic contraction in the presence of L-NAME and indomethacin may more correctly reflect the complete absence of a direct effect of hypoxia on the smooth muscle of

equine pulmonary artery. One method to determine whether a direct effect of hypoxia exists would be to isolate smooth muscle cells from the wall of the pulmonary artery and determine whether individual muscle cells can contract under hypoxic conditions.

4.4 Future studies

4.4.1 Endothelial cell studies

The work of this thesis suggests that ET production by endothelial cells is unaffected by acute (4 h or less) exposure to hypoxia. However, given the view that ET release has a role in the long term regulation of vascular tone, these experiments should be extended to include hypoxic incubations for up to 12 h for both equine and bovine cells.

Measurements of $PGF_{1\alpha}$ release suggested that equine cells had a quite different time course of PGI_2 release under control condition when compared to bovine vessels. Moreover, the results suggested that equine and bovine PGI_2 release was modulated differently by hypoxia. Further studies should be carried out to investigate these differences more fully. The work would be enhanced by the inclusion of simultaneous measurements of TxA_2 release. PGI_2 and TxA_2 are two vasoactive agents produced from the metabolism of arachidonic acid, and the relative proportions of PGI_2 and TxA_2 will be important in determining the response of the blood vessel to hypoxia.

An improved method of measuring total protein is required to improve the reproducibility of the measurements from cultured endothelial cells. NO release will continue to be difficult to measure, however it may be possible to develop methods to allow the quantification of the NADPH diaphorase stain. This would allow measurement of NOS activity under a range of conditions including hypoxia. Measurements could be repeated in the presence of a volatile anaesthetic such as halothane to establish whether anaesthetics effect the release of these vasoactive agents.

Culture techniques could be extended by the development of co-cultures of endothelial cells and smooth muscle cells to allow the interactions between these two cell types to be studied in more detail. This type of preparation has been successfully applied to endothelial and smooth muscle cells isolated from rat aorta (Johns 1995).

4.4.2 Isolated blood pulmonary blood vessels

The pharmacological characterisation of equine pulmonary vessels for each of the agonists used in this study is required since no published work exists. As highlighted in this thesis, equine vessels can respond quite differently from bovine vessels, thus these studies would be designed to determine the predominant adrenoreceptor or 5-HT receptor on the smooth muscle and endothelium of the vessel. A similar pharmacological study should be undertaken to identify the Bk and muscarinic receptors present on the endothelium of equine pulmonary arteries. In particular, studies should focus on the mechanisms underlying the poor ACh response in equine vessels compared to the almost complete relaxation seen in the presence of Bk. For example, ligand binding experiments would determine whether the poor ACh response is due to lower receptor number in equine vessels compared to bovine pulmonary arteries.

The cell physiology of the hypoxic contraction could be further studied by dissociating smooth muscle cells from equine and bovine vessels. By measuring cell shortening and intracellular [Ca^{2+}], the response of the isolated cells to hypoxia could be studied to determine whether the weaker response to hypoxia noted in equine vessel has a basis in the response of individual smooth muscle cells.

4.4.3 Summary

In summary, *in vivo* work on the pulmonary vasculature of the horse is particularly difficult due to the size and temperament of the animal. Furthermore, *in vitro* work on viable isolated vessels is hampered by the scarcity of suitable post mortem tissue, and the particularly fragile nature of isolated pulmonary vessels. These issues are borne out by the lack of published material on the pulmonary physiology and

pharmacology of this animal, yet the work makes important contributions to comparative physiology and pharmacology and is vital for the design of clinical treatments.

References

ADAM, L.P., GAPINSKI, C.J. & HATHAWAY, D.R. (1992) Phosphorylation sequences in h-caldesmon from phorbol ester-stimulated canine aortas. *FEBS Letters* **302**, 223-226.

AMATYRA, M.P., DEMIRYUREK, A.T., KANE, K.A., WADSWORTH, R.M. & ZEITLIN, I.J. (1989) The role of prostacyclin and other mediators in hypoxic constriction of sheep isolated coronary artery rings. *Journal of Vascular Medicine and Biology* **1**, 341-346.

AMIS, T.C., PASCOE, J.R. & HORNOF, W.J. (1984) Topographic distribution of pulmonary ventilation and perfusion in the horse. *American Journal of Veterinary Research* **45**, 1597-1601.

ARAI, H., HORI, S., ARAMORI, I., OHKUBO, H. & NAKANISHI, S. (1990) Cloning and expression of a cDNA encoding an endothelin receptor. *Nature* **348**, 730-732.

ATTENBURROW, D.P. (1983) Respiration and locomotion. In *Equine Exercise Physiology*, eds. SNOW, D. H., PERSSON, S. G. B. & ROSE, R. J. pp. 17-22. Cambridge: Burlington Press.

BARER, G.R., HOWARD, P. & SHAW, J.W. (1970) Stimulus-response curves for the pulmonary vascular bed to hypoxia and hypercapnia. *Journal of Physiology* **211**, 139-155.

BARMAN, S.A., SENTENO, E., SMITH, S. & TAYLOR, A.E. (1989) Acetylcholine's effect on vascular resistance and compliance in the pulmonary circulation. *Journal of Applied Physiology* **67**, 1495-1503.

BARNARD, J.W., BARMAN, S.A., ADKINS, W.K., LONGENECKER, G.L. & TAYLOR, A.E. (1991) Sustained effects of endothelin-1 on rabbit, dog, and rat pulmonary circulations. *American Journal of Physiology* **261**, H479-H486.

BARNARD, J.W., WARD, R.A., ADKINS, W.K. & TAYLOR, A.E. (1992) Characterization of thromboxane and prostacyclin effects on pulmonary vascular resistance. *Journal of Applied Physiology* **72**, 1845-1853.

BAYLY, W.M., SHULTZ, D.A., HODGSON, D.R. & GOLLNICK, P.D. (1987) Ventilatory responses of the horse to exercise: effect of gas collection systems. *Journal of Applied Physiology* **63**, 1210-1217.

BENHAM, C.D., MERRITT, J.E. & RINK, T.J. (1989) Receptor-mediated calcium entry. In *Ion Transport*, pp. 197-213. Academic Press Ltd.

BENNIE, R.E., PACKER, C.S., POWELL, D.R., JIN, N. & RHOADES, R.A. (1991) Biphasic contractile response of pulmonary artery to hypoxia. *American Journal of Physiology* **261**, L156-L163.

BERRIDGE, M.J. & IRVINE, R.F. (1989) Inositol phosphates and cell signalling. *Nature* **341**, 197-205.

BERRIDGE, M.J. (1993) Inositol triphosphate and calcium signalling. *Nature* **361**, 315-325.

BISGARD, G.E., ORR, J.A. & WILL, J.A. (1975) Hypoxic pulmonary hypertension in the pony. *American Journal of Veterinary Research* **36**, 49-52.

BLAISE, G.B., TO, Q., PARENT, M., LAGARDE, B., ASENJO, F. & SAUVE, R. (1994) Does halothane interfere with the release, action, or stability of endothelium-derived relaxing factor/nitric oxide? *Anesthesiology* **80**, 417-426.

BOCHSLER, P.N., SLAUSON, D.O., CHANDLER, S.K. & MITSU SUYEMOTO, M. (1989) Isolation and characterization of equine microvascular endothelial cells in vitro. *American Journal of Veterinary Research* **50 (10)**, 1800-1805.

BONAGURA, J.D. & MUIR, W.W. (1991) The cardiovascular system. In *Equine anesthesia: monitoring and emergency therapy*, eds. MUIR, W. W. & HUBBELL, J. A. E. pp. 39-104. St Louis: Mosby year Book.

BOOYSE, F.M., SEDLAK, B.J. & RAFELSON, M.E. (1975) Culture of arterial endothelial cells: characterization and growth of bovine aortic cells. *Thrombosis Et Diathesis Haemorrhagica* **34**, 825-839.

BOTTOMS, G.D., JOHNSON, M.A., LAMAR, C.H., FESSLER, J.F. & TUREK, J.J. (1985) Endotoxin-induced eicosanoid production by equine vascular endothelial cells and neutrophils. *Circulatory Shock* **15**, 155-162.

BOULANGER, C.M. & LUSCHER, T.F. (1990) Release of endothelin from porcine aorta. Inhibition by endothelium-derived relaxing factor. *Journal of Clinical Investigation* **85**, 587-590.

BOYLE, W.A. & MAHER, G.M. (1995) Endothelium-independent vasoconstricting and vasodilating actions of halothane on rat mesenteric resistance vessels. *Anesthesiology* **82**, 221-235.

BREDT, D.S. & SNYDER, S.H. (1990) Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proceedings of National Academy of Science of the United States of America* **87**, 682-685.

BUCHAN, K.W., MAGNUSSON, H., RABE, K.F., SUMNER, M.J. & WATTS, I.S. (1994) Characterisation of the endothelin receptor mediating contraction of human pulmonary artery using BQ123 and Ro 46-2005. *European Journal of Pharmacology* **260**, 221-226.

BUSSE, R., FLEMING, I. & HECKER, M. (1993) Signal transduction in endothelium-dependent vasodilation. *European Heart Journal* **14 Suppl I**, 2-9.

BUSSE, R., FORSTERMANN, U., MATSUDA, H. & POHL, U. (1984) The role of prostaglandins in the endothelium-mediated vasodilatory response to hypoxia. *Pflugers Archiv* **401**, 77-83.

BUSSE, R., LUCKHOFF, A. & MULSCH, A. (1991) Cellular mechanisms controlling EDRF/NO formation in endothelial cells. *Basic Research in Cardiology* **86 Suppl 2**, 7-16.

CALIXTO, J.B. & MEDEIROS, Y.S. (1992) Effect of protein kinase C and calcium on bradykinin-mediated contractions of rabbit vessels. *Hypertension* **19**, II-87-II-93.

CAO, W.B., ZENG, Z.P., ZHU, Y.J., LUO, W.C. & CAI, B.Q. (1994) Inhibition of nitric oxide synthesis increases the secretion of endothelin-1 in vivo and in cultured endothelial cells. *Chinese Medical Journal* **107**, 822-826.

CHARD, T. (1990) *An introduction to radioimmunoassay and related techniques*. Amsterdam: Elsevier.

CHUNG-WELCH, N., SHEPRO, D., DUNHAM, B. & HECHTMAN, H.B. (1988) Prostacyclin and prostglandin E_2 secretions by bovine pulmonary microvessel endothelial cells are altered by changes in culture conditions. *Journal of Cell Physiology* **135**, 224-234.

CLARKE, K.W. & HALL, L.W. (1990) A survey of anaesthesia in small animal practice. *Journal of the Association of Veterinary Anaesthetists* **17**, 4-10.

CLARKE, W.R., HABERKERN, C.M., ZEH, J., POWERS, K., SHARAR, S.R. & SOLTOW, L.O. (1993) The HPV response is different with constant pressure vs constant flow perfusion. *Respiration Physiology* **94**, 75-90.

COLYER, J. & WANG, J.H. (1991) Dependence of cardiac sarcoplasmic reticulum calcium pump activity on the phosphorylation status of phospholamban. *Journal of Biological Chemistry* **266**, 17486-17493.

CORNWELL, T.L. & LINCOLN, T.M. (1989) Regulation on intracellular Ca^{2+} levels in cultured vascular smooth muscle cells. *Journal of Biological Chemistry* **264**, 1146-1155.

CREMONA, G., HIGGENBOTTOM, T.W., WOOD, A.M., PEPKE-ZABA, J., BUTT, A.Y. & DINH-XUAN, A.T. (1992) Endothelium-dependent relaxation induced by acetylcholine is impaired in isolated perfused lungs from patients with chronic lung disease. *Journal of Vascular Research* **29**, 66P(Abstract)

DANTHULURI, N.R. & DETH, R.C. (1984) Phorbol ester-induced contraction of arterial smooth muscle and inhibition of alpha-adrenergic response. *Biochemical and Biophysical Research Communications* **125**, 1103-1109.

DARLING, D.C. & MORGAN, S.J. (1994) *Animal cells, culture and media, essential data.* Chichester: John Wiley & Sons.

DAUNT, D.A. (1990) Supportive therapy in the anesthetized horse. *Veterinary Clinics of North America, Equine Practice* **6**, 557-574.

DAWSON, C.A. (1984) Role of pulmonary vasomotion in physiology of the lung. *Physiological Reviews* **64**, 544-616.

DE NUCCI, G., GRYGLEWSKI, R.J., WARNER, T.D. & VANE, J.R. (1988) Receptor-mediated release of endothelium-derived relaxing factor and prostacyclin from bovine aortic endothelial cells is coupled. *Proceedings of National Academy of Science of the United States of America* **85**, 2334-2338.

DEMIRYUREK, A.T., MACLEAN, M.R., WADSWORTH, R.M. & KANE, K.A. (1994) Effects of hypoxia on endothelin-1 release from human, sheep and rabbit pulmonary artery rings. *Endothelium* **1**, 251-258.

DEMIRYUREK, A.T., WADSWORTH, R.M. & KANE, K.A. (1991) Effects of hypoxia on isolated intrapulmonary arteries from the sheep. *Pulmonary Pharmacology* **4**, 158-164.

DEMIRYUREK, A.T., WADSWORTH, R.M., KANE, K.A. & PEACOCK, A.J. (1993) The role of endothelium in hypoxic constriction of human pulmonary artery rings. *American Review of Respiratory Disease* **147**, 283-290.

DETAR, R. & GELLAI, M. (1971) Oxygen and isolated vascular smooth muscle from the main pulmonary artery of the rabbit. *American Journal of Physiology* **221**, 1791-1794.

DETWEILER, D.K. (1993) Control mechanisms of the circulatory system. In *Dukes' Physiology of Domestic Animals*, eds. SWENSON, M. J. & REECE, W. O. pp. 184-226. Ithaca, NY: Comstock Publishing Associates.

DOUGLAS, S.A., VICKERY CLARK, L.M. & OHLSTEIN, E.H. (1993) Endothelin-1 does not mediate hypoxic vasoconstriction in canine isolated blood vessels: effect of BQ-123. *British Journal of Pharmacology* **108**, 418-421.

DRUMMOND, R.M. & WADSWORTH, R.M. (1994) Contraction of the sheep middle cerebral, pulmonary and coronary arteries initiated by release of intracellular calcium. *Journal of Autonomic Pharmacology* **14**, 109-121.

DUCHARME, N.G., HACKETT, R.P., DUCHARME, G.R. & LONG, S. (1983) Surgical treatment of colic: results in 181 horses. *Veterinary Surgery* **12**, 206-209.

DYCE, K.M., SACK, W.O. & WENSING, C.J.G. (1987) *Textbook of Veterinary Anatomy.* Philadelphia: W.B.Saunders.

ELLIOTT, A.R., STEFFEY, E.P., JARVIS, K.A. & MARSHALL, B.E. (1991) Unilateral hypoxic pulmonary vasoconstriction in dog, pony and miniature swine. *Respiration Physiology* **85**, 355-369.

ELLIS, E.S., BYRNE, C., MURPHY, O.E., TILFORD, N.S. & BAXTER, G.S. (1995) Mediation by 5-hydroxytryptamine$_{2B}$ receptors of endothelium-dependent relaxation in rat jugular vein. *British Journal of Pharmacology* **114**, 400-404.

EMORI, T., HIRATA, Y. & MARUMO, F. (1990) Specific receptors for endothelin-3 in cultured bovine endothelial cells and its cellular mechanism in action. *FEBS Letters* **263**, 261-264.

EMORI, T., HIRATA, Y., AHTA, K., SHICHIRI, M. & MARUMO, F. (1989) Secretory mechanism of immunoreactive endothelin in cultured bovine endothelial cells. *Biochemical and Biophysical Research Communications* **160**, 93-100.

ERICKSON, B.K., ERICKSON, H.H. & COFFMAN, J.R. (1992) Pulmonary artery and aortic pressure changes during high intensity treadmill exercise in the horse: effect of frusemide and phentolamine. *Equine Veterinary Journal* **24**, 215-219.

ERICKSON, B.K., SEAMAN, J., KUBO, K., HIRAGA, A., KAI, M., YAMAYA, Y. & WAGNER, P. (1994) Mechanism of reduction in alveolar-arterial PO_2 difference by helium breathing in the exercising horse. *Journal of Applied Physiology* **76**, 2794-2801.

ERICKSON, H.H. (1993) Exercise physiology. In *Dukes' Physiology of Domestic Animals*, eds. SWENSON, M. J. & REECE, W. O. pp. 303-324. Ithaca, NY: Comstock Publishing Associates.

FEDDERSEN, C.O., CHANG, S., CZARTALOMNA, J. & VOELKEL, N.F. (1990) Arachidonic acid causes cyclooxygenase-dependent and -independent pulmonary vasodilation. *Journal of Applied Physiology* **68**, 1799-1808.

FISHMAN, A.P. (1976) Hypoxia on the pulmonary circulation: how and where it acts. *Circulation Research* **38**, 221-231.

FISHMAN, A.P. (1985) Pulmonary circulation. In *Handbook of Physiology*, eds. FISHMAN, A. P. & FISHER, A. B. pp. 93-165. Bethesda, MD: American Physiological Society.

FRESHNEY, R.I. (1994) *Culture of animal cells*. New York: Wiley-Liss.

FROSTELL, C.G., BLOMQVIST, H., HEDENSTIERNA, G., LUNDBERG, J. & ZAPOL, W.M. (1993) Inhaled nitric oxide selectively reverses human hypoxic pulmonary vasoconstriction without causing systemic vasodilation. *Anesthesiology* **78**, 427-435.

FUJIMOTO, K., SAKAI, A., YOSHIKAWA, S., SHINOZAKI, S., MATSUZAWA, Y., KUBO, K., KOBAYASHI, T., UEDA, G., SEKIGUCHI, M. & VOELKEL, N.F. (1990) Effect of cyclic guanosine monophosphate on hypoxic and angiotensin- II-induced pulmonary vasoconstriction. *Lung* **168**, 333-343.

FURCHGOTT, R.F. & ZADAWZKI, J.V. (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288**, 373-376.

FURCHGOTT, R.F. (1983) Role of endothelium in responses of vascular smooth muscle. *Circulation Research* **53**, 557-573.

FURCHGOTT, R.F. (1984) The role of endothelium in the responses of vascular smooth muscle to drugs. *Annual Review of Pharmacological Toxicology* **24**, 175-197.

FURCI, L., FITZGERALD, D.J. & FITZGERALD, G.A. (1991) Heterogeneity of prostaglandin H_2/thromboxane A_2 receptors: distinct subtypes mediate vascular smooth muscle contraction and platelet aggregation. *Journal of Pharmacology and Experimental Therapeutics* **258**, 74-81.

GERTLER, J.P. & OCASIO, V.H. (1993) Endothelin production by hypoxic human endothelium. *Journal of Vascular Surgery* **18**, 178-182.

GILLESPIE, J.R., TYLER, W.S. & HALL, L.W. (1969) Cardiopulmonary dysfunction in anesthetized, laterally recumbent horses. *American Journal of Veterinary Research* **30**, 61-72.

GLEED, R.D. & DOBSON, A. (1990) Effect of clenbuterol on arterial oxygen tension in the anaesthetised horse. *Research in Veterinary Science* **48**, 331-337.

GLEED, R.D. (1988) Improvement in arterial oxygen tension with change in posture in anaesthetised horses. *Research in Veterinary Science* **44**, 255-259.

GORFIEN, S., SPECTOR, A., DELUCA, D. & WEISS, S. (1993) Growth and physiological functions of vascular endothelial cells in a new serum-free medium (SFM). *Experimental Cellular Research* **206**, 291-301.

GRANDY, J.L., STEFFEY, E.P., HODGSON, D.S. & WOLINER, M.J. (1987) Arterial hypotension and the development of postanesthetic myopathy in halothane-anesthetized horses. *American Journal of Veterinary Research* **48**, 192-197.

GRASER, T. & VANHOUTTE, P.M. (1991) Hypoxic contraction of canine coronary arteries: role of endothelium and cGMP. *American Journal of Physiology* **261**, H1769-H1777.

GRAVEN, K.K., ZIMMERMAN, L.H., DICKSON, E.W., WEINHOUSE, G.L. & FARBER, H.W. (1993) Endothelial cell hypoxia associated proteins are cell and stress specific. *Journal of Cell Physiology* **157**, 544-554.

GREEN, L.C., WAGNER, D.A., GLOGOWSKI, J., SKIPPER, P.L., WISHNOK, J.S. & TANNENBAUM, S.R. (1982) Analysis of nitrate, nitrite and [^{15}N] nitrate in biological fluids. *Analytical Biochemistry* **126**, 131-138.

GREENBERG, B. & KISHIYAMA, S. (1993) Endothelium-dependent and -independent responses to severe hypoxia in rat pulmonary artery. *American Journal of Physiology* **265**, H1712-H1720.

GRUETTER, C.A. & LEMKE, S.M. (1986) Comparison of endothelium-dependent relaxation in bovine intrapulmonary artery and vein by acetylcholine. *Journal of Pharmacology and Experimental Therapeutics* **238(3)**, 1055-1064.

GRYGLEWSKI, R.J., BUNTING, S., MONCADA, S., FLOWER, R.J. & VANE, J.R. (1976) Arterial walls are protected against deposition of platelet thrombi by a substance (prostaglandin X) which they make from prostaglandin endoperoxides. *Prostaglandins* **12**, 685-714.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985) A new generation of Ca indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry* **260**, 3440-3450.

GUIMARAES, S., PAIVA, M.Q. & MOURA, D. (1987) $Alpha_2$-adrenoceptor-mediated responses to so-called selective $alpha_1$-adrenoceptor agonists after partial blockade of $alpha_1$-adrenoceptors. *Archives of Pharmacology* **335**, 397-402.

HAI, C. & MURPHY, R.A. (1989) Ca^{2+}, crossbridge phosphorylation, and contraction. *Annual Review of Physiology* **51**, 285-298.

HALL, L.W. (1979) Oxygenation of pulmonary vein blood in conscious and anaesthetised ponies. *Equine Veterinary Journal* **11**, 71-75.

HALL, L.W. (1984) Cardiovascular and pulmonary effects of recumbency in two conscious ponies. *Equine Veterinary Journal* **16 (2)**, 89-92.

HALL, L.W., GILLESPIE, J.R. & TYLER, W.S. (1968) Alveolar-arterial oxygen tension differences in anaesthetized horses. *British Journal of Anaesthesia* **40**, 560-567.

HARDER, D.R., MADDEN, J.A. & DAWSON, C.A. (1985) Hypoxic induction of Ca^{2+}-dependent action potentials in small pulmonary arteries of the cat. *Journal of Applied Physiology* **59**, 1389-1393.

HASSOUN, P.M., THAPPA, V., LANDMAN, M.J. & FANBURG, B.L. (1992) Endothelin 1: mitogenic activity on pulmonary artery smooth muscle cells and release from hypoxic endothelial cells. *Proceedings of the Society for Experimental Biology and Medicine* **199**, 165-170.

HEWETT, P.W. & MURRAY, J.C. (1993) Human microvessel endothelial cells: isolation, culture and characterization. *In Vitro Cellular Developmental Biology in Animals* **29A**, 823-830.

HICKEY, K.A., RUBANYI, G.M., PAUL, R.J. & HIGHSMITH, R.F. (1985) Characterization of a coronary vasoconstrictor produced by cultured endothelial cells. *American Journal of Physiology* **248**, C550-C556.

HIEDA, H.S. & GOMEZ-SANCHEZ, C.E. (1990) Hypoxia increases endothelin release from bovine endothelial cells in culture, but epinephrine, norepinephrine, serotonin, histamine and angiotensin II do not. *Life Sciences* **47**, 247-251.

HIGGINS, A.J. & LEES, P. (1984) Arachidonic acid metabolites in carrageenin-induced equine inflammatory exudate. *J.Vet.Pharmacol.Ther.* **7**, 65-72.

HIGHSMITH, R.F., AICHHOLZ, D., FITZGERALD, O., PAUL, R., RUBANYI, G.M. & HICKEY, G. (1988) Endothelial cells in culture and production of endothelium-derived constricting factors. In *Relaxing and contracting factors*, ed. VANHOUTTE, P. M. pp. 137-158. Clifton: Humana.

HIGHSMITH, S., BLOEBAUM, P. & SNOWDOWNE, K.W. (1986) Sarcoplasmic reticulum interacts with the Ca^{2+}-indicator precursor fura 2-AM. *Biochemical and Biophysical Research Communications* **138**, 1153-1162.

HIRATA, Y., EMORI, T., EGUCHI, S., KANNO, K., IMAI, T., OHTA, K. & MARUMO, F. (1993) Endothelin receptor subtype B mediates synthesis of nitric oxide by cultured bovine endothelial cells. *Journal of Clinical Investigation* **91**, 1367-1373.

HODGSON, D.S. & DUNLOP, C.I. (1990) General anaesthesia for horses with specific problems. *Veterinary Clinics of North America, Equine Practice* **6**, 625-650.

HOLDEN, W.E. & MCCALL, E. (1984) Hypoxia-induced contractions of porcine pulmonary artery strips depend on intact endothelium. *Experimental Lung Research* **7**, 101-112.

HOOD, D.M., GROSENBAUGH, D.A., MOSTAFA, M.B., MORGAN, S.J. & THOMAS, B.C. (1993) The role of vascular mechanisms in the development of acute equine laminitis. *Journal of Veterinary Internal Medicine* **7**, 228-234.

HORNICKE, H., MEIXNER, R. & POLLMANN, U. (1983) Respiration in exercising horses. In *Equine Exercise Physiology*, eds. SNOW, D. H., PERSSON, S. G. B. & ROSE, R. J. pp. 7-16. Cambridge: Burlington Press.

HOSHINO, Y., MORRISON, K.J. & VANHOUTTE, P.M. (1994) Mechanisms of hypoxic vasoconstriction in the canine isolated pulmonary artery: role of endothelium and sodium pump. *American Journal of Physiology* **267**, L120-L127.

HOSHINO, Y., OBARA, H., KUSUNOKI, M., FUJII, Y. & IWAI, S. (1988) Hypoxic contractile response in isolated human pulmonary artery: role of calcium ion. *Journal of Applied Physiology* **65(6)**, 2468-2474.

HOYER, D., CLARKE, D.E., FOZARD, J.R., HARTIG, P.R., MARTIN, G.R., MYLECHARANE, E.J., SAXENA, P.R. & HUMPHREY, P.P.A. (1994) International union of pharmacology classification of receptors for 5-hydroxytryptamine (serotonin). *Pharmacological Reviews* **46**, 157-203.

HOYER, L.W., DE LOS SANTOS, R.P. & HOYER, J.R. (1973) Antihemophilic factor antigen: localization in endothelial cells by immunofluorescent microscopy. *Journal of Clinical Investigation* **52**, 2737-2744.

IGNARRO, L.J. & KADOWITZ, P.J. (1985) The pharmacological and physiological role of cyclic GMP in vascular smooth muscle relaxation. *Annual Review of Pharmacological Toxicology* **25**, 171-191.

IGNARRO, L.J. (1989) Biological actions and properties of endothelium-derived nitric oxide formed and released from artery and vein. *Circulation Research* **65**, 1-21.

IGNARRO, L.J., BUGA, G.M., WOOD, K.S., BYRNS, R.E. & CHAUDHURI, G. (1987) Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proceedings of National Academy of Science of the United States of America* **84**, 9265-9269.

IGNARRO, L.J., LIPPTON, H., EDWARDS, J.C., BARICOS, W.H., HYMAN, A.L., KADOWITZ, P.J. & GRUETTER, C.A. (1981) Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside, and nitric oxide: evidence for the involvement of s-nitrosothiols as active intermediates. *Journal of Pharmacology and Experimental Therapeutics* **218**, 739-749.

ISHIKAWA, K., IHARA, M., NOGUCHI, K., MASE, T., MINO, N., SAEKI, T., FUKURODA, T., FUKAMI, T., OZAKI, S., NAGASE, T., NISHIKIBE, M. & YANO, M. (1994) Biochemical and pharmacological profile of a potent and selective endothelin B-receptor antagonist, BQ-788. *Proceedings of National Academy of Science of the United States of America* **91**, 4892-4896.

JAFFE, E.A., NACHMAN, R.L., BECKER, C.G. & MINICK, C.R. (1973) Culture of human endothelial cells derived from umbilical veins: identification by morphologic and immunologic criteria. *Journal of Clinical Investigation* **52**, 2745-2756.

JIANG, H., COLBRAN, J.L., FRANCIS, S.H. & CORBIN, J.D. (1992) Direct evidence for cross-activation of cGMP-dependent protein kinase by cAMP in pig coronary arteries. *Journal of Biological Chemistry* **267**, 1015-1019.

JIN, N., PACKER, C.S. & RHOADES, R.A. (1992) Pulmonary arterial hypoxic contraction: signal transduction. *American Journal of Physiology* **263**, L73-L78.

JOHNS, R.A., LINDEN, J.M. & PEACH, M.J. (1989) Endothelium-dependent relaxation and cyclic GMP accumulation in rabbit pulmonary artery are selectively impaired by moderate hypoxia. *Circulation Research* **65**, 1508-1515.

JOHNS, R. A., TICHOTSKY, A., MURO, M., SPAETH, J. P., LE CRAS, T. D. & RENGASAMY, A. (1995). Halothane and isoflurane inhibit endothelium-derived relaxing factor-dependent cyclic guanosine monophosphate accumulation in endothelial cell-vascular smooth muscle co-cultures independent of an effect on guanylyl cyclase activation. *Anesthesiology* **83**, 823-834.

JOHNSTON, G.M., TAYLOR, P.M., HOLMES, M.A. & WOOD, J.L.N. (1995) Confidential enquiry of perioperative equine fatalities (CEPEF-1): preliminary results. *Equine Veterinary Journal* **27**, 193-200.

JONES, J.H., LONGWORTH, K.E., LINDHOLM, A., CONLEY, K.E., KARAS, R.H., KAYAR, S.R. & TAYLOR, C.R. (1989) Oxygen transport during exercise in large mammals. I. Adaptive variation in oxygen demand. *Journal of Applied Physiology* **67**, 862-870.

KAKU, S., TANAKA, M., MURAMATSU, M. & OTOMO, S. (1994) Determination of nitrite by high-performance liquid chromatography system with electrochemical detector: measurement of nitric oxide synthase activity in rat cerebellum cytosol. *Biomedical Chromatography* **8**, 14-18.

KHALIL, R.A. & MORGAN, K.G. (1992) Phenylephrine-induced translocation of protein kinace C and shortening of 2 types of vascular cells of the ferret. *Journal of Physiology* **455**, 585-599.

KITAZAWA, T., MASUO, M. & SOMLYO, A.P. (1991) G protein-mediated inhibition of myosin light-chain phosphatase in vascular smooth muscle. *Proceedings of National Academy of Science of the United States of America* **88**, 9307-9310.

KLEIN, L. (1990) Anesthetic complications in the horse. *Veterinary Clinics of North America, Equine Practice* **6**, 665-692.

KLEMM, P., HECKER, M., STOCKHAUSEN, H., WU, C.C. & THIEMERMANN, C. (1995) Inhibition by N-acetyl-5-hydroxytryptamine of nitric oxide synthase expression in cultured cells and in the anaesthetized rat. *British Journal of Pharmacology* **115**, 1175-1181.

KOBAYASHI, S., KITAZAWA, T., SOMLYO, A.V. & SOMLYO, A.P. (1989) Cytosolic heparin inhibits muscarinic and alpha-adrenergic calcium release in smooth muscle. *Journal of Biological Chemistry* **264**, 17997-18004.

KOTERBA, A.M., KOSCH, P.C., BEECH, J. & WHITLOCK, T. (1988) Breathing strategy of the adult horse (*Equus caballus*) at rest. *Journal of Applied Physiology* **64**, 337-346.

KOVITZ, K.L., ALESKOWITCH, T.D., SYLVESTER, J.T. & FLAVAHAN, N.A. (1993) Endothelium-derived contracting and relaxing factors contribute to hypoxic responses of pulmonary arteries. *American Journal of Physiology* **34**, H1139-H1148.

LAMAR, C.H., TUREK, J.J., BOTTOMS, G.D. & FESSLER, J.F. (1986) Equine endothelial cells in vitro. *American Journal of Veterinary Research* **47**, 956-958.

LAVOIE, J.P., PASCOE, J.R. & KUPERSHOEK, C.J. (1995) Partitioning of total pulmonary resistance in horses. *American Journal of Veterinary Research* **56**, 924-929.

LEACH, R.M., ROBERTSON, T.P., TWORT, C.H. & WARD, J.P. (1994) Hypoxic vasoconstriction in rat pulmonary and mesenteric arteries. *American Journal of Physiology* **266**, L223-L231.

LEESON, C.R., LEESON, T.S. & PAPARO, A.A. (1985) *Textbook of Histology*. Philadelphia: W.B.Saunders Company.

LEKEUX, P. (1993) *Pulmonary function in healthy, exercising and diseased animals*. Gent: Flemish Veterinary Journal.

LEVESQUE, L., DRAPEAU, G., GROSE, J.H., RIOUX, F. & MARCEAU, F. (1993) Vascular mode of action of kinin B_1 receptors and development of a cellular model for the investigation of these receptors. *British Journal of Pharmacology* **109**, 1254-1262.

LINCOLN, T.M., CORNWELL, T.L. & TAYLOR, A.E. (1990) cGMP-dependent protein kinase mediates the reduction of Ca^{2+} by cAMP in vascular smooth muscle cells. *American Journal of Physiology* **258**, C399-C407.

LOEB, A.L., LONGNECKER, D.E. & WILLIAMSON, J.R. (1993) Alteration of calcium mobilization in endothelial cells by volatile anesthetics. *Biochemical Pharmacology* **45**, 1137-1142.

LOEB, A.L., O'BRIEN, D.K. & LONGNECKER, D.E. (1994) Halothane inhibits bradykinin-stimulated prostacyclin production in endothelial cells. *Anesthesiology* **81**, 931-938.

LOESCH, A. & BURNSTOCK, G. (1995) Ultrastructural localization of nitric oxide synthase and endothelin in coronary and pulmonary arteries of newborn rats. *Cell & Tissue Research* **279**, 475-483.

LUNN, J.N. & MUSHIN, W.W. (1982) Mortality associated with anaesthesia. *Anaesthesia* **37**, 856

MACLEAN, M.R., CLAYTON, R.A., HILLIS, S.W., MCINTYRE, P.D., PEACOCK, A.J. & TEMPLETON, A.G.B. (1994) 5-HT_1-receptor-mediated vasoconstriction in bovine isolated pulmonary arteries: influences of vascular endothelium and tone. *Pulmonary Pharmacology* **7**, 65-72.

MACLEAN, M.R., CLAYTON, R.A., TEMPLETON, A.G.B. & MORECROFT, I. (1996) Evidence for 5-HT_1-like receptor-mediated vasoconstriction in human pulmonary artery. *British Journal of Pharmacology* **119**, 277-282.

MACLEAN, M.R., MCCULLOCH, K.M. & BAIRD, M. (1994) Endothelin ET_A- and ET_B-receptor-mediated vasoconstriction in rat pulmonary arteries and arterioles. *Journal of Cardiovascular Pharmacology* **23**, 838-845.

MACLEAN, M.R., MCCULLOCH, K.M. & MCGRATH, J.C. (1993) Influences of the endothelium and hypoxia on $alpha_1$- and $alpha_2$-adrenoceptor-mediated responses in the rabbit isolated pulmonary artery. *British Journal of Pharmacology* **108**, 155-161.

MACLEAN, M.R., MCCULLOCH, K.M., MACMILLAN, J.B. & MCGRATH, J.C. (1993) Influences of the endothelium and hypoxia on neurogenic transmission in the isolated pulmonary artery of the rabbit. *British Journal of Pharmacology* **108**, 150-154.

MADDEN, J.A., DAWSON, C.A. & HARDER, D.R. (1985) Hypoxia-induced activation in small isolated pulmonary arteries from the cat. *Journal of Applied Physiology* **59**, 113-118.

MADDEN, M.C., VENDER, R.L. & FRIEDMAN, M. (1986) Effect of hypoxia on prostacyclin production in cultured pulmonary artery endothelium. *Prostaglandins* **31**, 1049-1062.

MAGNUSSON, A., HALLDORSSON, H., THORGEIRSSON, G. & KJELD, M. (1994) Endothelin secretion is regulated by cyclic AMP and phosphatase 2A in endothelial cells. *Journal of Cell Physiology* **161**, 429-434.

MALEK, A. & IZUMO, S. (1992) Physiological fluid shear stress causes downregulation of endothelin-1 mRNA in bovine aortic endothelium. *American Journal of Physiology* **263**, C389-C396.

MARSDEN, P.A., DANTHULURI, N.R., BRENNER, B.M., BALLERMANN, B.J. & BROCK, T.A. (1989) Endothelin action on vascular smooth muscle involves inositol triphosphate and calcium mobilization. *Biochemical and Biophysical Research Communications* **158**, 86-93.

MARSHALL, B.E., MARSHALL, C., MAGNO, M., LILAGAN, P. & PIETRA, G.G. (1991) Influence of bronchial arterial PO_2 on pulmonary vascular resistance. *Journal of Applied Physiology* **70**, 405-415.

MARSHALL, C. & MARSHALL, B.E. (1983) Site and sensitivity for stimulation of hypoxic pulmonary vasoconstriction. *Journal of Applied Physiology* **55**, 711-716.

MARSTON, S.B. & SMITH, C.W.J. (1985) The thin filaments of smooth muscle. *Journal of Muscle Research and Cell Motility* **6**, 669-708.

MARTIN, L.D., BARNES, S.D. & WETZEL, R.C. (1992) Acute hypoxia alters eicosanoid production of perfused pulmonary artery endothelial cells in culture. *Prostaglandins* **43**, 371-382.

MCCALL, T. & VALLANCE, P. (1992) Nitric oxide takes centre-stage with newly defined roles. *Trends in Pharmacological Science* **13**, 1-6.

MCCORMACK, D.G., BARNES, P.J. & EVANS, T.W. (1989) Purinoceptors in the pulmonary circulation of the rat and their role in hypoxic vasoconstriction. *British Journal of Pharmacology* **98**, 367-372.

MCDONELL, W.N. & HALL, L.W. (1974) Functional residual capacity in conscious and anaesthetized horses. *British Journal of Anaesthesia* **46**, 802-803.

MCDONELL, W.N., HALL, L.W. & JEFFCOTT, L.B. (1979) Radiographic evidence of impaired pulmonary function in laterally recumbent anaesthetised horses. *Equine Veterinary Journal* **11**, 24-32.

MCGRATH, J.C. (1985) Alpha-adrenoreceptor agonists and the Ca^{2+}-dependence of smooth muscle contraction: evidence for subtypes of receptors or for agonist-dependent differences in the agonist-receptor interaction? *Clinical Science* **68**, 55s-63s.

MCGRATH, J.C., BROWN, C.M. & WILSON, V.G. (1989) Alpha-adrenoceptors: a critical review. *Medical Research Reviews* **9**, 407-533.

MCLAUGHLIN, R.F., TYLER, W.S. & CANADA, R.O. (1961) A study of the subgross pulmonary anatomy in various animals. *American Journal of Anatomy* **108**, 149-165.

MCMURTRY, I.F., PETRUN, M.D. & REEVES, J.T. (1978) Lungs from chronically hypoxic rats have decreased pressor response to acute hypoxia. *American Journal of Physiology* **235**, H104-H109.

MITCHELL, B. & LITTLEJOHN, A. (1974) The effects of anaesthesia and posture on the exchange of respiratory gases and on the heart rate. *Equine Veterinary Journal* **6**, 177-179.

MONCADA, S. & VANE, J.R. (1979) Pharmacology and endogenous roles of prostaglandin and endoperoxidases, thromboxane A_2 and prostacyclin. *Pharmacological Reviews* **30**, 293-331.

MONCADA, S., GRYGLEWSKI, R.J., BUNTING, S. & VANE, J.R. (1976) An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature* **263**, 663-665.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacological Reviews* **43**, 109-142.

MOORE, J.N. (1994) Endotoxaemia: recent advances in pathophysiology and treatment. *Journal of Veterinary Anaesthesia* **21**, 77-81.

MORRIS, B.J., SIMPSON, C.S., MUNDELL, S., MACEACHERN, K.E., JOHNSTON, H.M. & NOLAN, A.M. (1997) Dynamic changes in NADPH-diaphorase staining reflect activity of nitric oxide synthase: evidence for a dopaminergic regulation of striatal nitric oxide release. *Neuropharmacology* **In Press.**

MUIR, W.W. (1991) Complications: induction, maintenance, and recovery phases of anesthesia. In *Equine anesthesia: monitoring and emergency therapy*, eds. MUIR, W. W. & HUBBELL, J. A. E. pp. 419-443. St. Louis: Mosby Year Book, Inc.

MURRAY, T.R., CHEN, L., MARSHALL, B.E. & MACARAK, E.J. (1990) Hypoxic contraction of cultured pulmonary vascular smooth muscle cells. *American Journal of Respiratory Cells and Molecular Biology* **3**, 457-465.

NAKAMICHI, K., IHARA, M., KOBAYASHI, M., SAEKI, T., ISHIKAWA, K. & YANO, M. (1992) Different distribution of endothelin receptor subtypes in pulmonary tissue revealed by the novel selective ligands BQ-123 and [$Ala^{1,3,11,15}$]ET-1. *Biochemical and Biophysical Research Communications* **182**, 144-150.

NAKAMURA, S., NARUSE, M., NARUSE, K., DEMURA, H. & UEMURA, H. (1990) Immunocytochemical localization of endothelin in cultured bovine endothelial cells. *Biochemistry* **94**, 475-477.

NAKASHIMA, M. & VANHOUTTE, P.M. (1993) Endothelin-1 and -3 cause endothelium-dependent hyperpolarization in the rat mesenteric artery. *American Journal of Physiology* **265**, H2137-H2141.

NELSON, M.T., STANDEN, N.B., BRAYDEN, J.E. & WORLEY, J.F. (1988) Noradrenaline contracts arteries by activating voltage-dependent calcium channels. *Nature* **336**, 382-385.

NISHIZUKA, Y. (1984) The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature* **308**, 693-698.

NYMAN, G. & HEDENSTIERNA, G. (1989) Ventilation-perfusion relationships in the anaesthetised horse. *Equine Veterinary Journal* **21**, 274-281.

NYMAN, G., FROSTELL, C., HEDENSTIERNA, G., FUNKQUIST, B., KVART, C. & BLOMQVIST, H. (1987) Selective mechanical ventilation of dependent lung regions in the anaesthetized horse in dorsal recumbency. *British Journal of Anaesthesia* **59**, 1027-1034.

OBI, T., KABEYAMA, A. & NISHIO, A. (1994) Characterization of muscarinic receptor subtype mediating contraction and relaxation in equine coronary artery in vitro. *Journal of Veterinary Pharmacology and Therapeutics* **17**, 226-231.

OKADA, T. (1991) Hypoxia-induced change in prostanoids production and coronary flow in isolated rat heart. *Journal of Molecular and Cellular Cardiology* **23**, 939-948.

OSIPENKO, O.N., EVANS, A.M. & GURNEY, A.M. (1997) Regulation of the resting potential of rabbit pulmonary artery myocytes by a low threshold, O_2-sensing potassium current. *British Journal of Pharmacology* **120**, 1461-1470.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987) Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **327**, 524-526.

PANEK, B.L., MAJOR, T.C., HINGORANI, G.P., DOHERTY, A.M., TAYLOR, D.G. & RAPUNDALO, S.T. (1992) Endothelin and structurally related analogues distinguish between endothelin receptor subtypes. *Biochemical and Biophysical Research Communications* **183**, 1566-1571.

PARUMS, D.V., CORDELL, J.L., MICKLEM, K., HERYET, A.R., GATTER, K.C. & MASON, D.Y. (1990) JC70: a new monoclonal antibody that detects vascular endothelium associated antigen on routinely processed tissue sections. *Journal of Clinical Pathology* **43**, 752-757.

PEARSON, P.J. & VANHOUTTE, P.M. (1993) Vasodilator and vasoconstrictor substances produced by the endothelium. *Reviews in Physiological and Biochemical Pharmacology* **122**, 1-67.

PELLETIER, N. & LEITH, D.E. (1993) Cardiac output but not high pulmonary artery pressure varies with FIO_2 in exercising horses. *Respiration Physiology* **91**, 83-97.

PETERSON, G.L. (1977) A simplification of the protein assay method of Lowry et al which is more generally applicable. *Analytical Biochemistry* **83**, 346-356.

POST, J.M., GELBAND, C.H. & HUME, J.R. (1995) $[Ca^{2+}]_i$ inhibition of K^+ channels in canine pulmonary artery. Novel mechanism for hypoxia-induced membrane depolarization. *Circulation Research* **77**, 131-139.

PUTNEY, J. W. (1986). A model for receptor-regulated calcium entry. *Cell Calcium* **7**, 1-12.

RABINOVITCH, M., BOUDREAU, N., VELLA, G., COCEANI, F. & OLLEY, P.M. (1989) Oxygen-related prostaglandin synthesis in ductus-arteriosus and other vascular cells. *Pediatric Research* **26**, 330-335.

RANG, H.P. & DALE, M.M. (1991) *Pharmacology*. Edinburgh: Churchill Livingstone.

RENGASAMY, A. & JOHNS, R.A. (1991) Characterization of endothelium-derived relaxing factor/nitric oxide synthase from bovine cerebellum and mechanism of modulation by high and low oxygen tensions. *Journal of Pharmacology and Experimental Therapeutics* **259**, 310-316.

RHODIN, J.A. (1980) Architecture of the vessel wall. In *Handbook of Physiology, The Cardiovascular System*, eds. BOHR, D. F., SOMYLO, A. P. & SPARKS, JR. pp. 1-31. Bethesda,MD: American Physiological Society.

RICHARDS, J.M., GIBSON, I.F. & MARTIN, W. (1991) Effects of hypoxia and metabolic inhibitors on production of prostacyclin and endothelium-derived relaxing factor by pig aortic endothelial cells. *British Journal of Pharmacology* **102**, 203-209.

RIEZEBOS, J., WATTS, I.S. & VALLANCE, P.J.T. (1994) Endothelin receptors mediating functional responses in human small arteries and veins. *British Journal of Pharmacology* **111**, 609-615.

ROBERTSON, B.E., PATERSON, D.J., PEERS, C. & NYE, P.C. (1989) Tolbutamide reverses hypoxic pulmonary vasoconstriction in isolated rat lungs. *Quarterly Journal of Experimental Physiology* **74**, 959-962.

ROBERTSON, T.P., AARONSON, P.I. & WARD, J.P. (1995) Hypoxic vasoconstriction and intracellular Ca^{2+} in pulmonary arteries: evidence for PKC-independent Ca^{2+} sensitization. *American Journal of Physiology* **268**, H301-H307.

ROBINSON, N.E. (1982) Some functional consequences of species differences in lung anatomy. *Advances in Veterinary Science and Comparative Medicine* **26**, 1-33.

ROBINSON, N.E. (1991) The respiratory system. In *Equine anesthesia: monitoring and emergency therapy*, eds. MUIR, W. W. & HUBBELL, J. A. E. pp. 7-38. St Louis: Mosby Year Book.

ROBINSON, N.E. (1992) Pulmonary blood flow. In *Textbook of Veterinary Physiology*, ed. CUNNINGHAM, J. G. pp. 547-554. Philadelphia: W.B. Saunders.

RODMAN, D.M., YAMAGUCHI, T., HASUNUMA, K., O'BRIEN, R.F. & MCMURTRY, I.F. (1990) Effects of hypoxia on endothelium-dependent relaxation of rat pulmonary artery. *American Journal of Physiology* **258**, L207-L214.

RODMAN, D.M., YAMAGUCHI, T., O'BRIEN, R.F. & MCMURTRY, I.F. (1989) Hypoxic contraction of isolated rat pulmonary artery. *Journal of Pharmacology and Experimental Therapeutics* **248**, 952-959.

RUBANYI, G.M. & VANHOUTTE, P.M. (1985) Hypoxia releases a vasoconstrictor substance from the canine vascular endothelium. *Journal of Physiology* **364**, 45-56.

RUDOLPH, A.M. & YUAN, S. (1966) Response of the pulmonary vasculature to hypoxia and H^+ ion concentration changes. *Journal of Clinical Investigation* **45**, 399-411.

RUGH, K.S., GARNER, H.E., HATFIELD, D.G. & HERROLD, D. (1984) Arterial oxygen and carbon dioxide tensions in conscious laterally recumbent ponies. *Equine Veterinary Journal* **16**, 185-188.

RYAN, U.S., CLEMENTS, E., HABLISTON, D. & RYAN, J.W. (1978) Isolation and culture of pulmonary artery endothelial cells. *Tissue and Cell* **10 (3)**, 535-554.

SAIJONMAA, O., RISTIMAKI, A. & FYHRQUIST, F. (1990) Atrial natriuretic peptide, nitroglycerin and nitroprusside reduce basal and stimulated endothelin production from cultured endothelial cells. *Biochemical and Biophysical Research Communications* **173**, 514-520.

SAKATA, K., OZAKI, H., KWON, S.-C. & KARAKI, H. (1989) Effects of endothelin on the mechanical activity and cytosolic calcium levels of various types of smooth muscle. *British Journal of Pharmacology* **98**, 483-492.

SAKURAI, T. (1992) Molecular characterizations of endothelin receptors. *Trends in Pharmacological Science* **13**, 103-108.

SAKURAI, T., YANAGISAWA, M., TAKUWA, Y., MIYAZAKI, H., KIMURA, S., GOTO, K. & MASAKI, T. (1990) Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature* **348**, 732-735.

SCHMIDT, H.H.H.W. (1995) Determination of nitric oxide via measurement of nitrite and nitrate in culture media. *Biochemica* **2**, 30

SCHOTT, C.A., GRAY, G.A. & STOCLET, J.C. (1993) Dependence of endotoxin-induced vascular hyporeactivity on extracellular L-arginine. *British Journal of Pharmacology* **108**, 38-43.

SCHROR, K. (1993) The effect of prostaglandins and thromboxane A_2 on coronary vessel tone - mechanisms of action and therapeutic implications. *European Heart Journal* **14**, 34-41.

SEDMAK, J.J. & GROSSBERG, S.E. (1977) A rapid, sensitive, and versatile assay for protein using Coomassie Brilliant Blue G250. *Analytical Biochemistry* **79**, 544-552.

SHAWLEY, R.V. & MANDSAGER, R.E. (1990) Clinical use of positive-pressure ventilation in the horse. *Veterinary Clinics of North America, Equine Practice* **6**, 575-585.

SIME, A., NOLAN, A.M. & MCKELLAR, Q.A. (1993) Studies on the role of endothelin on equine smooth muscle in vitro. *Proceedings of the 32nd International Union of the Physiological Society* (Abstract)

SIMONEAU, C., THURINGER, D., CAI, S., GARNEAU, L., BLAISE, G. & SAUVE, R. (1996) Effects of halothane and isoflurane on bradykinin-evoked Ca^{2+} influx in bovine aortic endothelial cells. *Anesthesiology* **85**, 366-379.

SIROIS, E., COTE, M.F. & DOILLON, C.J. (1993) Growth factors and biological supports for endothelial cell lining: in vitro study. *The International Journal of Artificial Organs* **16**, 609-619.

SMITH, J.M., MEINKOTH, J.H., HOCHSTATTER, T. & MEYERS, K.M. (1996) Differential distribution of von Willebrand factor in canine vascular endothelium. *American Journal of Veterinary Research* **57**, 750-755.

SOMA, L.R. (1980) Equine anesthesia: causes of reduced oxygen tension and increased carbon dioxide tensions. *The Compendium on Continuing Education* **2**, S57-S63.

SOMLYO, A.P. & SOMLYO, A.V. (1968) Vascular smooth muscle. *Pharmacological Reviews* **20**, 197-272.

SOMLYO, A.P. & SOMLYO, A.V. (1990) Flash photolysis studies of excitation-contraction coupling, regulation, and contraction in smooth muscle. *Annual Review of Physiology* **52**, 857-874.

SOMLYO, A.P. & SOMLYO, A.V. (1994) Signal transduction and regulation in smooth muscle. *Nature* **372**, 231-236.

SOMLYO, A.P., WALKER, J.W., GOLDMAN, Y.E., TRENTHAM, D.R., KOBAYASHI, S., KITAZAWA, T. & SOMLYO, A.V. (1988) Inositol trisphosphate, calcium and muscle contraction. *Philosophical Transactions of the Royal Society* **320**, 399-414.

STADDON, G.E. & WEAVER, B.M.Q. (1981) Regional pulmonary perfusion in horses: a comparison between anaesthetised and conscious standing animals. *Research in Veterinary Science* **30**, 44-48.

STAUB, N.C. (1985) Site of hypoxic pulmonary vasoconstriction. *Chest* **88**, 240s-245s.

STEFFEY, E.P. & HOWLAND, D. (1978) Cardiovascular effects of halothane in the horse. *American Journal of Veterinary Research* **39,** 611-615.

STEFFEY, E.P. (1991) Inhalation anesthetics and gases. In *Equine anesthesia: monitoring and emergency therapy*, eds. MUIR, W. W. & HUBBELL, J. A. E. pp. 352-379. St Louis: Mosby Year Book.

STOLK, P.W.T. (1982) The effects of anaesthesia on pulmonary blood flow in the horse. *Proceedings of the 1st International Congress of Veterinary Anaesthesia* 119-129.

SUMNER, M.J. & HUMPHREY, P.P.A. (1990) Sumatriptan (GR43175) inhibits cyclic-AMP accumulation in dog isolated saphenous vein. *British Journal of Pharmacology* **99**, 219-220.

SUNG, Y.J., HOTCHKISS, J.H., AUSTIC, R.E. & DIETERT, R.R. (1992) Direct measurement of nitric oxide in headspace gas produced by a chicken macrophage cell line in a closed culture system. *Biochemical and Biophysical Research Communications* **184**, 36-42.

SYLVESTER, J.T., HARABIN, A.L., PEAKE, M.D. & FRANK, R.S. (1980) Vasodilator and constrictor responses to hypoxia in isolated pig lungs. *Journal of Applied Physiology* **49**, 820-825.

TALLARIDA, R.J. & JACOB, L.S. (1979) *The dose-response relation in pharmacology*. New York: Springer-Verlag.

TAYLOR, P.M. (1984) Risks of recumbency in the anaesthetised horse. *Equine Veterinary Journal* **16**, 77-78.

TOD, M.L. & RUBIN, L.J. (1992) Pulmonary vasculature responses to thromboxane and prostacyclin. *Journal of Applied Physiology* **73**, 2717-2719.

TOMASIC, M. (1996) Evidence suggesting a role for a-dendrotoxin sensitive potassium channels and cGMP in the hypoxic contractile response of equine pulmonary arteries. *Anesthesiology* **85**, A570(Abstract)

TRIM, C.M. & MASON, J. (1973) Post-anaesthetic forelimb lameness in horses. *Equine Veterinary Journal* **5**, 71-76.

TSENG, C.M., MCGEADY, M., PRIVETT, T., DUNN, A. & SYLVESTER, J.T. (1990) Does leukotriene C4 mediate hypoxic vasoconstriction in isolated ferret lungs? *Journal of Applied Physiology* **68**, 253-259.

TSUCHIDA, S., HIRAOKA, M., SUDO, M., KIGOSHI, S. & MURAMATSU, I. (1994) Attenuation of sodium nitroprusside responses after prolonged incubation of rat aorta with endotoxin. *American Journal of Physiology* **267**, H2305-H2310.

TUCKER, A., MCMURTRY, I.F., REEVES, J.T., ALEXANDER, A.F., WILL, D.H. & GROVER, R.F. (1975) Lung vascular smooth muscle as a determinant of pulmonary hypertension at high altitude. *American Journal of Physiology* **228**, 762-767.

TUREK, J.J., LAMAR, C.H., FESSLER, J.F. & BOTTOMS, G.D. (1987) Ultrastructure of equine endothelial cells exposed to endotoxin and flunixin meglumine and equine neutrophils. *American Journal of Veterinary Research* **48 (9)**, 1363-1366.

TYLER, W.S., GILLESPIE, J.R. & NOWELL, J.A. (1971) Symposium on pulmonary and cardiac function. I Modern Functional Morphology of the Equine Lung. *Equine Veterinary Journal* **3**, 84-94.

VALTSCHANOFF, J.G., WEINBERG, R.J. & RUSTIONI, A. (1992) NADPH diaphorase in the spinal cord of rats. *Journal of Comparative Neurology* **321**, 209-222.

VANE, J.R. & BOTTING, R.M. (1993) Formation by the endothelium of prostacyclin, nitric oxide and endothelin. *Journal of Lipid Mediators* **6**, 395-404.

VANHOUTTE, P.M. (1988) The endothelium - modulator of vascular smooth-muscle tone. *New England Journal of Medicine* **319**, 512-513.

VOELKEL, N.F. (1986) Mechanisms of hypoxic pulmonary vasoconstriction. *American Review of Respiratory Disease* **133**, 1186-1195.

VON EULER, U.S. & LILJESTRAND, G. (1946) Observations on the pulmonary arterial blood pressure in the cat. *Acta Physiologica Scandinavica* **12**, 301-320.

WAGNER, P.D. (1995) Determinants of VO_{2max}: man vs horse. *Journal of Equine Veterinary Science* **15**, 398-404.

WAGNER, P.D., GALE, G.E., MOON, R.E., TORRE-BUENO, J.R., STOLP, B.W. & SALTZMAN, H.A. (1986) Pulmonary gas exchange in humans exercising at sea level and simulated altitude. *Journal of Applied Physiology* **61**, 260-270.

WAGNER, P.D., GILLESPIE, J.R., LANDGREN, G.L., FEDDE, M.R., JONES, B.W., DEBOWES, R.M., PIESCHL, R.L. & ERICKSON, H.H. (1989) Mechanisms of exercise-induced hypoxemia in horses. *Journal of Applied Physiology* **66**, 1227-1233.

WALSH, M. P., ANDREA, J. E., ALLEN, B. G., CLEMENTCHOMIENNE, O., COLLINS, E. M. & MORGAN, K. G. (1994). Smooth-muscle protein-kinase C-1. *Canadian Journal of Physiology and Pharmacology* **72**, 1392-1399.

WARREN, J.B., MALTBY, N.H., MACCORMACK, D. & BARNES, P.J. (1989) Pulmonary endothelium-derived relaxing factor is impaired in hypoxia. *Clinical Science* **77**, 671-676.

WATANABE, T.X., ITAHARA, Y., NAKAJIMA, K., KUMAGAYE, S.I., KIMURA, T. & SAKAKIBARA, S. (1991) The biological activity of endothelin-1 analogues in three different assay systems. *Journal of Cardiovascular Pharmacology* **17**, S5-S9.

WEISS, D.J., GEOR, R.J., JOHNSTON, G. & TRENT, A.M. (1994) Microvascular thrombosis associated with onset of acute laminitis in ponies. *American Journal of Veterinary Research* **55**, 606-612.

WEST, J.B. (1977a) *Regional differences in the lung*. New York: Academic Press, Inc.

WEST, J.B. (1977b) State of the art: ventilation-perfusion relationships. *American Review of Respiratory Disease* **116**, 919-943.

WEST, J.B. (1982) Ventilation-perfusion relationships. In *Scientific foundations of anaesthesia*, eds. SCURR, C. & FELDMAN, S. pp. 217-222. Bath: Pitman.

WEST, J.B. (1990) *Respiratory physiology - the essentials*. Baltimore, MD USA: Williams & Wilkins.

WEST, J.B., DOLLERY, C.T. & NAIMARK, A. (1964) Distribution of blood flow in isolated lungs; relation to vascular and alveolar pressures. *Journal of Applied Physiology* **19**, 713-724.

WIENER, C.M., BANTA, M.R., DOWLESS, M.S., FLAVAHAN, N.A. & SYLVESTER, J.T. (1995) Mechanisms of hypoxic vasodilation in ferret pulmonary arteries. *American Journal of Physiology* **269**, L351-L357.

WINDER, S. & WALSH, M. (1990) Inhibition of the actomyosin MgATPase by chicken gizzard calponin. *Frontiers in smooth muscle research* 141-148.

YANAGISAWA, M., KURIHARA, H., KIMURA, S., TOMOBE, Y., KOBAYASHI, M., MITSUI, Y., YAZAKI, Y., GOTO, K. & MASAKI, T. (1988) A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* **332**, 411-415.

YOSHIZUMI, M., KURIHARA, H., SUGIYMA, T., TAKAKU, F., YANAGISAWA, M., MASAKI, T. & YAZAKI, Y. (1989) Hemodynamic shear stress stimulates endothelin production by cultured endothelial cells. *Biochemical and Biophysical Research Communications* **161**, 859-864.

YOUNG, S.S. & TAYLOR, P.M. (1993) Factors influencing the outcome of equine anaesthesia: a review of 1,314 cases. *Equine Veterinary Journal* **25 (2)**, 147-151.

YUAN, X., GOLDMAN, W.F., TOD, M.L., RUBIN, L.J. & BLAUSTEIN, M.P. (1993) Hypoxia reduces potassium currents in cultured rat pulmonary but not mesenteric arterial myocytes. *American Journal of Physiology* **264 (2 Pt 1)**, L116-L123.

ZHANG, F., WOODMANSEY, P.A. & MORICE, A.H. (1995) Acute hypoxic vasoconstriction in isolated rat small and large pulmonary arteries. *Physiological Research* **44**, 7-18.

ZUO, Z., TICHOTSKY, A. & JOHNS, R.A. (1996) Halothane and isoflurane inhibit vasodilation due to constitutive but not inducible nitric oxide synthase. *Anesthesiology* **84**, 1156-1165.