Endothelin-Receptor Mediated Responses In Pulmonary Resistance Arteries: Effect of Developmental Age and Left Ventricular Dysfunction.

by

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To my Mum and Dad, Lorraine and Thomas

With the greatest gratitude for their boundless love and support

Abstract

(1) The potent vasoconstrictor endothelin-1 (ET-1) is thought to regulate pulmonary blood flow and play a role in the aetiology of pulmonary hypertension (PHT), as well as in the transition of the pulmonary circulation from fetal to neonatal life. Responses to ET-1, and the receptor subtypes involved, were studied in isolated pulmonary resistance arteries (PRAs) from a rabbit coronary ligation model of left ventricular dysfunction (LVD), and also from fetal and neonatal rabbits.

(2) The rabbit coronary ligation model of LVD displayed right ventricular hypertrophy and significant increase in lung weight in animals with coronary artery ligation for 8 weeks compared to age matched sham-operated animals. Also consistent with the development of PHT was significant structural alteration demonstrated in the pulmonary vasculature of the LVD group animals. Small muscular pulmonary arteries were studied 8, 16 and 32 weeks after coronary artery ligation or sham operation.

(3) In the rabbit coronary-ligation model, investigation of ET-receptor mediated responses showed an agonist potency profile, according to pEC_{50} values, of SXS6c > ET-3 = ET-1 in all PRAs from all 8, 16 and 32 week procedure groups. This is indicative of a predominant role of contractile ET_B receptor subtypes in these vessels.

(4) ET receptor subtypes in this preparation were examined further with the use of several selective antagonists. The results demonstrated a biphasic response to ET-1 in all vessels. In sham-operated rabbit PRAs, the shallow component of the response at lower ET-1 concentrations was resistant to the effects of the non-selective ET_A/ET_B receptor antagonist SB209670 but sensitive to BQ788, a selective ET_B receptor antagonist. The steeper component of the ET-1 response, observed at higher peptide concentrations, was resistant to BQ788 but sensitive to SB209670. These differential effects of the antagonists may provide evidence for a heterogeneous population of ET_B-like-receptors. Furthermore, competition radioligand binding studies in rabbit pulmonary artery membranes provided a best fit for a two-site model, thus indicating the existence of two distinct ET receptor populations in this preparation. K_i values of 6.43 $\times 10^{-14}$M and 4.43

$x10^{-10}$M were calculated for the first ultra-high affinity site and the second high affinity site respectively.

In PRAs from 8 week coronary-ligated animals, responses to ET-1 concentrations less than 1nM were potentiated in the presence of BQ788 and SB209670. This suggests that, with the development of PHT secondary to LVD, the BQ788-sensitive-receptor-mediated response has changed in that it is now potentiated by both BQ788 and SB209670; thus may indicate an alteration in the contribution of ET_B receptor subtypes in this model. FR139317 did not inhibit ET-1-induced responses in any PRAs. Indeed this selective ET_A antagonist tended to augment the ET-1 responses; this was apparent at higher ET-1 concentrations in sham-operated rabbit PRAs and was also observed at lower ET-1 concentrations in vessels from LVD animals. Hence ET-1 does not appear to be acting at a typical ET_A receptor and suggests the presence of a putative inhibitory ET_A receptor, which may have a greater role at lower, pathophysiologically relevant ET-1 concentrations in PRAs from coronary-ligated animals.

(5) The role of intracellular cyclic nucleotides was examined in pulmonary arteries from the rabbit coronary ligation model. Basal levels and ET-1-induced levels of cGMP tended to be greater in 8 week coronary-ligated preparations compared to sham-operated vessels. Similarly, basal and forskolin-stimulated levels of cAMP tended to be greater in arteries from LVD animals compared to control preparations. Unfortunately however, due to the variability noted between tissues in individual groups and lack of statistical significance, no definite conclusions can be made regarding the involvement of cAMP and cGMP in this preparation.

(6) The influence of NO on ET-receptor mediated responses was examined in PRAs from the rabbit coronary-ligation model. The nitric oxide synthase inhibitor L-NAME caused a marked potentiation in responses to ET-1 and SXS6c in PRAs from rabbits with LVD but not in vessels from age-matched control animals. The increase in sensitivity to these peptides was most pronounced in the 8 week group, was still evident in vessels from 16 week coronary-ligated rabbits whereas in the 32 week LVD group,

was no longer evident. These results suggest an increase in NO production associated with the development PHT in these animals. This may represent an early compensatory mechanism in response to elevation in pulmonary pressure with LVD. In addition, the potency of SNP, the endothelium-independent relaxatory agent, was significantly reduced following 8 week of coronary-artery ligation. This may be related to reduced NO sensitivity of the vascular smooth muscle in the face of increased NO production.

(7) KCl-induced responses were similar in all isolated pulmonary resistance arteries (PRAs) from 8, 16 and 32 week control and experimental groups, indicated the integrity and contractility of the vascular smooth muscle. 5-HT had a similar potency in stock/sham-operated rabbit PRAs and in preparations from LVD rabbits both after 8 and 32 weeks of coronary artery ligation. However, the magnitude of the maximal response to 5-HT and the 5-HT_{1D} receptor agonist sumatriptan was decreased in the 8 week ligated compared to sham-operated group, whilst the non-selective 5-HT_1 receptor agonist 5-CT evoked similar responses in both groups. These findings indicate a functional population of 5-HT_1-like receptors in this preparation, and a possible alteration in 5-HT_{1D}-like receptors which may occur in the PHT state secondary to LVD in these animals.

(8) The potency of ET receptor agonists was altered with developmental age in isolated rabbit PRAs. In particular, there was a hypersensitivity to contractile ET_B-receptor stimulation in PRAs from newborn rabbits. The potency of ET-1 was similar (pEC_{50} ~8.7) in fetal, 0-24 hour and 4 day old rabbit PRAs. These were all significantly more sensitive to ET-1 than vessels from 7 day old rabbits, which in turn, had similar sensitivity to adult rabbit PRAs (pEC_{50} ~8.0). According to pEC_{50} values, the order of potency for SXS6c was 7 days = 4 days > 0-24 hours > fetal rabbit PRAs. The pEC_{50} for SXS6c in 7 day vessels was ~11.1 and this was markedly greater than that noted in adult PRAs, ~8.6. The magnitude of SXS6c-induced vasoconstriction was significantly greater at 0-24 hours and 4 days after birth, compared to other age points studied.

(9) BQ788 inhibited ET-1-induced responses in PRAs from fetal, 0-24 hour and 4 day old rabbits but, as was noted in the adult vessels from the rabbit coronary ligation model,

was ineffective against ET-1 responses of 7 day old preparations. FR139317, inhibited ET-1 induced responses of 0-24 hour and 4 day old rabbit PRAs, however was ineffective against these responses in fetal and 7 day old vessels, thus similar to that noted in adult PRAs. These differential antagonist results indicate that in the fetus and newborn less than 7 days old, antagonism of either ET_A or ET_B receptor alone is sufficient to inhibit ET-1 mediated responses, whereas with increasing age, blockade of both receptors is necessary. Thus "crosstalk" between ET-receptor subtypes may develop in this preparation later than 4 days after birth.

(10) In rabbit PRAs, the ability of noradrenaline (NA) to evoke vasoconstriction was greatest 0-24 hours after birth, it then decreased during the first week of life and was non-existent in adulthood. Alterations in vasodilator responses to acetylcholine (ACh) were also observed. These increased with the transition from fetal to newborn state and maximal endothelium-dependent relaxatory responses were evident in PRAs from 4 and 7 day old rabbits. In comparison, ACh evoked a vasoconstrictor response in adult PRAs.

(11) The influence of L-NAME on ET-receptor mediated responses was also examined in PRAs from fetal and neonatal rabbits. The most pronounced effects were shown in vessels from 7 day old rabbits, where L-NAME potentiated responses to ET-1. This finding, together with the greatest vasodilator response to ACh being observed in the 7 day vessels, suggests increased NO level in the 7 day old compared to the newborn preparation, and suggests that sensitivity to ET-1 is normally reduced by NO in these neonatal vessels. In comparison, the lack of effect of L-NAME on ET-1- and SXS6c-induced responses of fetal PRAs provides evidence for diminished NO levels in the pulmonary vasculature in fetal compared with neonatal life.

(12) ET-1 was equipotent in human PRAs and in adult rabbit PRAs of a similar internal diameter. The response to lower ET-1 concentrations were inhibited by BQ788, indicating the involvement of vasoconstrictor ET_B receptors. A markedly greater antagonism of ET-1-induced contraction was evident with SB209670. This suggests that ET_{B2}-like receptors of human PRAs are not as sensitive to the prototypical ET_B receptor antagonist BQ788 as they are to the non-selective antagonist SB209670.

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This thesis is entirely my own composition and the experimental work detailed within was undertaken wholly by myself, with the exception of figures 3.3 and 3.4 which were produced in collaboration with Dr. I. Montgomery; and figures 3.5 and 3.6 which were produced in collaboration with Mr. C. Daly.

Signed

Some of the results within this thesis have been published, details of which are given below.

Publications

Full papers

McCulloch, K.M., Docherty, C.C., Morecroft, I. & MacLean, M.R. (1996). ET_B receptors mediate vasoconstriction in human pulmonary resistance arteries. *British Journal of Pharmacology*, **119**, 1125-1130.

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Chapter 1

Introduction and Literature Review

Introduction to Research

Extensive morphological and functional studies of the pulmonary circulation over the last five decades have demonstrated the strikingly complex systems for the regulation of blood flow in the lung and its numerous important non-respiratory functions. This refutes earlier notions which regarded the pulmonary circulation as merely a passive conduit for the purpose of gas exchange of the continual flow of blood. Likewise, the vascular endothelium can no longer be regarded as only a selective barrier. The vast amount of evidence for the endothelium's paracrine and autocrine function, by its ability to release vasoactive agents, highlights a crucial role in the understanding of local mechanisms of vascular tone in both the systemic and pulmonary circulation, and in both health and disease.

My research has focused on the vascular reactivity of pulmonary resistance arteries and in particular on the role of endothelin, a potent endothelium-derived peptide. Of the vast amount of previous *in vitro* studies already carried out on the pulmonary circulation, the majority of these have been on relatively large calibre pulmonary vessels. There has been comparatively little investigation of the small pulmonary arteries yet indirect evidence suggests that they may have an important role in the control of pulmonary vascular tone (e.g. Leach, *et al.*, 1989; Leher & Bevan, 1985). In addition, Leach *et al.* (1992) demonstrated a number of important differences between the responses of large (1-2 mm internal diameter) and small (100-300μm internal diameter) pulmonary arteries to various agents. Thus it may be unwise to extrapolate from results obtained in either large pulmonary artery preparations or in perfused lungs to what may occur in the functionally important resistance arteries. In addition, using a vascular occlusion technique ET-1 has been shown to preferentially increase small artery resistance in the intact lung, and ET-receptor mediated responses have been demonstrated to vary depending on the pulmonary artery size *in vitro* (Barnard, *et al.*, 1991; MacLean, *et al.*, 1994). Hence taking these findings into consideration, characterisation of vascular reactivity in pulmonary resistance arteries is of great importance.

The physiology and pharmacology of endothelin and its role in the pulmonary circulation is reviewed in sections 1.4 and 1.5, respectively, of this introduction. The vascular responsiveness of pulmonary resistance arteries was investigated under two different states; (i) in the transition from fetal to neonatal life and (ii) secondary to left ventricular dysfunction and in the transition to right ventricular dysfunction. Before we can examine the pulmonary vasculature in transitional states it is important to understand the pulmonary circulation which exists under normal conditions.

1.1 The Pulmonary Circulation

The adult pulmonary circulation is characterised as a low pressure, low resistance vascular bed that accommodates the entire cardiac output of the right ventricle to the gas exchanging surface at less than 20% of the systemic vascular pressure. Vasodilators have little or no effect on the pulmonary vascular pressures suggesting that under normal conditions, there is little or no resting vascular tone (Fishman, 1985). The pulmonary vasculature is highly compliant and this prime feature, along with capillary recruitment, ensures maintenance of low pressure even in the face of increased flow rate, such as increased cardiac output during exercise. This ensures that the perfusion pressure of the lungs always remains low and that the thin walled right ventricle is not stressed.

From the extensive studies of the pulmonary circulation of many species including man over numerous decades, a vast amount of information is now available on its various aspects. However, since I primarily examined the pulmonary resistance arteries of the rabbit in this thesis, I will focus this literature review on the pulmonary vasculature of this species where appropriate.

1.1.1 Functions of the Pulmonary Circulation

The continual flow of the cardiac output through the lungs is essential for the purpose of gas exchange. Deoxygenated blood is delivered, via the pulmonary arterial system, to sheets of pulmonary capillaries in the alveoli to allow gas exchange to occur.

The oxygenated blood returns to the left side of the heart via the pulmonary venous system. Moreover, it has become more clear in recent years that the pulmonary vascular bed is the site also of many non-respiratory functions including vital metabolic processes. The pulmonary microvasculature represents an enormous surface area for processing of circulating vasoactive substances (Vane, 1969). Several mediators including noradrenaline (NA), 5-hydroxytryptamine (5-HT) and endothelin (ET) are removed from the blood upon passing through the pulmonary vasculature (Albaster & Bakhle, 1970; Said, 1982; De Nucci, *et al.*, 1988). In addition, pulmonary endothelial cells contain angiotensin converting enzyme, which catalyses formation of AII and degrades bradykinin (Johnson & Erdos, 1977; Said, 1982).

1.1.2 Structure of the pulmonary arterial system

The main pulmonary trunk leaving the right ventricle passes upwards through the heart and rapidly divides into two daughter branches. The left pulmonary artery extends to the hilum of the left lung where it divides into two branches, one passing to each lobe. The right pulmonary artery divides into two branches also, the larger of which extends to the middle and lower lobes, and the smaller to the upper lobe of the right lung. The pulmonary arteries form a rapidly branching structure with daughter branches occurring generally at the distal end of the parent vessels although an artery may have a number of side branches often coming off at right angles to the parent branch. Most of the pulmonary arteries follow the branching pattern of the airways. Singhal *et al.* (1973) estimated 17 orders in the human pulmonary arterial tree. The pulmonary arteries from the main trunk down to a diameter of approximately 1 mm have the structure of typical conducting arteries with a media consisting of several elastic laminae with a small quantity of interposed smooth muscle, a thin intima and a thick adventitia. These have been defined as elastic pulmonary arteries. With continued branching of the human pulmonary arterial tree, the number of elastic fibrils gradually decreases and as the arteries diminish in size the proportion of muscle in the media and its thickness relative to diameter increases (Daly & Hebb, 1966). The predominantly muscular arteries (100

μm-1 mm internal diameter (i.d.)) accompanying the terminal bronchioles have four to six layers of obliquely arranged smooth muscle cells bound by distinct internal and external elastic laminae (Brenner, *et al.*, 1935; Heath & Edwards, 1958). At the level of the respiratory bronchioles, this layer is abruptly reduced and the more distal branches are only partially muscular or non muscular (Heath & Edwards, 1958; Meynick & Reid, 1983). In the human lung, pulmonary arterioles can generally be classed as arterial vessels less than 100μm i.d. (Brenner, 1935). These vessels consist of a single elastic lamina, with extremely sparse smooth muscle (Heath & Edwards, 1958); this is the region most significantly affected in pulmonary hypertension and this is discussed in section 1.3.1 of this introduction. At the level of the respiratory bronchioles, the arterioles give off one branch to each alveolar duct and this divides into an number of smaller arterioles which supply capillaries to the alveolar sac in which the duct terminates.

Considerable variation in the structure of the pulmonary vessels exists not only between species (Kay, 1983) but also within species (Heath & Williams, 1981). In general the structure of the pulmonary arteries is similar in man, monkey and ferret with most other mammals demonstrating more muscular pulmonary arteries (Kay, 1983). In the human pulmonary vasculature, vessels below 100 μm i.d. are normally non muscular, whereas in some animals muscular pulmonary arteries can extend to vessels smaller than 100 μm i.d.. The pulmonary arteries of the rabbit have a consistently thick muscle coat although in some places it is much thicker than in others (Daly & Hebb, 1966).

1.1.3 Pulmonary Pressures and Vascular Resistance

The pulmonary circulation is a low pressure system. In humans, the systolic pulmonary arterial pressure averages approximately 22 mmHg, the diastolic pulmonary arterial pressure approximately 8 mmHg and the mean arterial pressure 13mmHg. This low pressure condition is associated with the structural characteristics of the pulmonary arteries, which have a luminal diameter significantly larger than systemic arteries of the

same size. Despite equal blood flow rates, the mean pulmonary arterial-to-venous pressure difference is about one-tenth that in the systemic circulation, hence the pulmonary vascular resistance (PVR) is approximately one-tenth of the total (systemic) peripheral resistance (Fishman, 1985). In the systemic circulation the arterioles represent 70% of the resistance to blood flow. In contrast an even distribution of PVR throughout the lung is indicated by the similar pressure drop occurring across the arterial, capillary and venous beds. However, evidence from morphometric studies in human lung suggests small muscular pulmonary arteries (100 μm-1 mm i.d.) and arterioles (<100 μm i.d.) as being the major sites of PVR (Horsefield, 1978; Singhal, 1973), particularly in PHT.

1.1.4 Regulation of low pressure vascular tone

The pulmonary circulation is under the control of both active and passive factors (Daly & Hebb, 1966). Active factors alter pulmonary vascular resistance and tone by causing contraction or relaxation of vascular smooth muscle. These factors include autonomic nerves, humoral factors, and gasses. Passive factors include changes in airway and interstitial pressure, gravitational force, and vascular obstruction or recruitment. Alterations in cardiac output and left atrial pressure are also passive factors that influence pulmonary vascular pressure. These variables in particular are of important consideration in the pulmonary circulation in conditions of heart abnormalities; this is discussed in section 1.3.2.2. Passive factors change pulmonary vascular resistance and/or blood flow independently of the changes in vascular tone. I study both active and passive factors. Active regulation by oxygen is studied at birth and the passive effect of left atrial pressure is studied in the rabbit model of left ventricular dysfunction.

1.1.4.1 Neural regulation

The autonomic nervous system may modify the pulmonary blood flow under physiological conditions and may be involved in the pathophysiology of pulmonary

vascular diseases. The pulmonary vasculature is innervated primarily from the anterior and posterior pulmonary plexi (Downing & Lee, 1980). The density and type of innervation appears to be strongly species dependent and varies with the location and size of the vessel (Downing & Lee, 1980, Barnes & Liu, 1995). In general it would appear that extrapulmonary arteries and large muscular pulmonary arteries are more densely innervated, whereas the smaller pulmonary arteries and arterioles tend to have sparse innervation. However, some species including rabbit (Cech & Dolezel, 1967) and man (McLean, 1986), have an extensive and dense adrenergic innervation, which extends to arteries < 70μm and < 60μm outer diameter, respectively. The pulmonary circulation receives little cholinergic input compared with adrenergic innervation. Again there is a marked species-dependent variation in the distribution of cholinergic nerve fibres. Pulmonary arteries of the rabbit are extensively innervated with AChE-positive nerve fibres which extend down to arterioles (Barnes & Liu, 1995). Stimulation of sympathetic (adrenergic) nerve fibres in the pulmonary vasculature mediates predominantly vasoconstrictor responses, whereas stimulation of parasympathetic (cholinergic) nerve fibres mediates vasodilation (Downing & Lee, 1980; Barnes & Liu, 1995). Muscarinic and adrenergic receptors are heterogeneous and the receptor subtypes on vascular smooth muscle seem to have a variable regional distribution, this factor along with the level of pre-existing tone will determine the overall effect, i.e. vasoconstriction or vasodilation (Hyman & Kadowitz, 1988; 1989). The contribution of autonomic innervation to basal tone is uncertain, but may be involved during conditions of stress (Fishman, 1985).

1.1.4.2 Humoral regulation

Many circulating mediators and hormones have effects on pulmonary vascular tone that are mediated via multiple receptors, and vary with species, age, and pre-existing tone (Barnes & Liu, 1995). To name a few agents, in general, AII, thromboxane, and prostaglandins (PGs) D_2, E_2 and $F_{2\alpha}$, are pulmonary vasoconstrictors, whereas ANP and PGs E_1 and I_2 are pulmonary vasodilators. Mediators such as

bradykinin, histamine, substance P, 5-HT, arachadonic acid and endothelins have dual effects on the pulmonary vascular tone, generally causing contraction when vascular tone is low but relaxation when vascular tone is high.

1.1.4.3 Endothelial regulation

Despite intensive research carried out over many years, the factors that maintain low pulmonary vascular tone remain enigmatic. It seems increasingly likely, however, that the state of tone of vascular smooth muscle depends on endothelial factors.

It has been widely demonstrated that the endothelium plays an important role in both short- and long-term regulation of vascular homeostasis as well as in the modulation of several physiological functions, including inflammation, platelet aggregation, fibrinolysis and angiogenesis. The vascular endothelium also participates in the metabolism of blood-borne compounds such as 5-HT. Endothelial cells synthesise and release a great variety of paracrine substances which have vasoactive properties. Among these are the vasoconstrictor factor endothelin, endothelium-derived relaxing factor (EDRF), endothelium-derived hyperpolarising factor (EDHF), arachadonic acid (AA) metabolites, the prostaglandins and leucotrienes. Endothelium-derived factors can have profound influences on vascular tone in both the systemic and pulmonary vasculature (Luscher, *et al.*, 1989). In keeping with the nature of the studies shown in this thesis, I will focus on the role of EDRF and endothelin in particular. This latter endothelial derived peptide and its role in the pulmonary circulation is introduced in sections 1.4 and 1.5.

EDRF is a diffusible factor discovered by Furchgott and Zawadski in 1980 when they demonstrated acetylcholine (ACh)-induced relaxations in rabbit isolated aortic strips were dependent upon an intact vascular endothelium. Ignarro *et al.* (1987a) suggested that EDRF is nitric oxide (NO), based on pharmacological similarities between EDRF and NO generated from either acidified NO_2^- or NO gas. Evidence now strongly supports the identification of EDRF as NO (Moncada, *et al.*, 1991; Nathan, 1992). Many substances including bradykinin, substance P, 5-HT and histamine were

also shown to mediate vasodilation via the release of EDRF (NO) (Luscher, 1989; Furchgott, 1990).

NO is synthesised by a number of cells from the terminal guanidino nitrogen atom of L-arginine in the presence of molecular oxygen to yield L-citrulline and NO, by means of either constitutive (cNOS) or inducible (iNOS) NO synthases (Palmer, *et al.*, 1987; Angus & Cocks, 1089; Palmer & Moncada, 1989). Three isoforms of this enzyme have been identified and named isoform I, II, and III; I and III are constitutively expressed, whilst II is induced by bacterial lipopolysaccaride and cytokines (Marin & Rodriguez-Martinez, 1995). cNOS was first described in vascular endothelium and in neurones but has also been detected in other cell types (Palmer & Moncada, 1989; Mayer, *et al.*, 1989), whilst iNOS is primarily located in macrophages and neutrophils (Marletta, *et al.*, 1988; Moncada, *et al.*, 1991). cNOS is strongly regulated by calcium/calmodulin, is continually expressed, and once activated will produce picomolar amounts of NO until calcium levels decrease (Moncada, *et al.*, 1991). In contrast, iNOS is regulated at the level of transcription, requiring the action of inducers such as cytokines for expression, and is calcium independent (Moncada, *et al.*, 1991). After induction iNOS remains active for 4-24 hours, yielding nanomolar concentrations of NO, 100 fold greater than those of cNOS. The synthesis of NO is stereo-specifically inhibited by various L-arginine analogues which act as competitive inhibitors of NOS, such as L-N^{ω}-arginine methyl ester (L-NAME) (Rees, *et al.*, 1990). In addition to chemical activation, physical forces such as pulsatile flow and sheer stress mediate EDRF production (Tesfamariam & Halpern, 1987; Tesfamariam & Cohen, 1988).

Figure 1.1 schematically summarises endogenous NO production and illustrates the interaction between endothelial cell and smooth muscle cell in effecting vasorelaxation. Upon release NO diffuses freely through the endothelial cell to the smooth muscle where it activates soluble guanylate cyclase, stimulating the production of cyclic 3', 5' guanosine monophosphate (cGMP) from guanosine-5'-triphosphate (GTP) (Ignarro, 1989). cGMP is hydrolysed by cGMP-specific phosphodiesterase enzymes (PDEs) to inactive metabolite 5'GMP (see also section 1.1.4.4). Moreover, recent work

has demonstrated that NO may have a direct effect on the smooth muscle cell, independent of guanylate cyclase activation (Bolotina, *et al.*, 1994).

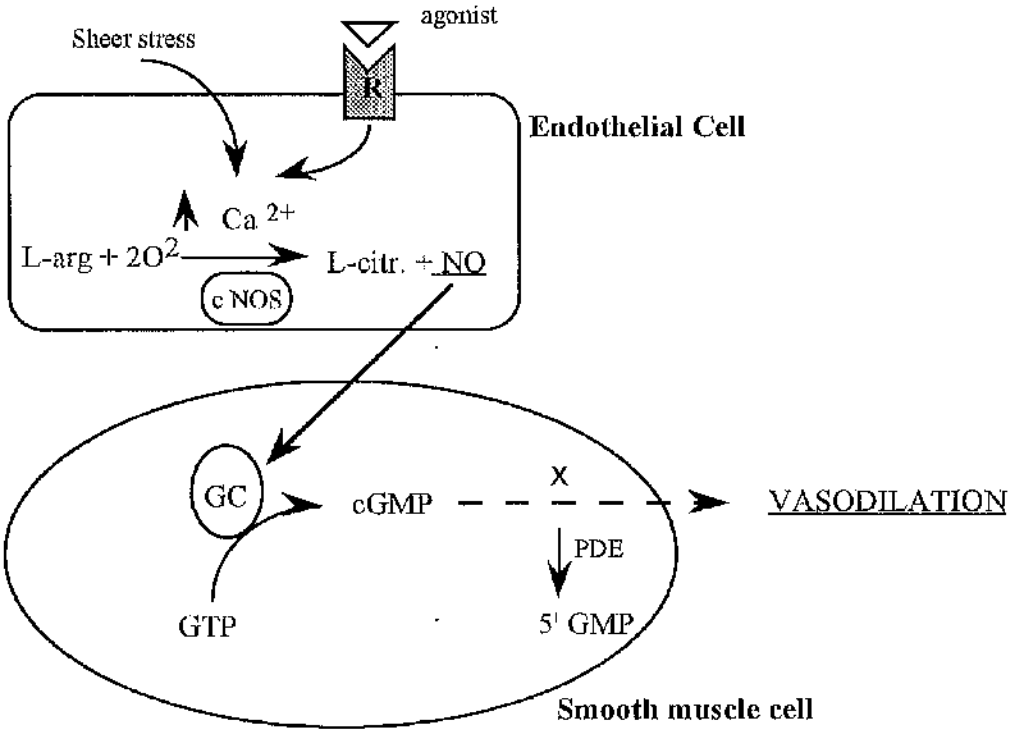


Figure 1.1 Simplified illustration of formation of endogenous NO and mechanism of action. See text for details. R=receptor; L-arg=L-arginine; L-citr.=L-citrilline; cNOS=constitutive NOS; GTP=guanosine-5'-triphosphate; GC=soluble guanylate cyclase enzyme; cGMP=cyclic 3' 5' guanosine monophosphate; PDE=phosphodieterase enzyme(s); X=see section 1.1.4.4 for possible mechanisms of action.

The activity of NO is short lived, approximately 3-30 seconds, and decomposes rapidly to form mixtures of nitrate and nitrite in oxygenated solutions (Tolins, *et al.*, 1991). The short half life of NO in plasma is reflective of the existence of several reactants in the plasma milieu that causes inactivation, including haemoglobin and superoxide anions (Moncada, *et al.*, 1991). In accordance with the interaction between NO and superoxide anion, Rubanyi and Vanhoutte (1986) demonstrated that the half life of NO was prolonged in the presence of superoxide dismutase (SOD), a scavenger of superoxide anions.

1.1.4.4 Intracellular cyclic nucleotides

Cyclic nucleotides are important in the regulation of pulmonary vascular tone. Vasoactive compounds can stimulate or inhibit the activity of the enzymes adenylate cyclase or guanylate cyclase, there by altering intracellular concentrations of cAMP and cGMP, respectively, and these second messengers in turn can regulate pulmonary vascular tone. cGMP is the key second messenger of NO-induced pulmonary vasodilation (see section 1.1.4.3), whereas cAMP plays a central role in the pulmonary vasodilator response to many direct acting vasodilator agents, including β–adrenoceptor agonists, PGI_2 and vasoactive intestinal polypeptide (VIP). Exogenous cGMP and cAMP themselves are potent pulmonary vasodilators (Haynes, *et al.*, 1992; McMahon, *et al.*, 1992; 1993). Ignarro *et al.* (1987b) demonstrated a basal level of cGMP in isolated bovine endothelium-denuded pulmonary artery rings.

The mechanisms underlying cGMP- and cAMP-induced vasodilations are incompletely understood. Nevertheless, evidence suggests that cGMP mediated relaxations are related to activation of protein kinase G, inhibition of IP_3, dephosphorylation of myosin light chain kinase, stimulation of Ca^{2+}-ATPase, opening of K^+ channels, and inhibition of Ca^{2+} influx (Lincoln, 1989). Likewise several mediators may mediate cAMP-induced vasorelaxations, including activation of cAMP-dependent protein kinase, thus resulting in a reduced myosin light chain kinase activity, inhibition of Ca^{2+} influx, stimulation of Ca^{2+} efflux, and opening of Ca^{2+}-dependent K^+ channels (Murray, 1990). Cells inactivate cyclic nucleotides by the action of the enzymes phosphodiesterases (PDEs). At present there are over 30 recognised PDE isoforms which, on the basis of their amino acid sequence, substrate specificity and sensitivity to pharmacological agents, can be classified into seven main families (Beavo, *et al.*, 1994). cAMP and cGMP are degraded by PDE-mediated hydrolysis to the corresponding 5' nucleotide.

1.1.4.5 Regulation by respiratory gasses

The relative composition of the respiratory gas has a profound effect on pulmonary vascular tone, with both hypoxia and hypercapnia inducing pulmonary vasoconstriction (Fishman, 1961). The phenomenon of hypoxic pulmonary vasoconstriction (HPV) was first described by Von Euler and Liljestrand (1947) and is unique to the pulmonary vasculature; a vasodilator response to hypoxia is noted in the systemic circulation. This pulmonary response is of great physiological importance in both fetal and adult life. In the fetus HPV, which exists due to the relatively hypoxic conditions *in utero*, acts to divert blood away from the as yet non functioning lungs (see section 1.2.1). Whilst in the adult lung, HPV functions to redirect circulating blood away from less well ventilated (hypoxic) alveoli toward better ventilated areas of the lung, thus optimising the matching of ventilation and perfusion and maximising arterial oxygenation. Mixed venous PO_2 contributes to HPV however, the main stimulus seems to be alveolar hypoxia (Marshall & Marshall, 1983). Pulmonary arteries of different sizes exert HPV though most investigators have concluded that the small muscular pulmonary arteries are the major site of HPV. For example, Nagasaka *et al.* (1984) demonstrated by direct micropuncture measurements of intrapulmonary vascular pressure, that the predominant site of HPV is the precapillary arteries in the hypoxic cat. Despite many years of investigation, the mechanism(s) responsible for HPV remains controversial, with evidence both for and against (i) reduced release of relaxants from endothelial cells, (ii) via release of mediators from other cells and (iii) by a direct effect of hypoxia on the smooth muscle cell, being reported (Voelkel, 1986).

An acute hypoxia produces a HPV which is maintained only for the duration of the stimulus. However, the HPV reflex appears to be malign following a chronic exposure to hypoxia as it is maintained even upon return to normoxia (see also section 1.3.4 (i)).

1.2 The pulmonary circulation of the fetus and neonate

1.2.1 Fetal pulmonary circulation

The fetal pulmonary circulation exists as a high-resistance, low-flow circuit, accepting less than 10% of the combined ventricular output. This unique physiologic state ensures sufficient pulmonary blood flow to provide for lung growth and development, yet allows blood to shunt right to left to perfuse the placenta, the fetal organ of gas exchange. The lungs do not have a physiological role apart from possible metabolic functions which include secretion and activation of hormones and degradation of active agents. Most of the systemic and venous and umbilical venous blood returning to the heart is shunted through the foramen ovale to the left atrium and ventricle, and approximately 85-90% of the blood ejected by the right ventricle flows through the ductus arterious directly from the pulmonary trunk to the descending aorta, thus diverting the lungs (Rudolph, 1979). The low fetal pulmonary blood flow has been explained on the basis of a high pulmonary vascular resistance. In fetal lambs, calculated pulmonary vascular resistance was shown to be extremely high at 6 mmHg/ml/min at 0.4 gestation and fell progressively to 0.3-0.35 mmHg/ml/min at term (Rudolph, 1977). This decrease in pulmonary vascular resistance presents a 17-20 fold increase in the cross sectional area of the pulmonary vascular bed. This appears to result from a decrease in the resting vascular vasoconstriction and an increase in individual vessel diameter and growth of new vessels.

The mechanisms that maintain vasoconstriction *in utero* and permit vasodilation after birth are not fully understood. The pulmonary vasoconstriction in the fetal lung has been related to the phenomenon of hypoxic pulmonary vasoconstriction, due to the low pO_2 of blood perfusing the lungs (see section 1.1.4.5), autonomic nervous influences, and circulating hormones (Randolph, 1979). Maintenance of high resting tone also reflects a different "balance", compared with the postnatal lung, between vasoconstrictor and vasodilator mediators. As already stated the vascular endothelium is able to influence vascular tone via the release of several vasoactive agents. Amongst these are NO (see section 1.1.4.3) and the potent vasoconstrictor ET-1. The potential role of this

latter peptide in the transitional pulmonary circulation is reviewed in section 1.5.3.1. The high pulmonary vascular resistance during fetal life and the greater responsiveness in the lung as compared with adult animals have been explained by greater muscularity of the arteries (O'Neal, *et al.*, 1957).

1.2.2 Transition of the pulmonary circulation at birth

At birth, normal transition of gas exchange from the placenta to the lungs depends on a 10-fold increase in the pulmonary blood flow and a sharp decline in the pulmonary arterial pressure. These events are dependent on a dramatic decrease in pulmonary vascular resistance within the first few hours after birth, due to structural (Haworth & Hislop, 1981; Hall & Haworth, 1986) and functional (Zellers & Vanhoutte, 1991; Liu, *et al.*, 1992) remodelling.

Haworth and Hislop (1981) examined the normal adaptation of the pulmonary circulation in the pig, from fetal to six months of age and the following was shown. Associated with the rapid fall in pulmonary arterial pressure is dilatation and recruitment of small arteries within the acinar region. This was observed during the first 5 minutes and continued during the first 24 hours, associated with a loss of arterial smooth muscle. Muscularity also decreases in the hilar and lobar elastic arteries during the first week of life. This structural change is associated with a reduction in arterial elasticity, compatible with the reduced pressure at which the vessels are now operating. The significant reduction in the amount of arterial smooth muscle was related to a reduction in the pulmonary : systemic vascular resistance ratio from 0.58-0.18, observed between 24 hours and 2 weeks after birth. Growth and development of the lung continued until an adult pattern was reached by 6 months of age. Furthermore, the haemodynamic and structural adaptation of the pulmonary occurring in the first two weeks of life were shown to follow a similar time course to those in the human infant.

Although the processes occurring in the transition of the pulmonary circulation at birth involve multiple factors, growing evidence suggests that NO may be, at least in part, a mediator for these processes (Xue, *et al.*, 1994) and that NO may also play a role

in angiogenesis during lung development (Halbower, *et al.*, 1994). The vasodilatory response to birth-related stimuli for the postnatal decline in pulmonary vascular resistance, such as increased oxygenation and shear stress, have been shown to be markedly attenuated by pretreatment with the NOS inhibitor L-NN (Shaul, *et al.*, 1992; Cornfield, *et al.*, 1992). Xue *et al.* (1996), using immunohistochemical labelling in fetal and neonatal rat lung, demonstrated endothelial NOS (eNOS) immunoreactivity 14 -day fetal lung cells and the quantity of immunopositive cells increased as gestation proceeded, to coalesce to form an inner (endothelial) layer of pulmonary vessels. This progress of angiogenesis was seen from 15 days of gestation to at least 7 days postnatally. Levy and co-workers (1995) examined the influence of the endothelium in the contractility of newborn pig intrapulmonary arteries. It was found that at birth, when measured plasma ET-1 levels were shown to be at their greatest, the endothelium enhanced the contractile response to KCl and PGF2α. Whilst these responses were inhibited by the endothelium from 10 day onwards. However the NOS inhibitor L-NMMA augmented the contractile response at all ages. This suggests a complex change in the functional properties of both pulmonary arterial endothelial and smooth muscle cells, and the interaction between these cell types during development.

Numerous lines of evidence implicate the possible involvement of ET-1 in the extrauterine adaptation of the pulmonary circulation and this is reviewed in section 1.5.3.1. A third vasoactive endothelial-derived mediator with a possible role is the eicosanoids. It appears that the cyclooygenase pathway is the predominant pathway for arachadonic acid (AA) metabolism in the fetal and transitional pulmonary circulations, producing prostaglandins. Prostacyclin causes a potent vasodilation when infused in the fetal lung *in vivo* (Cassin, *et al.*, 1981). Davidson (1988) demonstrated that acute cyclooxygenase inhibition attenuated the drop in pulmonary vascular resistance at birth in term animals. However this attenuation was modest and postnatal adaptation was not changed significantly, suggesting that, although prostacyclin contributes to the pulmonary vascular changes during the transition, it does not appear to be essential for the postnatal adaptation.

1.3 Pulmonary Hypertension

Pulmonary hypertension (PHT) is a condition which exists when mean pulmonary arterial pressure increases by 10-15 mmHg (Fishman, 1985). This condition can arise as a primary phenomenon (idiopathic PHT) where the cause is unknown. Primary PHT is a rare disease, it occurs most frequently in women of 30-40 years of age and after the onset of the symptoms, has an average survival rate of 2-3 years (Rich, 1988). More commonly, PHT occurs as a secondary phenomenon to other disease states. There are many mechanisms that appear to cause or contribute to the pathogenesis of PHT (Rich, *et al.*, 1987). The principal causes have 3 basic mechanisms; (i) passive type, produced by disease which increase pulmonary venous pressure, e.g. chronic left ventricular failure, and mitral stenosis. (ii) Reactive or hyperkinetic type, produced by congenital heart defects that cause an increased blood blow through the pulmonary vasculature; e.g. persistent pulmonary hypertension of the newborn caused by a patent ductus arteriosus (see section 1.3.2) and atrial or ventricular septal defects. (iii) Vaso-occlusive type, which can be: obstructive e.g. multiple emboli, thrombosis and chronic obstructive lung disease (COLD); obliterative, due to a reduction in vascular bed by chronic parenchymal disease such as emphysema or advanced fibrosis; and vasoconstrictive which is usually related to hypoxia. The form of secondary PHT occurs most commonly in response to hypoxic lung disease, but can also develop as a result of exposure to low inspired O_2 such as displayed in people with long term residence at altitudes over 2500m above sea level (Aldashev, *et al.*, 1989). Hence the main distinction is between those that cause a direct physical change in the pulmonary vascular bed due to structural or functional alterations, and those which affect other determinants of the pulmonary vascular resistance, such as flow and airway pressure, and contribute to exacerbate the process. Despite the differential aetiologies of PHT, this condition is often progressive and is characterised by a relentless increase in pulmonary vascular resistance which eventually leads to right heart failure and death.

The low pulmonary artery pressure sustained in a normal subject depends on two components: firstly, the high compliance of the major pulmonary blood vessels and,

secondly, the state of tone of the peripheral precapillary resistance vessels. With disease the structure of the pulmonary circulation may change markedly. In pulmonary hypertension there is thickening of the major vessels and a narrowing of the peripheral vessels (see section 1.3.1). Constriction of the pulmonary arteries results in elevated pulmonary artery pressure, which increases the pressure on the right side of the heart, whereas constriction of the pulmonary veins increases pulmonary capillary pressure, and this could result in pulmonary oedema. The association of vasoconstriction and anatomical restriction results in PHT.

The mechanism(s) underlying the development of PHT has not yet been clarified, although some evidence suggests an important role of the endothelium in its pathogenesis (Reid, 1986). The importance of the vascular endothelium in the regulation of pulmonary vascular tone is introduced in section 1.1.4.3. The role of the ET-1 (and NO) in the pulmonary circulation and its putative involvement in PHT is introduced in section 1.5.

Recent studies have shown that inhalation of nitric oxide (NO) in patients with chronic lung disease and PHT relieves the hypertension by a selective effect on the pulmonary circulation (Adnot, *et al.*, 1992). Nitric oxide was also shown to reverse PHT in children with neonatal PHT and may provide permanent benefit (Kinsella, *et al.*, 1992). Inhaled NO therapy was subsequently applied to the treatment of various PHT states including secondary to congenital heart disease (Roberts, *et al.*, 1993) and acquired heart disease (Girard, *et al.*, 1992). This success eased a major problem facing clinicians caused by the lack of selectivity of most vasodilators used in the treatment of PHT. Due to the pulmonary vascular remodelling (see section 1.3.1), diseased lungs are relatively resistant to vasodilator therapy and the standard vasodilators exert greater effects on the systemic circulation hence leading to unwanted systemic hypotension. At present oxygen is the only generally available selective pulmonary vasodilator but NO is likely to become more commonly used. Moreover, from the many studies of ET-receptor mediated responses in the pulmonary circulation under both normal and abnormal conditions, an increasing amount of evidence indicates a possible therapeutic

use of ET-receptor antagonists in the treatment of PHT; this is discussed in section 1.5.3.2. In addition, 5-HT receptor antagonists present another possible therapeutic intervention. For example, Herve *et al.* (1990) reported the case of a patient with primary PHT in the presence of familial platelet storage pool disease and elevated plasma 5-HT levels. Administration of a 5-HT_2 receptor antagonist, ketanserin, was shown to substantially reduce the PHT.

1.3.1 Pulmonary vascular remodelling

Despite the differences in the origin of PHT states, there are many common features of vascular remodelling which are present in all forms of hypertensive pulmonary vascular disease, whatever the aetiology. Functionally, the important changes are those in the muscular arteries, which can undergo progressive medial hypertrophy until there is virtually no lumen. Generally the thickness of the media of the muscular pulmonary arteries is proportional to the level of the pulmonary arterial pressure (Yamaki & Wagenvoort, 1981). In the early stages of both primary and secondary PHT, a progression of muscularisation into the non-muscular terminal portion of the pulmonary arteriole tree is observed (Heath, *et al.*, 1987, 1993). This occurs as a result of hyperplasia of vascular smooth muscle cells which extend distally in a layer internal to the original internal elastic lamina (Heath, *et al.*, 1987), thus forming an inner layer of longitudinal smooth muscle. The media also extends peripherally along the arterial tree to the elastic arteries. In the latter stages of remodelling the pattern of cell migration and proliferation depends on the type of PHT. For example, in secondary PHT the muscular artery hyperplasia is not so advanced and dilatation lesions which occur in primary PHT are not seen. Plexogenic pulmonary hypertension in which distinct plexiform lesioning develops is typical of the primary form and Eisenmenger's syndrome. In this latter mentioned condition, the contributing factor leading to PHT is increased pulmonary flow due to intracardiac shunt.

Although a vast amount of attention has focused on the medial hypertrophy and hyperplasia, in PHT, the pulmonary arteries respond with thickening of all three

vascular layers, namely intima, media and adventitia, but the pattern of thickening varies depending on the size of the artery (Peacock, *et al.*, 1993). In the smallest arteries the dominant change is in the media. Whilst in the major arteries, the dominant change is in the adventitia with collagen deposition and contraction (Reid, 1990). With a chronic increase in pulmonary vascular pressure, there is structural remodelling with fibrosis, particularly in the intima layer. Smooth muscle cells migrate from the tunica media through fenestra in the internal elastic lamina into the intima where they proliferate and secrete proteoglycans and matrix proteins; this cellular intima eventually matures into a less collagenous layer (Edwards, 1957). Endothelial cell disruption has been observed in experimental monocrotaline PHT and in patients with congenital heart defects and PHT (Rosenberg & Rabinovitch, 1986; Rabinovitch, *et al.*, 1986). The resulting intimal and medial hypertrophy may reduce the calibre of the resistance arteries and occlude small vascular channels, resulting in increased vascular resistance. Once PHT has developed, the associated structural vascular changes renders the pulmonary circulation relatively resistant to standard vasodilators (see section 1.3).

1.3.2 Persistent pulmonary hypertension of the newborn

Failure of the pulmonary circulation to adapt normally to extrauterine life is a common cause of mortality and morbidity in the newborn (Hageman, 1984). Pulmonary vascular resistance remains high after birth and a right to left shunt persists across the ductus arteriosus and foramen ovale. Persistence of the fetal circulation is most commonly associated with asphyxia, is often seen in premature infants suffering from the respiratory distress syndrome, and is occasionally found in term babies following a normal delivery (Gersony, 1973).

The vascular remodelling associated with pulmonary hypertensive states has been introduced in the previous section (1.3.1). A stained section of lung from a human female baby born nine weeks premature is shown in figure 1.2. This newborn survived for only two days and suffered from PHT resulting from infantile respiratory distress syndrome. A persistence of morphological structure associated with fetal pulmonary

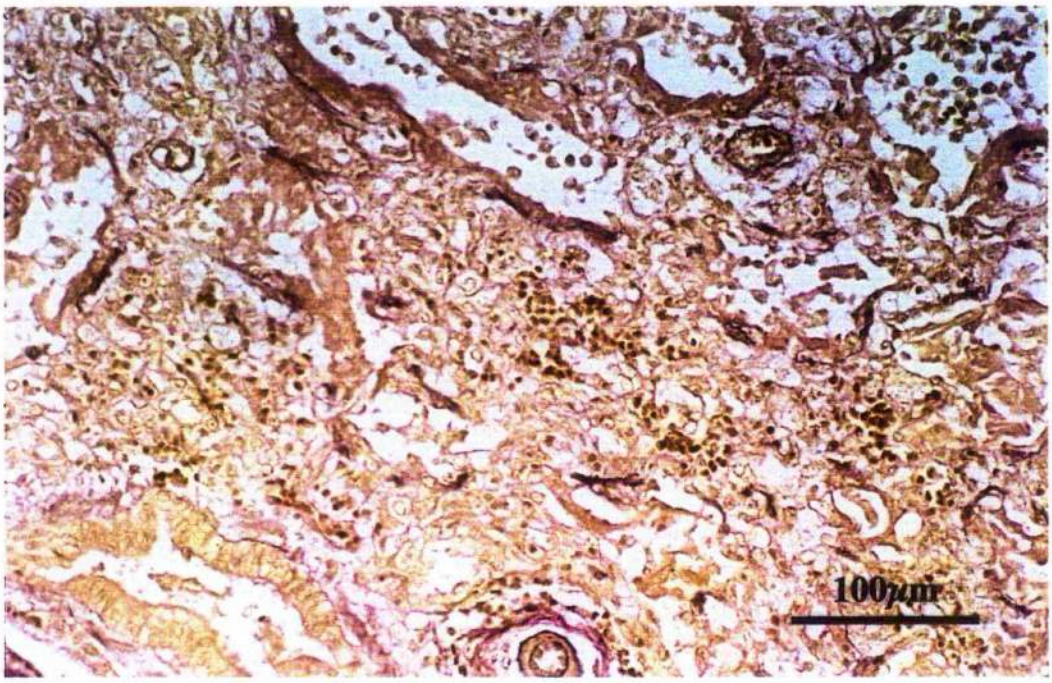


Figure 1.2

Section of lung (E.V.G. stain) from human female born 9 weeks premature with pulmonary hypertension resulting from Infantile Respiratory Distress Syndrome. (Newborn also suffered from bronchopneumonia and pleural effusions and died at 2 days old). Morphologically, this section shows persistence of fetal pulmonary vascular structure. See text for details.

vasculature is evident. The definitive histologic features lie in the pulmonary arterioles and the muscular pulmonary arteries (Heath & Edwards, 1958). Brenner (1935) defined pulmonary arterioles as arterial vessels in the lung less than 100 μm in diameter, but obviously this is only an approximation. In particular, figure 1.2 shows transverse section of pulmonary arterioles ~ 50 μm i.d. with muscular media and internal and external elastic lamina. The media is relatively thicker than found in muscular arteries. The thickened arterioles also bear a striking resemblance to the intralobar pulmonary arteries of the fetus (Heath & Edwards, 1958).

1.3.3 Pulmonary hypertension secondary to left heart disease

The heart and lungs function as an integrated unit and, consequently, heart disease frequently affects the lungs and vice versa. The pulmonary circulation acts as a passive system in most situations. In systemic hypertension, a proportional rise in blood pressure and vascular resistance in the pulmonary circulation are probable (Kobayashi & Amenta, 1994). Left sided heart failure interferes with pulmonary venous drainage and results in acquired congestive pulmonary vascular disease. Oedema is a frequent sequela and occurs not only since the alveolar capillaries are over-extended, but they also suffer from hypoxia and increased pressure, which promote escape of fluid across the vascular endothelium. In more severe cases of left-sided heart failure PHT results, leading to failure of the right side of the heart. Severe PHT may debar surgical correction of the cardiac defect since such procedures when the pulmonary vascular bed is in this "high resistance, low reserve" state may be fatal (Edwards, 1957).

Cardiac failure can be chronic or acute, with the latter being caused by such conditions as myocardial injury due to coronary occlusion. The rabbit coronary artery ligation model of left ventricular dysfunction (LVD) was first characterised by Pye *et al.* (1996) and is described in section 2.1.1 of this thesis. Recently, Deuchar *et al.* (1997) reported a marked elevation in pulmonary arterial pressure, pulmonary vascular remodelling and right ventricular hypertrophy in animals with LVD compared to sham-operated rabbits, thus providing evidence for the existence of PHT in this model.

Several other animal models of various forms of heart disease have been reported. These include a rat model of cardiac hypertrophy which was produced by placing a constricting clip around the suprarenal abdominal aorta (Arai, *et al.*, 1995); acute myocardial infarction using a rabbit model of regional myocardial ischaemia and reperfusion (Vitola, *et al.*, 1996); and a rat model of myocardial infarction produced by permanent ligation of left coronary artery (Stassen, *et al.*, 1997). However the majority of the studies in these models have been to examine alterations in the myocardium, coronary and systemic circulations.

1.3.4 Other animal models of pulmonary hypertension

(i) Chronic hypoxic animals (mainly rat and mice) is a well studied model of hypoxic PHT. Rats exposed to chronic (hypobaric or normobaric) hypoxia exhibit significant PHT, and develop similar morphological changes in the pulmonary vascular bed that are observed in human PHT (Hislop & Reid, 1976; Rabonitch, *et al.*, 1979). Chronic hypoxic animals also exhibit polycythaemia which exacerbates the development of PHT and right ventricular hypertrophy (Naeye, 1965; Hunter, 1974).

(ii) The leguminous plants *Crotalaria* are toxic due to their content of the alkolid monocrotoline. Ingestion of the seeds of this plant causes severe damage to the liver, lungs and central nervous system m in man (Fishman, 1985). However, in animals such as the rat, ingestion of *Crotolaria* or subcutaneous injection of monocrotoline results in the development of PHT within several days (Fishman, 1985; Olson, *et al.*, 1984). Monocrotoline has an indirect effect on the pulmonary circulation; it is converted by the liver to dehydromonocrotoline which is a substance highly toxic to the pulmonary circulation. Monocrotoline-induced PHT is characterised by early vascular endothelial damage, followed by increased vascular muscularisation, a rise in pulmonary arterial pressure and right ventricular hypertrophy (Rosenberg & Rabinovitch, 1988). This model is considered useful for studying both primary PHT and PHT associated with lung disease (Wanstall & O'Donnell, 1990).

(iii) The fawn hooded rat has an hereditary tendency to bleed due to platelet storage pool disease and has been shown to develop idiopathic PHT (Stelzner, *et al.*, 1992). Hence this may present a possible model of primary PHT.

1.4 Endothelin

1.4.1 Discovery

In 1985, Hickey *et al.* demonstrated that the culture medium of bovine aortic endothelial cells triggered a slowly developing and long lasting contraction of isolated pig coronary arteries, which could not be attributed to any known vasoconstrictor mediators and was shown to be peptidergic in nature. Subsequent studies confirmed this pioneering observation (Gillespie, *et al.*, 1986). By 1988, this potent endothelial derived constrictor factor (EDCF) had been isolated, purified, sequenced and cloned, and was named endothelin (ET) (Yanagisawa, *et al.*, 1988). Soon after its discovery, isotypes of ET ware identified. Analysis of human genomic sequences revealed the existence of three distinct ET genes for ET which encoded three distinct ET peptides, named endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3) (Inoue, et al., 1989).

1.4.2 Structure of the endothelins

The newly identified 21 amino acid peptide, ET, had no similarity in its sequence to the known peptides of mammalian origin. However in 1989, Kloog and Sokolovsky reported that the sequence of another family of peptides the sarafotoxins (SXS6a, SXS6b, SXS6c), found in the snake venom of the *Atractaspis engadennsis*, should a very similar sequence and structure to that of the ET family. The amino acid structural sequences of the ET isopeptides and that of SXS6c, a member of the SX family, are illustrated in figure 1.3. All family members contain 21 amino acid residues and show complete identity at 10 positions, including all four cysteine residues; these participate in two intrachain disulphide bridges formed by the bonding cys^{1}-cys^{15} and cys^{3}-cys^{11}. Human ET-2 and ET-3 have 2 and 6 substitutions, respectively, relative to ET-1. Human and porcine ET-1 have identical sequences, whilst the ET-3 sequence is

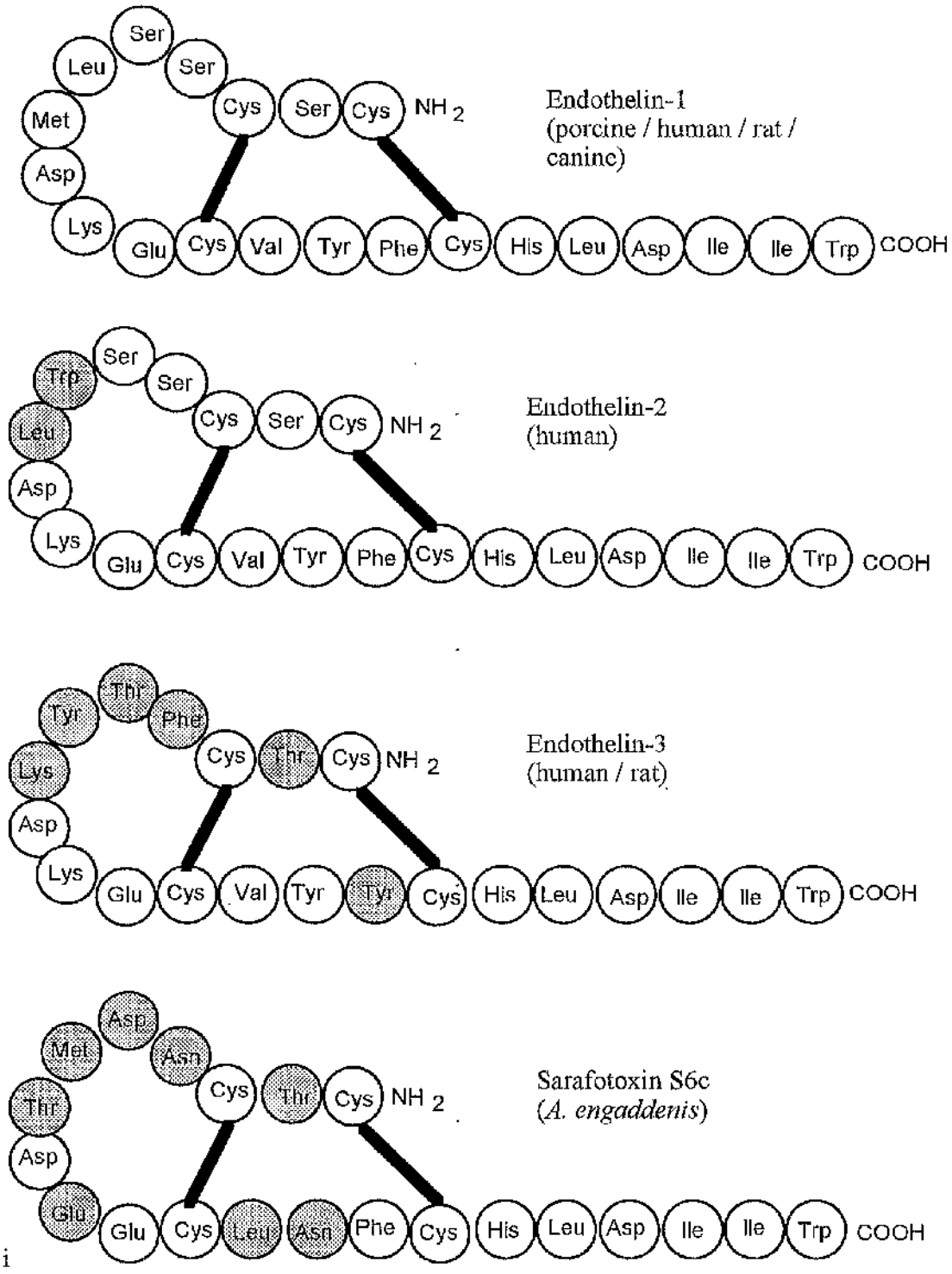


Figure 1.3 The amino acid sequences of the three isoforms of endothelin from different species and the related peptide sarafotoxin S6c. The dotted circles represent amino acid changes compared with the endothelin-1 sequence. Bold black lines indicate disulphide bonds between Cys^{1} - Cys^{15} and Cys^{3} - Cys^{11}.

identical in both human and rat. The sequence of the COOH-terminal hexapeptide region is conserved throughout the ET family, is greatly hydrophobic, and has been shown to have its own biological activity (Rovero, *et al.*, 1990). Numerous studies indicate that ETs exist in solution as highly compacted structures. The structures which have defined however differ depending on the technique used, although a good deal of consensus about certain structural elements does exist.

1.4.3 Endothelin genes: Regulation and tissue expression

The distinct genes for human ET-1, ET-2 and ET-3 have been mapped to chromosome 6, 1 and 20, respectively (Arinami, *et al.*, 1991). Each gene encodes for a large preproendothelin (preproET) mRNA from which the ET isotype is the product (see section 1.4.4). ET isoforms are now known to be produced in a variety of tissue and cell types and there are tissue-specific patterns of isoform expression. Endothelial cells regardless of their origin appear to express ET-1 mRNA; for example, umbilical vein (Inoue, *et al.*, 1989a; b) and aorta (Tokunaga, *et al.*, 1992). Evidence also indicates that vascular smooth muscle cells are capable of ET-1 mRNA expression (Yanagisawa, et al., 1988; Tokunaga, *et al.*, 1992). Northern analysis has suggested that ET-2 and ET-3 are not expressed in either vascular endothelium or smooth muscle (Bloch, *et al.*, 1989a; b). ET-1 expression also occurs in many other non-vascular cell types such as macrophages (Ehrenreich, *et al.*, 1990), cardiac myocytes (Suzuki, *et al.*, 1993) and neurones (Giaid, *et al.*, 1989).

Regulation of the transcription of ET mRNA plays an important role in the production of mature ET isoforms. Numerous studies show that several important stimuli enhance expression on the preproET-1 gene and de novo ET-1 synthesis. Increased message levels have been observed after treatment of endothelial cell with various growth factors, such as thrombin (Emori, *et al.*, 1992), and vasoactive substances such as angiotensin II (Dohi, *et al.*, 1992) and bradykinin (Marsden, *et al.*, 1991). Exposure of endothelial cells to physiologically low oxygen tension has also been shown to increase ET-1 mRNA expression (Kourembanas, *et al.*, 1991). Fluid sheer

stress may also influence expression and release of ET-1, however opposing findings have been reported. For example, Malek and Izumo (1992) demonstrated a dose dependent decrease in ET-1 expression with increasing sheer stress in bovine aortic endothelial cells. Whereas in cultured porcine aortic cells, low sheer stress was shown to increase ET-1 expression, whilst higher sheer rates had no effect (Yoshizumi, *et al.*, 1989). In addition, atrial natriuretic peptide (ANP) and NO have also been shown to inhibit ET-1 synthesis and release (Hu, *et al.*, 1992; Boulanger & Luscher, 1991).

1.4.4 Endothelin biosynthesis

All the ETs are derived from preproET precursors comprised of between 160 and 238 amino acid residues, depending on the isopeptide and the species; I will focus on ET-1. The amino acid sequences of prepro-, pro(big)-, and mature ET-1 predicted hypothetical biosynthetic pathway for the production of the mature 21 amino acid mature ET-1 (Yanagisawa, *et al.*, 1988); the proposed proteolytic pathway is shown in figure 1.4. The 203 residue prepro-form is initially processed by dibasic pair-specific endopeptidases and carboxypeptidases to yield a 39 amino acid intermediate, pro-ET-1 (or big ET-1), which is secreted and circulates in the plasma. In the final stage, big ET-1 is converted to the mature form via an unusual proteolytic processing between Trp^{21} and Val^{22} by specific endothelin converting enzymes (ECE) (Yanagisawa, *et al.*, 1988; Inoue, *et al.*, 1988a). This hypothesis was supported by the presence of big ET-1, and its carboxyl (COOH)-terminal fragment, in the conditioned medium of endothelial cells, also indicating that all of the biosynthetic stages can occur within the endothelial cell (Emori, *et al.*, 1989).

As the vasoconstrictor activity of big ET-1 is about 100-fold lower than that of ET-1 (Kimura, *et al.*, 1989), this step in the biosynthesis appears to be important for the biological significance of ET-1. Intravenous injection of big ET-1 in rats evoked a pressor effect with a potency similar to ET-1 (Sawamura, *et al.*, 1989), suggesting a rapid and efficient conversion of exogenous big ET-1 to biologically active ET-1 *in vivo*. ET-1 is not stored in secretory granules within endothelial cells (Nakamura, *et al.*,

1990), therefore active control of ET-1 release appears to depend upon de novo synthesis of the peptide.

1.4.4.1 Endothelin converting enzymes (ECE)

Early studies to characterise ECE activity revealed that the enzyme may be a membrane bound neutral metalloprotease, as the production of ET-1 or conversion of big ET-1 by cultured bovine carotid artery endothelial cells displayed a narrow pH

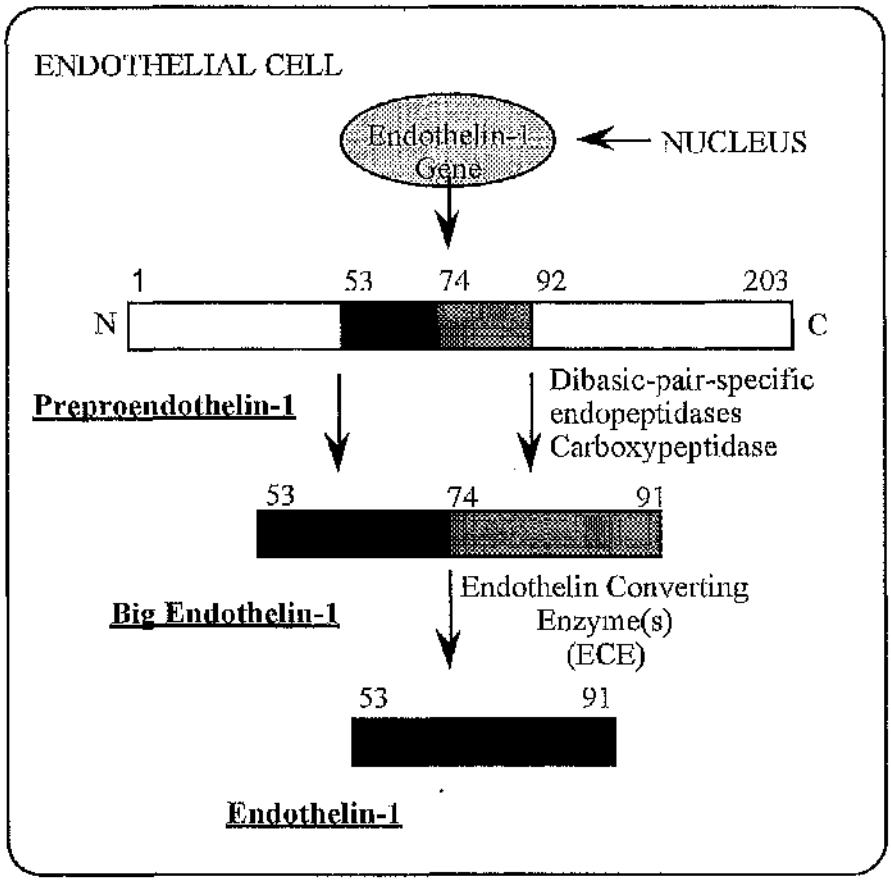


Figure 1.4 Biosynthetic pathway for ET-1 production.

optimum (pH 7.1) and could be selectively inhibited by phosphoramidon (Okada, *et al.*, 1990). Initial evidence suggested that the conversion of big ET-1 to ET-1 *in vivo* occurs by an ECE present on the vascular endothelium. For example, Fukuroda *et al.* (1990) showed that the conversion of big ET-1 to ET-1 in isolated blood vessels was dependent on the presence of an intact endothelium. Advances in ECE research demonstrated the

existence of more than one isoform of the enzyme. The first ECE isoform to be identified and cloned was termed ECE-1, this was described as a type II metalloprotease that processed endogenously produced ET-1 intracellularly and exogenously supplied big ET-1 on the cell surface (Xu, *et al.*, 1994). ECE-2 is structurally similar to ECE-1 but was found to have an unusually acidic pH 5.5 optimum, suggesting that the enzyme acts intracellularly in acidified compartments and not on the cell surface (Emoto & Yanagisawa, 1995). ECE-1 was found to be abundantly expressed in endothelial cells *in vivo*, but could not be detected in neurones (Xu, *et al.*, 1994). In contrast, ECE-2 is strongly expressed in neuronal cells (Emoto & Yanagisawa, 1995). Two isoenzymes of ECE-1 which are derived from the same gene have been described (Shimada, *et al.*, 1995); these were subsequently termed ECE-1a and ECE-1b. Recently, Emoto *et al.* (1997) demonstrated that ECE-1a, the predominant isoform in cultured vascular endothelial cell, resided strictly within intracellular secretory compartments. In contrast, ECE-1b, the isoform found in cultured smooth muscle cells, was expressed on the plasma membrane. The authors proposed the model that cleavage of big ET-1 by the "generator" cells takes place mostly inside the cell, provided that those cells express sufficient levels of ECE-1, as in the case of vascular endothelial cell. Whilst extracellular big ET-1 can be cleaved by cell-surface ECE-1 expressed by the "target" cells, with the resultant mature peptide acting locally on cell surface receptors.

1.4.5 Molecular cloning and characterisation of endothelin receptor subtypes

The discovery of various ET isoforms and the diverse biological functions exerted by them predicted the existence of more than one ET receptor. In 1990, two groups independently reported the cloning and sequencing of two different ET receptors. Arai *et al.*. (1990) isolated a receptor from bovine lung cDNA library; when this receptor was expressed in *Xenopus* oocytes, it showed extremely high selectivity for ET and SXS6 peptides. The second cloned receptor was isolated from rat lung cDNA library, and showed equal affinity for ET-1, ET-2 and ET-1 (Sakurai, *et al.*, 1990). These two cloned receptors are members of the rhodopsin superfamily, which have

seven transmembrane-spanning hydrophobic regions, an extracellular N terminus and a cytosolic C terminus. These receptors are coupled with guanine-nucleotide-binding (G) proteins and range from 45000 to 50000 daltons in size in various tissues.

1.4.5.1 Endothelin A receptors

The cloned receptor which showed selectivity for ETs in the following order, ET-1 = ET-2>ET-3, was subsequently named ET_A (Arai, *et al.*, 1990; Masaki, *et al.*, 1991). Cloned ET_A receptors show 1000 fold selectivity for ET-1 over ET-3. However, a selectivity for ET-1 in the range 10-100 fold is indicated by radioligand and functional studies of ET_A receptors. These discrepancies may be related to the tissues examined having a mixture of receptor subtypes.

The involvement of ET_A receptors in ET-1 mediated responses is generally classed by (i) the relative potencies of ET-1 over ET-1, (ii) the inability of selective ET_B receptor agonists to elicit equivalent responses, and (iii) effectiveness of selective ET_A receptor antagonists. In some tissues where agonist potencies indicated a role of ET_A receptors, it was found that the ET_A receptor antagonist was more effective against ET-3-induced compared with ET-1-induced responses (Sumner, *et al.*, 1992). Contractile responses in the rabbit saphenous vein are mediated by both ET_A and ET_B receptors, however the ET_A receptor mediated response could be subdivided into a BQ-123 sensitive and a BQ-123 insensitive component (Sudjarwo, *et al.*, 1994; Nishiyama, *et al.*, 1995). Thus ET_A receptors are occasionally subtyped with regard to there sensitivity to a selective antagonist. For example, ET_{A1} (BQ-123 sensitive) and ET_{A2} (BQ-123 insensitive) (Sudjarwo, *et al.*, 1994).

1.4.5.2 Endothelin B receptors

The ET_B receptor is non-isopeptide selective and therefore has the following rank order of sensitivity, ET-1=ET-2=ET-3 (Sakurai, *et al.*, 1990; Masaki, *et al.*, 1991). Considerable homologies exist between ET_A and ET_B receptors in a given species,

approximately 50% identity at the amino acid level, and between individual subtypes across mammalian species (85%-90%).

Numerous lines of evidence now indicate ET_B receptors to be present on the endothelium, evoking vasodilator responses (referred to as ET_{B1} receptors), and also on the vascular smooth muscle, inducing vasoconstriction on activation (referred to as ET_{B2} receptors) (see section 1.4.8.3). Although ET_B receptors are anatomically and functionally distinct, it is still uncertain as to whether these receptors are structurally distinct subtypes. Radioligand binding studies in canine coronary artery membranes indicated possible ET_B receptor subtypes, exhibiting either high or low affinity for ET-1 and ET-3 (Teerlink, *et al.,* 1994b). A functional correlate could be identified for the high affinity site however, none could be found for the low affinity site. Like the ET_A receptor, ET_B receptors have also been proposed to be subtyped with reference to antagonist sensitivity. The nonselective ET receptor antagonist PD 142893 may selectively inhibit ET_{B1} receptor mediated vasodilation but be ineffective against ET_{B2} receptor mediated vasoconstrictions (Warner, *et al.*, 1993; Douglas, *et al.*, 1995).

Recently, Mizuguchi, *et al.* (1997) performed a study to clarify whether the ET receptor subtypes mediating the two pharmacologically heterogeneous responses were the products of a single ET_B receptor gene. This clever experiment used isolated aorta and gastric fundus from ET_B receptor gene knockout mice and control mice, and the results indicated that the vasodilator and vasoconstrictor responses to SXS6c, selective ET_B receptor agonist, was derived from the same gene.

1.4.5.3 Endothelin C receptors

Numerous studies revealed that vascular endothelial cells were particularly sensitive to the action of ET-3. Emori *et al.* (1991) showed that an ET receptor on bovine endothelial cells was functionally selective for ET-3 over ET-1. Whilst Warner *et al* (1992) demonstrated that release of NO by endothelial cells was particularly sensitive to ET-3 compared to ET-1. These findings may suggest a further receptor subtype. Recently, a cDNA was cloned from dermal melanphores of the frog *Xenopus laevis*

(Karne, *et al.*, 1993). This cDNA encoded a protein predicted to have a seven-transmembrane domain structure, thus typical of the superfamily to which ET_A and ET_B receptors belong. However, to date no putative third (ET_C) receptor has been cloned by screening cDNA libraries prepared from mammalian cells. Thus the existence of a mammalian ET_C-like receptor remains to be determined.

Possible non ET_A/non ET_B receptors

An increasing number of studies in vascular preparations have observed atypical ET receptor mediated responses and this has led to the proposal of possible novel subtypes of ET_A or ET_B receptors, or non ET_A/non ET_B receptors in these preparations. In human saphenous vein contractile responses to ET-1 were resistant to the actions of the ET_A receptor antagonist BQ 123 , whereas SXS6b-induced responses were partially inhibited by BQ 123 (Bax, *et al.*, 1993). From these observations the authors proposed that ET-1 evoked vasoconstriction via activation of a non ET_A/non ET_B receptor. As another example, the vasoconstrictor response of guinea-pig bronchus was shown to be mediated predominantly by ET_B receptors, yet these responses appeared to be insensitive to selective ET_B receptor antagonists (Hay & Luttman, 1997). Thus the ET-receptor mediated responses in this preparation seems not to be mediated by the classical ET_B receptor, but rather an atypical population.

In vivo studies have also suggested possible further ET receptors. Recently, Cirino *et al.* (1997) demonstrated that the haemodynamic and renal effects of ET-1 in the anaesthetised pig could not be blocked by BQ-123 or BQ-788, however BQ788 inhibited SXS6c-induced effects. The authors hypothesised the existence of an additional ET-receptor or a subtype of the ET_B receptor, activated by ET-1 but not SXS6c, which is insensitive to BQ788. Although no strong conclusions can be made concerning possible ET receptor subtypes, from the ever growing number of studies the

considerable heterogeneity in ET-receptor mediated responses between various tissues and species is becoming increasingly apparent (see sections 1.4.8 and 1.5.2).

1.4.5.4 Regulation of expression of endothelin receptors

Hirata *et al.* (1988) demonstrated that ET-1 pretreatment caused a substantial decrease in [^{125}I]-ET-1 binding sites in vascular smooth muscle cells. Subsequently, numerous studies have demonstrated that ET receptors, like many other cell surface peptide receptors, are subject to ligand-induced downregulation (e.g. Sakurai *et al.*, 1992). The regulation of the production of ET-receptors often parallels that of the ETs. For instance, hypoxia or cyclosporine rapidly stimulates the production of ET-1 and ET_A receptors in endothelial cells and vascular smooth muscle cells, respectively (Simonson, 1993). Epidermal growth factor, basic fibroblast growth factor, cAMP and oestrogen upregulate ET_A receptors in some tissues. Whilst a down regulation of ET_A receptors to angiotensin II, platelet derived growth factors, and transforming growth factor β, has been shown in various tissues (Levin, 1995). Upregulation of ET_B receptor subtype mRNA by angiotensin II and (dibutyryl) cAMP has also been reported (Kanno, *et al.*, 1993; Hama, *et al.*, 1992). However other studies suggest that cAMP and also catecholamines downregulate ET_B receptors (Levin, 1995).

1.4.5.5 Tissue-specific expression of endothelin receptors

A variety of different techniques and approaches have been used to localise ET receptors which appear to demonstrate tissue-specific expression. Variable results have been reported fro the various studies however, in general, ET_A receptor mRNA appears to be strongly associated with vascular tissue, and is particularly expressed in the heart and lungs (Arai, *et al.*, 1990). Whilst ET_B receptors appear to be more widely distributed on many different tissue types, e.g. brain, liver, kidney and uterus (Sakurai, *et al.*, 1990; Simonson, 1993). Hori *et al.* (1992) showed that in the brain, ET_A receptors appeared to be associated with blood vessels, and ET_B receptors with glial and epithelial cells. Regarding the vasculature in general, the majority of evidence suggests that ET_A

receptors are located on smooth muscle cells, whereas ET_B receptors have been shown to be expressed in endothelial cells. However ET_B receptor mRNA has been detected in vascular smooth cells of various vessels from several species, including human coronary, pulmonary and internammmary artery (Davenport, *et al.*, 1995a; b). Stimulation of vascular ET_A receptors mediates a slowly developing, long-lasting contraction (Rubanyi & Parker-Botelho, 1991). Activation of endothelial ET_{B1} receptors appears to mediate vasodilation, whereas stimulation of ET_{B2} receptors present on the vascular smooth muscle is generally thought to mediate vasoconstriction (Rubanyi & Polokoff, 1994). The vascular effects of ET-receptor agonists is discussed more in section 1.4.8.3-5.

1.4.6 Agonists and antagonists for endothelin receptor subtypes

1.4.6.1 Selective agonists for endothelin receptors

ET-2 appears to be approximately equipotent with ET-1 at most ET_B receptor binding sites or functional receptors and only slightly less potent than ET-1 at ET_B receptors. Since relatively little data is available for ET-2, only ET-3 could be considered a selective agent with preference for ET_B receptors. However it is important to consider that in some cases, ET-3 has a potency in the low nanomolar range for ET_A receptors. In addition, as stated in section 1.4.5.3, there is evidence for a putative ET_C receptor with selectivity for ET-3. Hence determination of the ET-1:ET-3 potency ratio is required to reliably identify the ET receptor subtype(s) in a given preparation. In addition to ET-3, SXS6c has become accepted as a selective ligand at ET_B receptors. Williams *et al.* (1991) showed that in rat aorta and atrium, tissues rich in ET_A receptors, SXS6c was at least 50000 times less potent than ET-1 at inhibiting the binding of [^{125}I]-ET-1. BQ-3020 is also a potent agonist of ET_B receptors, this was shown to have an ET_B:ET_A selectivity ratio of 4700 (Saeki, *et al.*, 1991). [Ala$^{1, 3, 11, 15}$]ET-1 (Hiley, *et al.*, 1990) and IRL 1620 (Takai, *et al.*, 1992) are other compounds which are also used as selective ET_B receptor agonists. However, a selective agonist for the ET_A receptor site remains elusive.

1.4.6.2 Peptide endothelin receptor antagonists

The first ET receptor antagonists to be described were peptide derivatives of the ETs or structurally related analogues. ET_A receptor antagonists include BQ-123 (Ihara, *et al.*, 1992) and PD 151242 (Davenport, *et al.*, 1994). This latter compound was initially used in an iodinated form as a selective radioligand of ET_A receptors in human tissue. In the studies shown in chapters 5 and 9 of this thesis, I used FR139317 as a selective ET_A receptor antagonist. This compound was shown to have approximately 90-fold selectivity for ET_A receptors, with a pA_2 value of 7.2 against ET-1-induced contractions of rabbit aorta (Sogabe, *et al.*, 1993).

Selective ET_B receptor antagonists described include RES-701-1 (Morishita, *et al.* 1994). This peptide is less potent than BQ788, a selective ET_B receptor antagonist which I used in my own studies (chapters 5 and 9). BQ788 was characterised by Ishikawa *et al.* (1994) who showed this compound to have a pA_2 against BQ-3020-induced contraction of rabbit pulmonary artery of 8.4. Non-selective peptide ET receptor antagonists include PD 142893 and the more potent PD 145065 (Doherty, *et al.*, 1993).

1.4.6.3 Non-peptide endothelin receptor antagonists

The first non-peptide ET receptor antagonists were described in 1993 and 1994; these represented possible therapeutic advantage over those of a peptide nature since they would be orally active. Bosentan (Ro-46-2005) (Clozel, *et al.*, 1994) was among the first examples of non-selective non-peptide antagonists. Although this compound inhibits both ET_A and ET_B receptors, it demonstrates approximately 20-fold selectivity for the ET_A receptor subtype. BMS 182894 is a selective non-peptide ET_A receptor antagonist; the pA_2 was 6.3 for ET_A receptor-mediated contraction of rabbit carotid artery (Stein, *et al.*, 1994).

The most potent of the non-peptide ET receptor antagonists classed as non-selective is SB 209670 (Ohlstein, *et al.*, 1994); this was a compound I utilised in my own studies. SB 209670 was shown to antagonise ET-1-induced contraction of rat aorta

(ET_A receptors) and rabbit pulmonary artery (ET_B receptors) with pA_2 values of 9.39 and 6.7, respectively. At least a 90 fold selectivity for ET_A receptor subtype was indicated by the comparative Ki values of 0.2 nM (ET_A receptors) and 18 nM (ET_B receptors). In addition to presenting possible therapeutic agents in the treatment of various disease states in which ET-1 is implicated, this continually increasing list of highly effective antagonists should assist in the further elucidation of the role of ET-1 in both physiology and pathophysiology.

1.4.7 Signal transduction mechanisms

Several receptor signal transduction mechanisms are suggested to be involved in ET-1-induced vasoconstriction. Generally, in most vascular preparations ET-1 mediates increases in cytosolic free Ca^{2+} in two distinct phases; a transient initial phase, which is the result of Ca^{2+} mobilisation from intracellular stores (see section 1.4.7.1), and a sustained phase which is dependent on extracellular Ca^{2+} (see section 1.4.7.2) (Rubanyi & Polokoff, 1994).

1.4.7.1 Activation of phospholipase C (PLC) protein kinase C (PKC).

Both subtypes of ET receptors are coupled to PLC via a G-protein (Takuwa, *et al.*, 1989; Sakamoto, *et al.*, 1993). PLC catalyses the generation of inositol phosphate (IP_3) and diacylglycerol (DAG) via the hydrolysis of phosphotidylinositol (4, 5) biphosphate (PIP_2). The intracellular cascade of events triggered by PLC activation by vascular ET receptors is shown in figure 1.5. IP_3 acts upon specific receptors to release intracellularly stored Ca^{2+}. The increase in intracellular Ca^{2+} activates the enzyme myosin light-chain kinase (MLCK), which leads to phosphorylation of myosin light-chain (MLC) protein, thus triggering contractile events. The second product DAG may in turn activate PKC (Emori, *et al.*, 1990). PKC was shown to be involved in ET-receptor mediated vasoconstriction of rabbit saphenous vein (Sujarwo & Karaki, 1995). From a study in isolated small branches of rabbit pulmonary artery, Yoshida *et al.*, (1995) proposed that in this preparation ET-1 activates both ET_A and ET_B receptor

subtypes to enhance Ca^{2+} influx and to release Ca^{2+} from intracellular storage sites, and that stimulation of ET_A receptor subtype by ET-1 induces contraction mainly through PKC activation. In cultured rabbit vascular smooth muscle cells, ET-1 induced a rapid (15 sec) transient formation of IP_3, suggesting that the PIP_2 pathway is an initial event following ET-receptor activation (Marsden, *et al.*, 1989). The irreversibility of the ET-1-induced responses is due to a late signalling event for which PKC may be partly responsible (Marsault, *et al.*, 1991). PKC activation also stimulates DNA synthesis, gene transcription and mitogenesis (Simonson, 1993), thus appears to mediate the long term effects of ET-receptor activation.

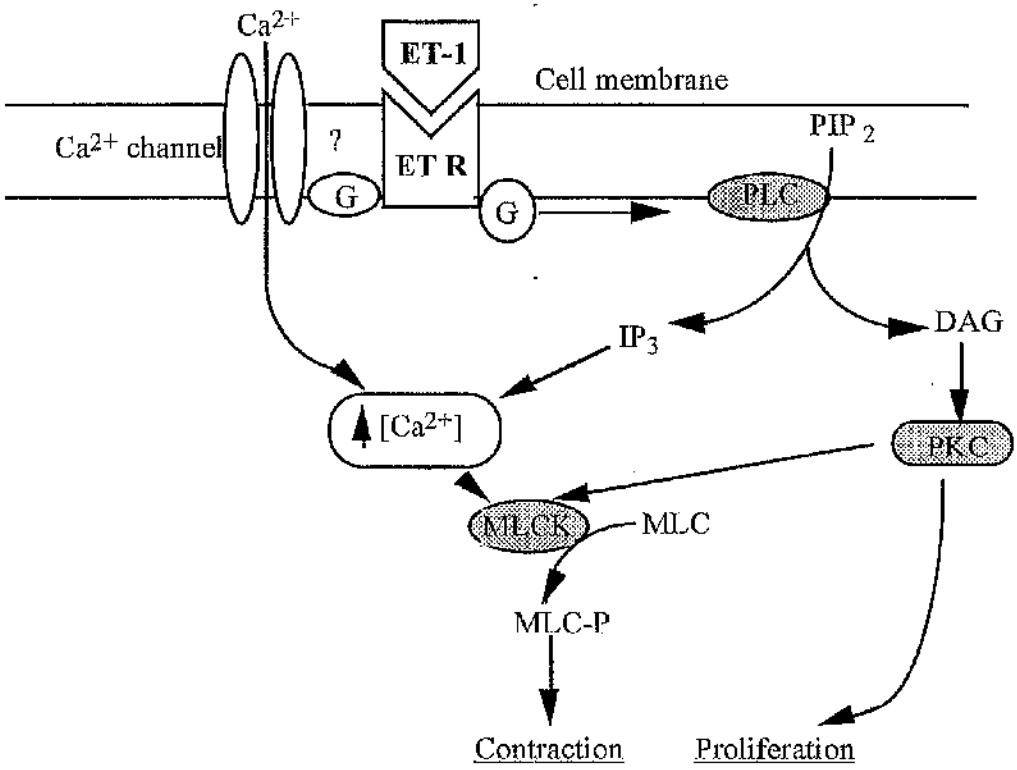


Figure 1.5 ET-1 intracellular signalling pathways. Main intracellular signalling events due to activation of ET_A and ET_B receptors. Refer to text for explanation. ET R = ET receptor; G = G-protein; PLC = phospholipase C; PIP_2 = phosphotidyl inositol bisphosphate; DAG = diacylglycerol; IP_3 = 1,4,5-inositol triphosphate; PKC = protein kinase C; MLCK = myosin light-chain kinase; MLC = myosin light-chain; MLC-P = phosphorylated myosin light-chain; ? = link between ET receptor and activation of Ca^{2+} channel still uncertain.

1.4.7.2 Increase of cytosolic calcium (Ca^{2+}) concentration

The first demonstration of the involvement of extracellular Ca^{2+} in ET-induced vasoconstriction was carried out by Hickey *et al.* (1995), who found that EDCF-induced coronary artery contraction was attenuated by removal of Ca^{2+} from the medium and by the L-type Ca^{2+} channel antagonist verapimil. Since then numerous studies have demonstrated that the many effects, vascular and non-vascular, of ET-1 to be dependent on extracellular Ca^{2+} and inhibited by Ca^{2+} channel antagonists, suggesting the importance of Ca^{2+} influx in the mechanism of action of ET-1 (Hirata, *et al.*, 1988b; Yoshida, *et al.*, 1995). The originally proposal that ET-1 was a possible endogenous direct activator of L-type Ca^{2+} channels (Yanagisawa, *et al.*, 1988), has since been discounted. Thus the observed activation of Ca^{2+} entry via VOC channels in some tissues must be the consequence of indirect gating by ET-1; several possible mechanisms have been proposed. Activation of VOC may be secondary to ET-1-ET-1-induced membrane depolarisation (Takenaka, *et al.*, 1992); however other reports indicate this to be unlikely (Wallnofer, *et al.*, 1989). The most convincing indirect mechanism to date is the involvement of Cl^- channels. Cl^- channel antagonists have been shown to inhibit the ET-1-induced sustained phase of $[Ca2+]_i$ increase and membrane depolarisation of cultured vascular smooth muscle cells (Takenaka, *et al.*, 1992).

1.4.7.3 Other intracellular pathways: ET-receptor mediated vasorelaxation

ET-1 may activate phospholipase A_2 in certain tissues to mediate the release of PGI_2 and TXA_2 through the metabolism of arachadonic acid (AA); this may be one of the mechanisms by which ETs evoke vasodilations. Numerous studies indicate that ET_B-receptor mediated vasodilator response involves activation of NO. (The mechanism of action of NO is discussed in section 1.1.4.3). For example, De Nucci *et al.* (1988) demonstrated the ability of ET to induce the release of EDRF (NO) from isolated perfused mesentery and PGI_2 and TXA_2 from isolated perfused lung. Cultured bovine

endothelial cells were shown to possess specific receptors coupled to PLC, which mediate vasodilation via the release of NO (Emori, *et al.*, 1990). Other studies in this preparation also indicate ET_B receptors to be functionally coupled PLC, and also to adenylate cyclase, possibly via an inhibitory G (G_i) protein (Eguchi, *et al.*, 1993). Activation of these receptors would result in a reduction in intracellular cAMP levels and hence a rise in intracellular free Ca^{2+}, and perhaps stimulate NOS activity resulting in NO production. In general, data suggests that in addition to coupling to PLC via G_q protein, ET_A and ET_B receptors subtypes are coupled to adenylate cyclase via G_s in smooth muscle cells and via G_i in endothelial cells (ET_B receptor).

The involvement of ATP-sensitive K^+ channels has also been indicated in ET_B-receptor mediated vasodilation, such as the vasodilator response to ET isopeptides in adult lung (Hasunma, *et al.*, 1990; Lippton, *et al.*, 1991). Distinct K^+ channels *in vitro* are coupled to pertussis toxin-sensitive G proteins. Other electrophysiological effects of ET-1 have recently been reported. Salter & Kozlowski (1996) demonstrated that in pulmonary arterial myocytes, ET_A receptor stimulation caused activation of Ca^{2+} activated Cl^- and K^+ currents, whereas stimulation of the ET_B receptor mediated a gradual inhibition of the delayed rectifier K^+ current.

A further mechanism for ET-induced vasorelaxation was suggested recently by the demonstration that activation of ET_B receptors can release adrenomedullin in canine aortic cells (Jougasaki, *et al.*, 1997). Adrenomedullin is a recently discovered natriuretic peptide which mediates vasorelaxations in various tissues including pulmonary vessels, and is of vascular smooth muscle and endothelial origin (Shirai, *et al.*, 1997). This finding suggests another important vasoactive system regulated by the ET_B receptor.

1.4.8 Endothelin and the cardiovascular system

1.4.8.1 Haemodynamic actions

Intravenous infusion of ET-1 into conscious, anaesthetised or pithed rats causes a biphasic response, comprising a rapid and transient vasodilation, followed by a profound and long lasting increase in blood pressure (Yanagisawa, *et al.*, 1988; Gardiner, *et al.*,

1990; MacLean, *et al.*, 1989). The initial transient depressor response has since been demonstrated to be mediated via the activation of ET_B receptors, due to the ability of ET_B receptor antagonists such as BQ788 to abolish the response (Ishikawa, *et al.*, 1994), and the ability of SXS6c and ET-3 to mediate the depressor response (Gardiner, *et al.*, 1990; Clozel, *et al.*, 1992). The depressor action of ET-receptor agonists may be due to release of prostacyclin and/or NO (De Nucci, *et al.*, 1988; Gardiner, *et al.*, 1990). The sustained pressor response in the pithed rat occurred with no alteration in cardiac output or heart rate, indicating an increase in total peripheral resistance (MacLean, *et al.*, 1989). Pressor responses in intact animals were initially thought to be mediated via ET_A receptor activation, however it is now known that vascular ET_B receptors contribute to the constrictor response *in vivo* (Clozel, *et al.*, 1992). More recent studies of the ET-receptor subtypes has provided further insight into the biphasic nature of the haemodynamic ET-1-induced response. The ET_B receptor has been shown to be strongly tachyphylactic (Henry, 1993; Sudjarwo, *et al.*, 1994). In addition, Schroeder *et al.* (1997) demonstrated in CHO cells that the ET_B receptor deactivated much more rapidly that the ET_A receptor subtype. Thus the transient vasodilator response noted *in vivo* may be due to rapid ET_B receptor desensitisation, with the sustained activation of ET_A receptors being responsible for the prolonged vasoconstrictor response. Furthermore, the interaction of ETs with their receptors is essentially irreversible (Marsault, *et al.*, 1991); this in part explains the well maintained vasoconstrictor response observed *in vivo* and in isolated vascular preparations (see section 1.4.8.3). The binding of ET-1 to its receptor is rapidly followed by internalisation (Resink, *et al.* 1990) and this may also be important for the protracted action of the peptide.

1.4.8.2 Cardiac actions

High affinity ET-binding sites are present in cardiac tissue (Nayler, 1990). Russell *et al.* (1997) demonstrated the existence of ET_A and ET_B receptors on the media and at the subcellular level of human coronary epicardial arteries, with the ET_A subtype being predominant, however no ET-receptors were detected on the endothelial cells.,

These receptors are likely to mediate the well established vasoconstrictor response to ET-1. ET-1 can evoke both inotropic and chronotropic effects on the heart. In isolated electrically driven rat and human cardiac myocytes, ET was shown to induce positive inotropic effects (Moravec, *et al.*, 1989). In anaesthetised pigs, ET-1 evoked the characteristic biphasic haemodynamic response, and reduced cardiac output and myocardial contractility (Cirino, *et al.*, 1997). SXS6c produced a marked transient reduction in MAP, cardiac output and myocardial contractility. From studying the effects of BQ788 on these responses, the results suggested the involvement of ET_B receptors in the initial transient depressor response and, due to the marked enhancement of the second sustained phase, it appears that ET_B receptors may also act to oppose the vasoconstrictor effects of ET_A receptor activation. Studies in other species such as the rat, however imply that ET_B receptors are not involved in the central cardiovascular actions of ET (Gulati, *et al.*, 1995); these differences in findings may be species related. In addition, both positive and negative chronotropic effects have been observed, which may be due to differences in experimental preparations and the site of ET-1 application (Rubanyi & Polokoff, 1994).

1.4.8.3 Vascular actions

This characteristic slowly developing and well maintained ET-1 induced vasoconstriction first reported by Hickey *et al* (1955) and Yanagisawa *et al.* (1988), has since been observed in vascular preparations of various anatomical origin isolated from a variety of animals and humans. Due to my interest in ET-1 being primarily in the pulmonary circulation, the pulmonary vasculature in particular is discussed in section 1.5.2.

In the majority of preparations the vasoconstrictor response could be attributed to the activation of ET_A receptors on the vascular smooth muscle. This was indicated by (i) the presence of ET_A receptors on these blood vessels (Arai, *et al.*, 1990), (ii) the order of potency of the isoforms (ET-1=ET-2>ET-3) (Masaki, *et al.*, 1994) and (iii) the ability of selective ET_A receptor antagonists to prevent or reverse the response (Cardell, *et al.*,

1993). However in some vascular preparations the ET-1-induced response was only partially inhibited or resistant to ET_A receptor antagonism and vasoconstrictor responses to selective ET_B receptor antagonists were observed; for example, saphenous vein (Sudjarwo, *et al.*, 1994), jugular vein (Sumner, *et al.*, 1992) and pulmonary artery (Warner, *et al.*, 1993) of the rabbit. *In vivo* studies confirmed *in vitro* observations, these included the demonstration of ET_B-mediated vasoconstriction in the pulmonary circulation of the rabbit (Clozel, *et al.*, 1992) and in the systemic circulation of the guinea-pig (Noguchi, *et al.*, 1993).

From studies comparing the effects of ETs on large systemic arteries and corresponding veins, it was generally found that ET-1 was 3- to 10-fold more potent in venous compared to the arterial preparations (e.g. Cocks, *et al.*, 1988a; b). It has been postulated that the greater sensitivity of the venous preparations may be due to activation of different receptor populations, as evidence for vascular ET_B receptors is commonly detected in systemic venous preparations, i.e. the low pressure side of the circulation (Moreland, *et al.*, 1994). Moreover the pulmonary circulation is also a low pressure system (see section 1.5.1). In addition, ET-1 has been shown to be a potent vasoconstrictor of systemic resistance arteries and the microcirculation (Rubanyi & Polakoff, 1994) and these vascular beds are important determinants of the total peripheral resistance.

1.4.8.4 Morphological effects on the vasculature

Substantial evidence indicates that ET-1 may also regulate growth and proliferation of vascular cell types. ET-1 stimulates DNA synthesis and cell proliferation of cultured pulmonary vascular smooth muscle cells (Janakidevi, *et al.*, 1992), and replication of pulmonary artery fibroblasts at high concentrations (Peacock, *et al.*, 1992). This response seems to be mediated by ET_A receptors, as BQ123 (a selective ET_A receptor antagonist) inhibits ET-1 mediated proliferation (Zamora, *et al.*, 1993). The mitogenic effects of ET-1 can be ascribed to its ability to stimulate PIP_2 pathway (see section 1.4.7.1) (Takuwa, *et al.*, 1989). It has been proposed that the growth and

migration of endothelial cells are the prerequisites for vascular remodelling. Morbidelli *et al.* (1995) demonstrated that ET-1 and ET-3 dose-dependently increased proliferation and migration of endothelial cells isolated from bovine adrenal capillaries and human umbilical veins. Furthermore this effect was inhibited by IRL-1038 whereas BQ-123 was ineffective, indicating ET_B receptor activation. In addition, treatment of vascular smooth muscle cells with ET-1 has been shown to induce the expression of other growth factors, suggesting that ET-1 can act by paracrine/autocrine regulatory mechanisms and thereby contribute to remodelling of surrounding tissues.

1.4.9 Clearance and metabolism of endothelin

The importance of pulmonary clearance of circulating ET-1 was initially reported by De Nucci *et al.* (1988). This group demonstrated that ET-1 was substantially removed by the pulmonary circulation in the rat *in vitro* and *in vivo* and by guinea-pig lungs *in vitro*. (~40% of infused amount detected in effluent). In 1994, Fukuroda *et al.* (1994b) reported that in isolated perfused rat lung, ~80% of bolus injected [^{125}I]ET-1 was retained by the lungs after one passage. Moreover, the importance of ET_B receptors in ET-1 clearance by the lungs and kidneys, but not liver, was demonstrated in this later study. More recently, ET_B receptors were shown to be completely and exclusively responsible for pulmonary ET-1 removal in the dog *in vivo*. (Dupius, *et al.*, 1996).

The binding of ET-1 to its receptor has been shown to be rapidly followed by an internalisation of the peptide (Resink, *et al.* 1990). Receptor internalisation may have an obvious function for clearance of ET-1 from the circulation (Ångaard, *et al.*, 1989). Mature ETs may be degraded by the actions of neutral endopeptidases (NEP), as infusion of the NEP inhibitor SQ-29072 significantly increased plasma levels and urinary excretion of ET-1 in the anaesthetised rat (Agassi, *et al.*, 1992).

1.4.10 Potential physiological and pathological role of endothelin in cardiovascular regulation

Several lines of evidence support the view that central ET plays an important role in cardiovascular regulation. ET receptors were shown to be present in high density in the cardiovascular regulatory areas of the brain in the rat, and these were down regulated in the hypothalamus and ventrolateral medulla of spontaneously hypertensive rats (Gulati & Rubello, 1992). Reports such as this and the evidence previously reviewed indicates a speculative role for ETs in the homeostasis of the cardiovascular system. ET-1 may act in a paracrine or autocrine fashion to regulate vascular tone via its direct effect on vascular smooth muscle or indirect interaction with the vascular endothelium. The ability of ET to induce release of the endothelial-derived relaxing factors NO and prostacyclin has been demonstrated (De Nucci, *et al.*, 1988). It may be postulated that the potent effects of ET-1 on the vasculature is modulated by the concomitant release of relaxing factors, with such interaction providing a feedback mechanism in the control of vascular tone. Indeed, previous studies indicate an attenuation of ET-1-induced arterial vasoconstrictions by NO (Raffestin, *et al.*, 1991). In addition, growth and migration of endothelial cells controls vascular remodelling necessary for the healing process and during vascular diseases. ET-1 induced endothelial cell proliferation and migration has been demonstrated (Morbidelli, *et al.*, 1995). The ability of ET-1 to stimulate proliferation of vascular smooth muscle cells and fibroblasts may contribute to the vascular remodelling associated with hypertension and atherosclerosis.

However a pathophysiological role of ETs has been postulated for numerous disease states including those of the cardiovascular system of various aetiologies; e.g. hypertension, myocardial ischaemia, congestive heart failure, stroke and atherosclerosis (Rubanyi & Polokoff, 1994). Thus selective ET-receptor antagonists or inhibitors of ECE may represent future therapeutic agents to alleviate or indeed prevent these conditions. The effect of several antagonists have been examined in various animal models. In a rabbit arterial-occlusion model, the administration of the selective ET_A

receptor antagonist FR139317 prior to but not after the coronary artery occlusion significantly reduced the infarct size (Burke & Nelson, 1997). Vitola *et al.* (1996) reported that exogenous ET-1 caused an elevation in coronary and systemic vascular resistance in a rabbit model of acute myocardial infarction,; these effects were significantly blocked by antagonism of the ET_A receptor with FR139317, however blockade of both ET_A and ET_B receptors, using the non-selective antagonist PD 145065, was required to significantly reduce infarct size.

Yoshimoto, *et al.* (1991) reported that as much as 75% of ET-1 secretion from cultured porcine cerebral endothelial cells was toward the vascular smooth muscle (abluminal) side. It is thought to be unlikely that this pool contributes to plasma ET-1 concentration, thus ET-1 may be regarded more as a paracrine than an autocrine hormone. Until the factors that regulate gene expression are fully elucidated it may be premature to link changes in the plasma ET-1 levels with the progress of a particular pathological disorder, as local regional ET-1 production is perhaps more responsible for the manifestation of effects. Plasma ET-1 levels are nevertheless useful, since plasma concentrations have been shown to correlate well with the severity of disease, such as chronic heart failure and pulmonary hypertension (Kiowski, *et al.*, 1995; Cody, *et al.*, 1992; Yoshibayashi, *et al.*, 1991), and may have prognostic and diagnostic value.

1.5 Endothelin in the pulmonary circulation

1.5.1 Pulmonary actions of endothelin

1.5.1.1 *In vivo* studies

In the pulmonary circulation, complex patterns of responses to ETs have been described. In conscious rats, an increase in pulmonary vascular resistance was detected only after high doses of ET-1 (Raffestin, *et al.*, 1991). Similarly, mild vasoconstriction of the pulmonary vascular bed was observed following i.v. infusion of ET-1 in the anaesthetised dog (Miller, *et al.*, 1989). Whilst in the anaesthetised cat, exogenous ET-1 has been shown to cause both mild (Lippton, *et al.*, 1989) and potent (Minkes, *et al.*,

1990) pulmonary vasoconstrictions. These differences may be due to the dose of ET-1 administered.

In some studies where pulmonary vascular tone was elevated experimentally, such as by increased airway hypoxia in the conscious rat and administration of U46619 in the rat *in vivo*, administration of ET-1 produced pulmonary vasodilation (Hasunma, *et al.*, 1990; Lippton, *et al.*, 1991). The relative potencies of ET isopeptides and the effects of the K^+ channel inhibitor glibenclamide, have indicated a role of ET_B receptors and K^+ Channel activation in the vasodilatory response (Lippton, *et al.*, 1991).

1.5.1.2 *In vitro* studies

ET-1 is a potent constrictor of animal and human pulmonary arteries *in vitro* (Hay, *et al.*, 1993; McKay, *et al*, 1991; Hay, *et al.*, 1996), and is particularly potent in small pulmonary resistance arteries (Leach, *et al.*, 1989; 1992). Similarly, ET-1 and ET-3 has a vasoconstrictor activity in lungs perfused under constant blood flow conditions (Raffestin, *et al.*, 1991; Lipton, *et al*, 1991). As observed in the systemic vasculature (see section 1.4.7), ET-1 causes a slowly developing and prolonged vasoconstriction, and the vasoconstrictor response of pulmonary vessels is thought to be partly mediated via an influx in Ca^{2+} ions via dihydropyridine-sensitive channels and partly via a mechanism that involves protein kinase C activation (Leach, *et al.*, 1990).

ET binding sites in human lung parenchyma have been shown to be localised to pulmonary vascular smooth muscle, particularly in arteries (Brink, *et al.*, 1991; McKay, *et al.*, 1991). The use of selective ET receptor agonists and antagonists has aided the progressive elucidation of ET receptor subtypes involved in the vascular effects. In guinea-pig pulmonary arteries functional responses to ET-1 were more potent than those to ET-2 and ET-3, and the selective ET_A receptor antagonists FR139317 and BQ-123 inhibited the ET-1-induced vasoconstriction, thus indicating ET_A receptor activation (Cardell, *et al.*, 1993). However, other studies indicate the involvement of vasoconstrictor ET_B receptors, such as in the isolated rabbit pulmonary artery (La

Douceur, *et al.*, 1993; Fukuroda, *et al.*, 1994a). In human pulmonary resistance (McCulloch & MacLean, 1995) SXS6c was shown to be more potent that ET-1. The selective ET_A receptor antagonist FR139317 was ineffective against ET-1-induced vasoconstrictions, whilst BMS 182874, another selective antagonist for the ET_A receptor, inhibited responses to only high ET-1 concentrations. These results may indicate a role for ET_B receptors in ET-1-mediated vasoconstriction in human pulmonary resistance arteries.

Evidence for a role endothelial ET_B receptors has also been reported in pulmonary vessels *in vitro*. For example, Fukuroda and co-workers (1992) showed that ET isopeptides evoked relaxation of isolated preconstricted porcine pulmonary arteries. (The possible mechanism of action of a vasorelaxatory response have been reviewed in section 1.4.7.3.) Furthermore, in several pulmonary vessels, including human and guinea-pig pulmonary artery and swine pulmonary vein, there is also evidence for an atypical receptor at which ET-3 is most active (Cardell, *et al.*, 1992; Hay, *et al.*, 1993; Sudjarwo, *et al.*, 1993). These findings have lead to the proposal of a possible existence of a novel atypical ET_B receptor subtype in these preparations; the possible existence of non ET_A / ET_B receptors is reviewed in section 1.4.5.3.

Alteration in ET-1-induced responses have been observed relative to the calibre of the pulmonary vessel examined (Leach, *et al.*, 1989; 1992). In rat pulmonary arteries, evidence has been shown for ET_A receptors mediating the vasoconstriction in the larger elastic pulmonary arteries whilst ET_B receptor activation appeared responsible for vasoconstriction in the pulmonary resistance arteries (Mac Lean, *et al.*, 1994). A similar situation is thought to occur in piglet pulmonary arteries (Perreault & Baribeau, 1995). In contrast to the large blood vessels of the systemic circulation (see section 1.4.8.3), arteries appear to be more sensitive than veins in the pulmonary circulation; this was demonstrated in the guinea pig by Cardell *et al.* (1990). These studies suggested that the increased sensitivity may be due to the differences in blood composition, i.e. comparatively low pO_2 of systemic venous and pulmonary arterial blood. Indeed hypoxia has been shown to increase ET-1 sensitivity in isolated rat mesenteric arterial

bed (Douglas, *et al.*, 1991) and in isolated rat perfused lungs (Eddahibi, *et al.*, 1991). All the aforementioned studies indicate apparent species- and preparation- related differences in the functional effects of ET-receptor agonists on the pulmonary vasculature, and also distinct differences compared to the systemic circulation.

As previously mentioned in section 1.4.8.4 , ET-1 is also a potent mitogenic and proliferative agent, and has also been shown to be a potent stimulus for DNA synthesis and proliferation of bovine, pig and human pulmonary vascular smooth muscle cells in culture (Hassoun, *et al.*, 1992; Janakiden, *et al.*, 1992; Zamora, *et al.*, 1993). In cultured human pulmonary artery smooth muscle cells, ET-1 was considerably more potent than ET-1 and SXS6c, and its effects were blocked by BQ-123, indicating the involvement of ET_A receptors. This receptor subtype has also been suggested to be involved in the chemotactic and mitogenic effects of ETs on rat pulmonary artery fibroblasts (Peacock, *et al.*, 1992).

1.5.2 Physiological role for endothelin in the pulmonary circulation

Besides the effects of ETs on the cardiovascular system, several lines of evidence suggest an important role for these peptides in regulating pulmonary circulatory function. From the marked vascular effects of ET-1 alluded to above, the influence of this peptide in the physiological regulation of pulmonary vascular tone seems plausible. Due to the variation in ET-1 mediated effects from the various studies which have been reported, the actual physiological role is difficult to determine. The vasodilator action of ET-1 could be implicated in the maintenance of low pulmonary vascular tone through interaction with the vascular endothelium, or through direct actions on vascular smooth muscle. However what may be of more physiological importance is that the pulmonary circulation appears to be an important site for ET-1 biosynthesis. ET-1 has been shown to be produced by a number of pulmonary cell types including vascular endothelium (Giaid, *et al.*, 1991), parenchymal cells (Marciniak, *et al.*, 1992), airway epithelial cells (Giaid, *et al.*, 1991) and tissue macrophages (Ehrenreich, *et al.*, 1990). Clearance of ET-1 from the plasma by the lungs may also be

of more physiological importance (see section 1.4.9) and this has been proposed to limit the pressor effects of circulating ET-1 in animal models (e.g. De Nucci, *et al.*, 1988). Furthermore, Stewart *et al.* (1991) demonstrated in normal humans that the arterial to venous ratio of ET-1 was less than unity, suggesting pulmonary clearance of ET-1 in the healthy human lung.

1.5.2.1 Extrauterine adaptation of the pulmonary circulation

Numerous studies provide physiologic support for a role of ET-1 in the maintenance of high pulmonary vascular resistance in the fetus and in the transition of the circulation at birth. Circulating immunoreactive levels of ET-1 are very high in the fetal and transitional circulations (Endo, *et al.*, 1996; Malamitsi Puchner, *et al.*, 1993). However variable haemodynamic and *in vitro* effects of ET-1 have been reported in the perinatal circulation. ET-1 has been demonstrated as a potent vasoconstrictor of isolated small pulmonary vessels from the fetal lamb (Wang & Coceani, 1992). In the ovine fetal pulmonary circulation *in vivo*, a dose-dependent increase in pulmonary blood flow and decrease in pulmonary vascular resistance was observed during blockade of the ET_A receptor with BQ-123 and also with stimulation of the ET_B receptor with SXS6c; both of these vasodilatory responses were inhibited by L-NNA (Ivy, *et al.*, 1994). These findings may suggest that selective ET_A receptor inhibition within the fetal lung allows for preferential binding of ET-1 to the endothelial ET_B receptor, resulting in vasodilation through the NO pathway. Cassin *et al.* (1991) demonstrated that a potent pulmonary vasodilation in response to a brief infusion of ET-1 in the sheep fetus, which has a very high pulmonary vascular resistance. Thus exogenous ET appears to have a complex effects on the perinatal pulmonary vasculature, which may be site, time and tone dependent.

1.5.3 Pulmonary hypertension (PHT)

ET-1 levels have been reported to be increased in several experimental and clinical settings, including enhanced pulmonary preproET-1 mRNA expression and

increased intrapulmonary ET-1 production in rats with idiopathic PHT (Stelzner, *et al.*, 1992), and increased pulmonary gene expression for ET-1 after chronic hypoxia in rats (Elton, et al., 1992). Also, increased plasma ET-1 levels have been found in the pulmonary circulation of patients with primary and secondary PHT (Stewart, *et al.*, 1991; Yoshibayashi, *et al.*, 1991) and in infants with persistent pulmonary hypertension of the newborn (Rosenberg, *et al.*, 1993; Kumar, *et al.*, 1996). These findings and the marked effects of ET-1 on the pulmonary circulation (section 1.5.1) suggests that ET-1 may contribute to the elevated pulmonary vascular resistance of this condition. The reported ability of ET-1 to stimulate proliferation of pulmonary vascular smooth muscle and fibroblasts suggests that it may also play role in the concomitant vascular remodelling (Hassoun, *et al.*, 1992; Janakiden, *et al.*, 1992). It is still unclear, however, if ET-1 is a mediator or modulator of PHT (Stewart, *et al.*, 1991).

Numerous investigations of the effects of ET-receptor antagonists in the pulmonary vasculature of animal and human models has brought about the prospective use of such compounds as possible therapeutic agents. For example, Chen and co-workers (1995; 1997) showed that both bosentan and the novel non-peptide ET_A receptor antagonist A-127722 were effective in preventing the pulmonary hypertensive effect of short-term hypoxia and attenuated the development of PHT. However, of important consideration was the significant elevation in systemic arterial pressure also observed. Due to these differential findings between the various experimental approaches in examining ET-1 in the pulmonary circulation, the question as to whether a selective ET_A or nonselective ET_A/ET_B receptor antagonist would provide a more effective treatment in pulmonary hypertensive diseases is a current topic of great debate. Moreover, the possible effectiveness of the various ET receptor antagonists in man will depend not only on their efficacy in the pulmonary circulation but also on a desirable influence on the systemic circulation.

1.6 Aims of project

In this thesis, I studied the pulmonary vasculature of the rabbit in two differing states; one being secondary to left ventricular dysfunction and hence involving "passive" regulation of the pulmonary vascular tone. In the other, the effect of the transition from fetal to extrauterine existence and during the early stages of neonatal life was studied, i.e. a study of "active" regulation due to changes in O_2. (These states have previously been introduced in this chapter.). These two paradigms in particular were examined since in both situations, the pulmonary vasculature undergoes marked alterations in similar aspects of structure and function. For example, the pulmonary circulation is normally a low pressure system, with low vascular resistance compared to that of the systemic circulation (see section 1.1.3). Whilst *in utero* the lungs do not function and the cardiac output bypasses the fetal pulmonary circulation which is under high pressure and resistance (section 1.2.1). At birth the lungs commence their functional role in life and this is accompanied by a marked reduction in the pulmonary pressure and thus resistance towards values of normal *in vivo* state (section 1.2.2). This variable is also altered but in the opposite direction in the disease state of pulmonary hypertension (PHT), such as secondary to left ventricular dysfunction (LVD), when a marked elevation of the pulmonary pressure/resistance occurs (section 1.3). Included in structural changes in these two states are the loss of pulmonary arterial muscularisation observed in small arteries and also in the elastic vessels during perinatal life (section 1.2.2), whilst in the pulmonary hypertensive lung, structural changes involve medial hypertrophy in the large and small arteries and functional vascular smooth muscle cells become evident in more distal branches of arteries, at the level of the terminal bronchioles, which are normally only partially muscular or non-muscular (section 1.3.1). Hence, although these two models are quite different in nature, the aspects of the pulmonary circulation which are affected are comparable in both.

ET-1 is yet another factor which is common to both these models, since the involvement of ET-1 has also been implicated in numerous conditions including the transitional pulmonary circulation of perinatal life (section 1.5.3.1), and disease states

such as heart failure (section 1.4.10) and PHT of various actiologies including secondary to myocardial infarction and left heart failure and persistent pulmonary hypertension of the newborn (section 1.5.3.2). Thus it is of interest to examine ET-receptor mediated responsiveness of pulmonary vessels from the same species in these two states. Chapters 3-7 show the results of the *in vitro* studies of pulmonary arteries in the rabbit coronary ligation model of left ventricular dysfunction, whilst the examination of the fetal and neonatal pulmonary resistance arteries are presented in chapters 8 and 9.

Chapter 2

Materials and Methods

2.1. Animal models used in studies

2.1.1 Rabbit coronary ligated model of left ventricular dysfunction

2.1.1.1 Introduction to model

Experimental pulmonary hypertension (PHT) can be induced by a variety of techniques, such as monocrotaline or chronic hypoxia. However, in the clinical setting the condition of the heart failure commonly occurs with associated PHT (Rabinovitch, *et al.*, 1978; Trell, 1973). Thus it is important to assess the structural and functional alterations of pulmonary vasculature which can arise from this source.

As with experimental models of PHT, experimental heart failure may also be induced by a variety of techniques (Smith & Nuttal, 1985; Elsner & Reiger, 1991). The rabbit coronary-ligated model of left ventricular dysfunction was first characterised by Pye *et al* (1996). The coronary artery ligations and sham-operations were performed by workers at The University's Department of Medical Cardiology at Glasgow Royal Infirmary. A brief description of this procedure is as follows. Adult male New Zealand White rabbit (2.5-3.5 kg body weight) received premedication using intramuscular fentanyl/fluanizone, 0.3-0.4 ml/kg (Hypnorm, Jansen). Anaesthesia was induced with midazolam (0.2-0.4 ml/kg) and, following intubation of the rabbits, was maintained with a mixture of nitrous oxide, oxygen (1:1 ratio) and 1% halothane.

A left thorocotomy was performed through the 4th intercostal space to expose the heart. Quinidine hydrochloride (3-5 mg/kg) was administered intravenously prior to coronary artery ligation to reduce the incidence of ventricular fibrillation. The major branch of the left coronary artery was occluded approximately midway between the left atrial appendage and the cardiac apex. This gives rise to a large homogeneous infarct due to the poor collateral circulation of the rabbit coronary system. In cases where ventricular fibrillation occurred, defibrillation was undertaken with an 8 joule epicardial shock. When an acceptable area of infarction (approx. 20% of the left ventricle) had been produced and the animal was haemodynamically and electrically stable, the thoracotomy was closed. In sham operated controls, hearts were manipulated as in the coronary ligated animals but the artery was left unoccluded.

Post-operative analgesia (buprenorphine 0.3 mg/kg) was administered every 8 hours for the first 24-48 hours together with a broad spectrum of antibiotics. The investigation conformed with the *Guide for care and use of laboratory animals* (NIH Publication No. 85-23, revised 1985) and with the provisions of the Animals (scientific procedures) Act 1986.

2.1.1.2 Echocardiography

The degree of left ventricular dysfunction in each animal was assessed using echocardiography as described by Pye *et al.* (1996). Briefly, measurements were made under light sedation (Hypnorm 0.3 mg/kg) using a 5 MHz focused paediatric transducer. M mode long axis measurements of left ventricular end diastolic and left atrial internal diameters were made. By rotating the transducer 90°, short axis end-diastolic and end-systolic frames were captured and traced onto the screen via an on-line cineloop computer analysis facility. The ejection fraction (EF) was calculated as:

end diastolic area - (end systolic area / end diastolic area)

Echocardiography was performed after the ligations / sham-operations and when the animals had been on procedure for the desired length of time, i.e. 8, 16 or 32 weeks. They were then transported to the Physiology department of Glasgow University, where they were sacrificed by overdose of sodium pentobarbitone (100 mg/kg), the heart and lungs were promptly removed *en bloc*.

2.1.1.3 Assessment of pulmonary hypertension (PHT)

(i) Using ventricular weight ratios

In both clinical and experimental forms of pulmonary hypertension, thickening of the right ventricular wall is observed. The degree of right ventricular hypertrophy gives an index of the development and the degree of pulmonary hypertension (Hunter, *et al.*, 1974).

Procedure for ventricular measurements

Rabbits from 8 week sham-operated and coronary-ligated groups were overdosed with sodium pentobarbitone (100 mg/kg). The thoracic cage was opened and the heart and lungs removed. Atria and associated large calibre vessels were dissected from the ventricular mass, and the heart was dissected so as to isolate the free wall of the right ventricle from the left ventricle plus septum as described by Fulton *et al.* 1952. The ventricles were washed in Krebs solution, blotted dry on a tissue and then wet weight were measured on a Mettler AT261 balance. As the model used was one of left ventricular dysfunction, hypertrophy of the left ventricle in the ligated animals was observed, thus the commonly used ratio of right ventricular (RV) free wall weight to left ventricle plus septum was not suitable. The ratio of RV weight to body weight was used as an index of right ventricular hypertrophy. (No difference in body weight was observed between the two animal groups). This has been shown to be a reliable index of the degree of PHT present in experimental animals (Hunter, *et al.*, 1974; Wanstall & O'Donnell, 1990).

(ii) Histological analysis of pulmonary vasculature

Pulmonary vascular remodelling can be assessed histologically, by estimation of the number of thick-walled peripheral pulmonary vessels. Thick walled peripheral vessels (TWPV) are generally characterised by their proximity to the alveolar ducts, their size, and the nature of their elastic coat.

Procedure for examination of TWPV

Lungs from the sham-operated and coronary-ligated rabbits were obtained as described in section (i) above. The lungs were washed in Krebs solution before being inflated and fixed in 10% formal saline. A transverse section of the middle right lobe was processed and embedded in paraffin wax under a vacuum. 5μm sections were cut using a microtome and mounted on slides. These sections were stained for elastic tissue and smooth muscle using elastic-Van Gieson (EVG) stain; this was as follows.

Sections were returned to water, soaked in 0.25% potassium permanganate for 1-5 minutes and then re-washed in water. The sections were then bleached in 1% oxalic acid for approximately 1 minute or until the tissue went white and again washed in water, before rinsing in 95% alcohol. Now rehydrated, the sections were soaked in Millers staining solution for 1 hour. Excess stain was removed by washing in 95% alcohol before being washed in water. Following this, sections were counterstained with van Gieson for 2 minutes and were then dehydrated, cleared and mounted.

The resulting sections were examined using a Zeiss Axiophot microscope. A double elastic lamina was said to be present when two laminae with a space between visible for at least half the diameter in cross section. The appearance of 100 vessels in sections obtained from an appropriate number of lungs was noted and quantified for the percentage of TWPV. Sections stained with EVG and also sections which were processed with a Haematoxylin/eison (H&E) stain, were photographed using Kodak Ektachrome 64 film.

Electron microscopy

This process was carried out by Dr Ian Montgomery, I.B.L.S.. Pulmonary resistance arteries were dissected from sham-operated and coronary-ligated rabbit lungs according to the methods stated in section 2.2.4.1. The vessels were fixed for 1 hour using 2% glutaraldehyde in 0.1M sodium cacodylate buffer at pH 7.2 (Sabatini, *et al.*, 1963). the specimens were then given three times 20 minute washes in 0.1M sodium cacodylate buffer at pH 7.2. Post fixation was carried out using 1% osmium tetroxide in 0.1M sodium cacodylate buffer at pH 7.2 . Following fixation, the specimens were dehydrated with graded alcohol and embedded in araldite.

Ultrathin sections were cut with a diamond knife on a L.K.B.3. Ultrotome and mounted on Formvar coated 1000μm aperture grids. The ultrathin sections were double stained with uranyl acetate (Stempak & Ward, 1964) and lead citrate (Reynolds, 1963). The stained sections were examined using J.E.O.L. 100S electron microscope at 80kV and electron micrographs taken on Kodak electron microscope film.

Confocal Microscopy

This process was carried out by Craig Daly, I.B.L.S.. Pulmonary resistance arteries were dissected from the lungs of 8 week sham-operated and coronary-ligated rabbits as described in section 2.2.4.1. Artery segments were incubated with the vital nuclear stain Hoechst 33342 (1 μg/ml) for 60 minutes in the dark at room temperature. After 4 washes in fresh Krebs solution, segments were cut open and placed lumen side up in the well of a glass slide. The grease well was flooded with fresh Krebs solution and a coverslip was placed on top. Slide mounted artery segments were then visualised using laser scanning confocal microscopy (LSCM, Odyssey XL, Noran Instruments). The Odyssey scan module was fitted to a Nikon Optiphot microscope (objective; 40x oil, NA 1.3). Excitation of Hoechst was achieved with the 364 nm line of a UV argon ion laser (em 400). For image collection, identical levels of laser intensity (100%), AOD power, and levels of brightness and contrast were used. Digital images were collected using a 64 frame average to reduce noise. Smooth muscle cells were identified as being those cells whose nuclei lay beneath the (autofluorescent) internal elastic lamina and whose orientation was perpendicular to the axis of the blood flow, as indicated by the long axis of the endothelial cell nuclei. Images of smooth muscle cell and endothelial cell nuclei were collected at intervals of 0.5μm along the axial plane (Z-axis) to produce an image stack. A series of images (the stack) was then combined to produce an extended focus view of all nuclei in all planes.

(iii) Measurements of lung weight

When left heart failure causes a rise in pulmonary venous pressure, this can lead to lung congestion resulting from oedema (Coalson, *et al.*, 1967; Kay & Edwards, 1973). This was examined in the rabbit LVD model by comparing lung weights of control animals and those of animals with left ventricular dysfunction. Lungs were obtained from sham-operated and coronary-ligated rabbits as stated above. Prior to the dissection of pulmonary arteries for experimental studies, all connective tissue and fat were removed and associated large calibre vessels were dissected from the lung mass.

This was to ensure that all lungs weighed only consisted of the actual lobes thus eliminating variability. Lungs were washed in Krebs solution, excess fluid was removed, and were weighed using a Mettler B3002 balance. The ratio of lung weight to body weight was assessed.

2.1.2 Fetal and neonatal rabbits

Female New Zealand White rabbits, pregnant with their first litter, were purchased from the credited commercial suppliers Harlan UK Ltd.. The animals were ordered in at approximately 23 days gestation, i.e. 7 days prior to their estimated date to litter down, and kept in the Central Animal Facility of the University of Glasgow. For the purpose of the experimental studies, the rabbit pups were used either 2 days preterm (fetal), 0-24 hours, 4 days or 7 days after birth. Animals were sacrificed with an overdose of sodium pentabarbitone (200 mg/kg). To obtain fetal pups, the mother was overdosed and a caesarean section was performed, fetal rabbits were then rapidly decapitated so as to ensure that a first breath was not taken. The lungs were then rapidly removed and placed in a beaker of ice cold Krebs solution. Pulmonary arteries were then dissected free, as described in section 2.2.4.2, for the experimental studies.

2.1.3 Human tissue

Whenever possible studies were carried out on human pulmonary arteries. As this tissue was only rarely available throughout my period of study, only limited investigation could be performed. Hence, I focused on key experimental results obtained in the animal models used. Macroscopically normal sections of lung tissue were obtained from patients undergoing surgery for bronchial carcinoma, who did not have evidence of any other chronic lung disease. Samples were supplied by the Royal and Western Infirmaries, Glasgow. Samples were refrigerated in fresh Krebs solution on site, collected and studied no longer than 12 hours post-operative. Details of individual patient histories are not known. The treatment provided for each patient is variable and depends strictly on individual needs.

2.2 Functional pharmacological studies of isolated vessels using wire myography

2.2.1 Background to technique

Until the mid-1970s, most of the information about the mechanical and pharmacological properties of vascular smooth muscle was confined to large diameter vessels, with the rat tail artery being the smallest vessel studied *in vitro*. Information regarding small vessels could only be inferred from perfusion experiments and histological examination. However a technique first described by Bevan and Osher (1972) for investigating smaller vessels with internal diameters own to 100 μm was developed and the small vessel wire myograph was first described by Mulvany and Halpern in 1976. This was an important advance in the investigations of blood vessels *in vitro* as it now allowed measurement of isometric responses of small resistance arteries. Measurements of pressures taken from various regions of the systemic vascular tree demonstrated that in some vascular beds, at least 50 % or more of the pre- capillary pressure drop occurs in vessels with diameter greater than 100 μm (Bohlen, 1986). Therefore vessels with internal diameters ranging from 100-400 μm must contribute substantially to peripheral vascular resistance. The technique which was developed is suited to vessels with internal diameters of 100-400 μm.

Initial studies involving wire myography concentrated on systemic resistance arteries, however the technique can be applied to any small tubular structures such as pulmonary vessels, small veins, bronchi or ureter (e.g. Leach *et al.*, 1992; Chopra, *et al.*, 1994).

2.2.2 Myograph equipment

Mulvany / Halpern small vessel wire myograph models 500A, 510A and 600M were used in the following experiments. The myographs consists of a stainless steel organ chamber, in models 500A and 510A, which can house 2 vessel preparations. Model 600M consists of 4 individual organ chambers and one vessel can be mounted in each. The vessels are mounted on the myograph by means wire (see mounting procedure section 2.2.5) to vessel support heads shown in figure 2.1.

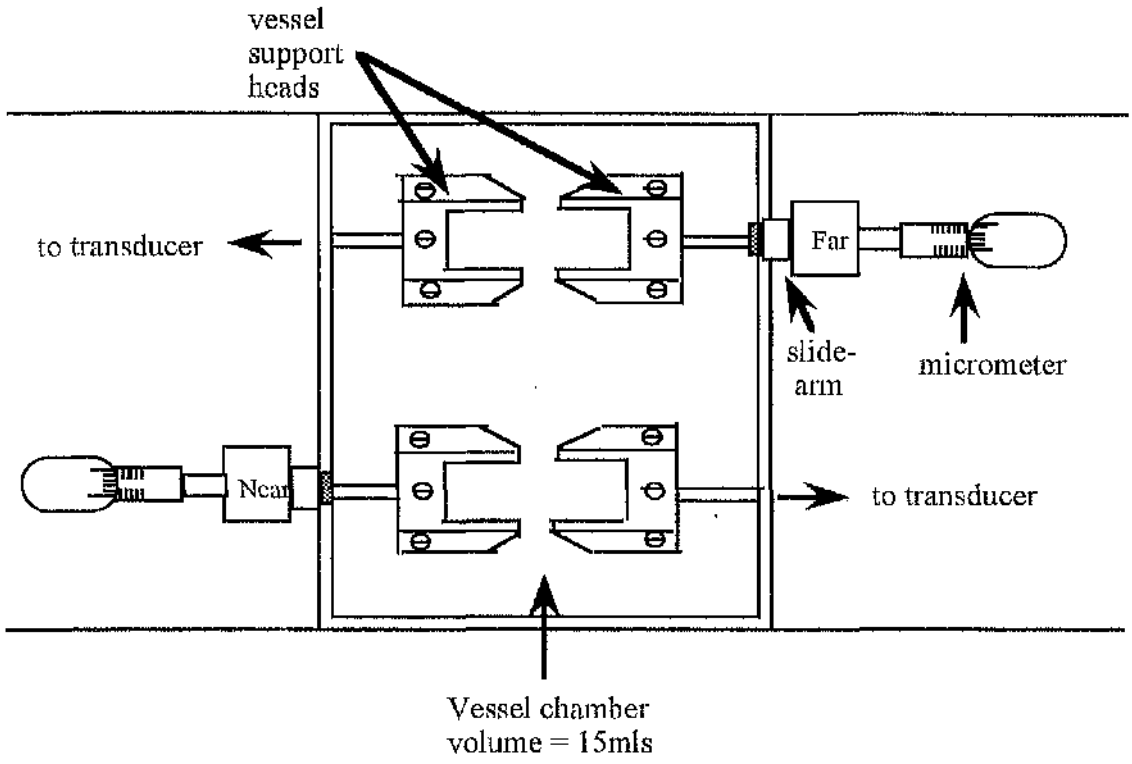


Figure 2.1 Illustration of Mulvany/Halpern wire myograph model 500A. An isolated vessel can be attached in between the vessel support heads by means of two wires inserted through vessel lumen, with either end secured around the top and bottom screw of the support head. Isometric tension is measured by the transducer.

One of the vessel supports is attached to a sensitive isometric force transducer. The other is attached to an adjustable support arm which is controlled by a micrometer. The micrometer allows the distance between the supporting heads to be accurately adjusted, allowing tension to be placed on the preparation.

The temperature of the chamber is electronically controlled via internal heating pads (within 0.1°C). A digital readout of force (all models) and temperature (models 500A and 510A) is displayed on a separate myograph controller module. This in turn has an output connection to a Linseis 4 channel chart recorder (model 6514B), to allow a hard copy of the force readout from each transducer.

2.2.3 Dissection of pulmonary resistance arteries

2.2.3.1 Adult rabbit

The left lung was cut free and pinned to the dissecting dish with its visceral surface exposed and parietal surface lying inferiorly. During the dissection, the preparation was frequently washed with ice cold Krebs solution. The dissection is illustrated in figure 2.2. An incision was made along the superficial aspect of the bronchus, cutting from the large proximal airway along the bronchial tree to the distal bronchus / bronchiole. Once completed, using a dissecting microscope, the associated pulmonary artery branching parallel to the bronchus in the bronchovascular bundle was then easily identified beneath the bronchiole wall. The bronchiole tissue was then gently dissected free and removed from the artery beneath. The lung tissue lateral to the artery was carefully dissected free, and the distal segment of the artery (~150 μm internal diameter) was removed. Isolated arteries were placed into a vial of ice cold Krebs solution, in preparation for mounting in the myograph.

2.2.3.2 Fetal and neonatal rabbit

This dissection was similar to that described for pulmonary arteries isolated from adult rabbit lung. However, due to the comparatively smaller size of the lung lobe and the associated arterial vascular tree, slight alterations were made. The left lung was prepared as for the adult preparation. Using a dissecting microscope, an incision was made along the superficial aspect of the bronchus and the lung tissue lateral to the associated pulmonary artery branching parallel to the bronchus was carefully dissected free, as previously described. This first branch of the main intrapulmonary artery (~300μm) was removed and placed into a vial of ice cold Krebs solution, in preparation for mounting in the myograph. Smaller vessels could not be used since the wall of these arteries were extremely delicate thus making them difficult to dissect free and clean of surrounding parenchymal tissue without damaging them.

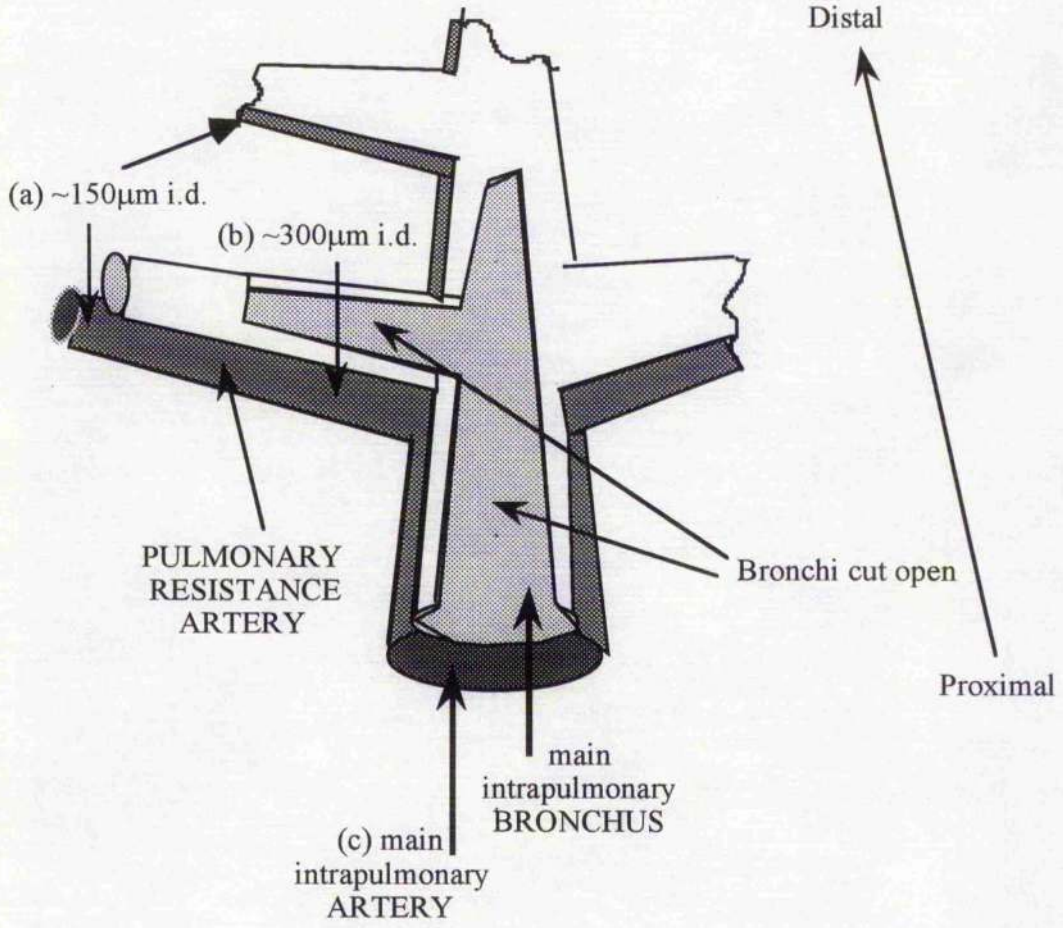


Figure 2.2 Diagram of dissection of pulmonary arteries. See text for details. (a) Distal segment (~150 μm) of pulmonary resistance arteries isolated from adult rabbit lungs. (b) Proximal segment (~300 μm) of first branch of main intrapulmonary artery isolated from fetal and neonatal rabbit lungs. (c) Main intrapulmonary artery and pulmonary resistance arteries were dissected free for biochemical studies (see section 2.3).

2.2.3.3 Human

Due to obvious differences in the surgery required for individual patients, the location of the area of lung removed during surgery, and the size of the section obtained varied between samples. However, great care was taken to ensure that vessels of the appropriate size were removed from each lung sample to minimise variation.

The lung samples were placed and pinned in a Krebs filled dissecting dish. and a large pulmonary artery segment was identified. The artery was followed along its

branching pathway. As the branches decreased in size, using a dissection microscope, the artery was followed further along its branching network until pulmonary resistance arteries of appropriate size (~200 μm internal diameter) were located. The identification of a resistance artery was verified by the proximity of the accompanying bronchiole. This was found to be a useful tool when only small sections of lung were obtained. The arteries were cleaned of surrounding tissue, removed and placed in a vial of ice cold Krebs solution in preparation for mounting in the wire myograph.

2.2.4 Mounting procedure

Two different models of Mulvany/Halpern small vessel wire myograph (J.P. Trading) were used. Vessels were either mounted as pairs in the same bath of the myograph models 500A and 510A, or singularly in the individual baths of the 600M model. The mounting procedure is similar to that described by Mulvany & Halpern (1977) for small systemic arteries but with a slight modifications.

The mounting procedure for pulmonary vessels is shown in figure 2.3.. Isolated small muscular pulmonary arteries were placed in a petri dish containing Krebs, and whilst gently holding onto the cut end of the artery, a 40μM stainless steel wire was carefully passed through the vessel lumen. **(1)** This was then transferred to the bath of the myograph which contained Krebs solution. The wire was secured between the mounting heads, such that the artery segment was positioned in the gap between the mounting jaws. **(2)** The free ends of the wire were then secured to the left mounting jaw by winding clockwise around the top and bottom screws which were then tightened. **(3)** The heads were then separated and any excess vessel that lay outside the jaw was carefully cut away. The length of the arterial segment in the myograph was ~2mm, the approximate length of the gap between the two heads. **(4)** A second 40μM wire was then carefully passed through the lumen of the vessel. **(5)** The mounting heads were then closed and the free ends of the second wire were secured around the screws of the right mounting head. **(6)** The mounting heads were then separated and, following adequate equilibration time period, the appropriate tension could now be placed on the vessel.

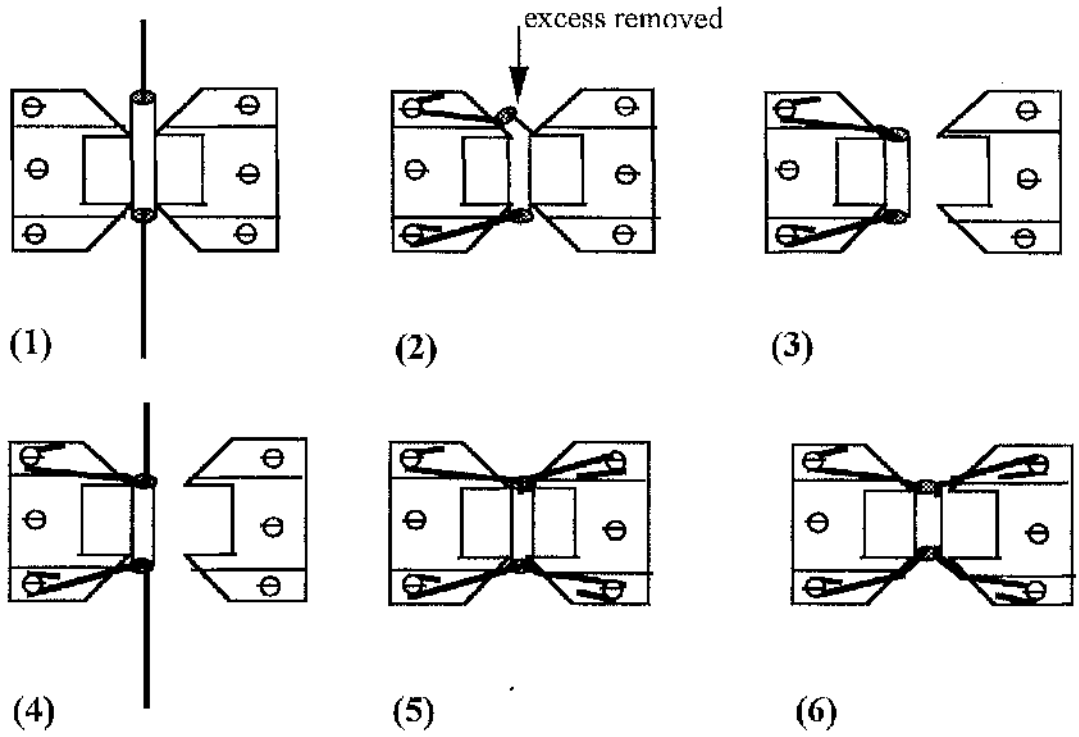


Figure 2.3 Mounting procedure for an isolated pulmonary resistance artery. Refer to text for description of points **(1)** to **(6).**

When the vessels were mounted, the Krebs was bubbled with the appropriate gas mixture (see section 2.2.8) and the heating system was activated to increase the temperature of the Krebs to 37°C.

2.2.5 Normalisation procedure

The normalisation procedure was originally described by Mulvany and Halpern in 1977. This procedure allows vessels to be stretched to a required resting transmural pressure. The Laplace relationship (*) can derive the wall tension in a cylinder if it's radius and the pressure within it are known. It can therefore be modified to give the relationship between these three variables for a vessel mounted on the wire myograph.

$$P_i = \textit{Wall tension} / (\textit{internal diameter} / 2\pi) \qquad (*)$$

Where,

P_i is the effective pressure. This is an estimate of the pressure which would be necessary to extend the vessel to the measured internal diameter,

Wall tension is the force divided by twice the vessel length (L). Twice the vessel length is used since there is both an "upper" and "lower" wall. i.e.

$$\text{Wall tension} = \text{Force (F)} / (\text{L} \times 2)$$

Internal circumference (IC) can be calculated after a given amount of stretch (1) has been placed on the vessel, as follows,

$$IC_1 = (\text{micrometer reading at 1 - micrometer reading at point B}) \times 2 + (IC_B)$$

Where point B is the point at which the two securing wires are just touching. When using 40μM diameter wires, IC_B would equal 205.6 μm. The difference in the micrometer readings between the two points will be equivalent to the distance between the two wires.

Rearranging equation (*) gives,

$$Pi = (2\pi) \times \text{wall tension} / IC$$

Thus substituting values for wall tension and internal circumference gives,

$$Pi = (2\pi) \times F / 2 \times L\,(205.6 + (2 \times \text{distance between wires}))$$

Therefore by using this relationship vessels could be stretched to mimic a required equivalent transmural pressure.

2.2.6 Tension applied to vessels

The majority of systemic arteries are normalised to a pressure of 0.9 of L100, where L100 is the diameter which the vessel would have if relaxed and under a pressure of 100mmHg. When set under these conditions the equivalent pressure of the vessel is normally in the range 60-70 mmHg, depending on the vessel type. As the pulmonary circulation normally operates under conditions of low pressure and resistance, it is clearly more suitable for these vessels to be set up under more appropriate conditions. It was therefore decided that arteries taken from the lungs would be tensioned to give an

equivalent transmural pressure of ~16 mmHg. This is approximately the pulmonary pressure which would be experienced *in vivo* (Fishman, 1976).

Note

In the rabbit coronary-ligation model (8 week), *in vivo* measurements of pulmonary arterial pressure demonstrated a marked elevation in coronary-ligated rabbits compared to sham-operated. However, these measurements were not available until near the end of the functional studies shown in this thesis. Thus, although it may have been more appropriate to set up vessels isolated from coronary-ligated animals under comparatively greater tension, the *in vivo* evidence for this was only hypothetical at the time when the majority of the functional studies were carried out.

2.2.7 Gas mixtures

As the pulmonary vasculature is actively sensitive to O_2 and CO_2 concentrations, a gas mixture that would mimic physiological conditions was chosen. The contents of the gas mixture used for "bubbling" tissues *in vitro* was as follows: 16% O_2, 5% CO_2 and balance N_2. Bubbling the Krebs bicarbonate solution with this mixture gives a final bath O_2 tension of approximately 100- 110 mmHg and CO_2 tension of 35- 36 mmHg, with pH 7.4 (measurements taken by oxygen electrode and blood gas analyser), which are values equivalent to those found in pulmonary arteriolar blood. This gas mixture was used when studying vessels from neonatal and adult rabbits, the rabbit coronary ligation model and those from human lung samples, thus minimising variables between the different groups.

Isolated arteries from fetal rabbits however were bubbled with a gas mixture containing 3% O_2, 5% CO_2, balance N_2, thus mimicking the hypoxic conditions which exist *in utero*. This is much more appropriate than the O_2 level used in the other preparations, considering that a major factor instigating the structural and functional alterations in the pulmonary circulation at birth is the loss of the hypoxic pulmonary vasoconstrictor response, as the newborn inhales the comparatively hyperoxic atmospheric air.

2.2.8 Calibration of equipment

The myograph equipment was calibrated on a regular basis using a calibration balance and 2g weight. The calibration device consists of a horizontal arm with a "pan" on each side and a vertical transducer arm attached to the mid point of the pan arm. This is carefully placed behind a wire which is secured to the mounting head which is connected to the isometric force transducer. The principle is that when a load W (e.g. 2 g weight) is placed on the appropriate load pan, the force transducer is subjected to a force (F) which is equal to

$$F = W \times g \times (\text{pan arm} / \text{transducer arm})$$

where, (1) g = 9.81 mN / gram and (2) pan arm / transducer arm = 2 cm /4 cm, thus "arm ratio" = 0.5.

Hence, calibration with a 2 gram weight gives

$$F = 2 \times 9.81 \times 0.5 = 9.81\text{mN}$$

Thus on calibrating the myographs, the magnitude of the deflection shown on the pen recorder readout was equivalent to 1 g weight or 9.81mN. This could then provide (1) a calibration factor (α), which is the value of the force expressed in mN for a given deflection, to be used in the calculation of force in the normalisation procedure (see section 2.2.6), and (2) the force in mg weight tension for a given deflection, thereby allowing the absolute magnitude of the functional responses to be calculated in the experiments.

Little variation was observed in the readings from calibration to calibration.

2.2.9 General experimental procedure

The general procedure for *in vitro* wire myography experiments are listed as follows. Details may vary between individual experiments, therefore the precise details are given in the methods section of each results chapter.

(1) After the arterial segments were mounted in the myograph, prior to the addition of any drugs, vessels were allowed to equilibrate for one hour in Krebs solution maintained at 37°C and bubbled with the appropriate gas mixture.

(2) Vessels were then stimulated with 50mM KCl to verify tissue viability. Once the contractile response reached a plateau, the vessels were then washed at least 6 times with fresh Krebs solution and allowed to return to baseline tension. The stimulation with 50mM KCl was repeated a second time and this provided a reference contractile response.

(3) The vessels were left for a further 30 minutes before the addition of any drugs.

(4) A cumulative concentration response curve (CCRC) was then constructed to the required agonist covering a range of concentrations which included a threshold and maximum response (if possible).

(5) For agonists which irreversibly bind to their receptor(s), only one CCRC could be conducted in each preparation. Therefore, separate vessels were used to perform experiments using inhibitors/ antagonists. In such experiments, the inhibitor or antagonist was left for it's required incubation period before the CCRC to the agonist was performed.

2.3 Measurement of intracellular cyclic nucleotide concentrations

2.3.1. Introduction

Intracellular cyclic AMP (cAMP) and cyclic GMP (cGMP) concentrations were quantified by using a modified version of the technique described by Brown *et al.* (1972) and Cailla *et al.* (1976), respectively. The principal of the cAMP assay is based on competition between unlabelled cAMP to be quantified and a fixed, known amount of labelled nucleotide ([^{3}H]cAMP) for a binding protein which has a high affinity and specificity for the cyclic nucleotide. Whereas the cGMP assay is based on competition between the unlabelled cGMP and a fixed, known amount of [^{125}I]cGMP for an antibody which has a high affinity and specificity for cGMP.

2.3.2 Preparation of samples

Lungs were obtained from 8 week coronary-ligated rabbits and age-matched sham-operated rabbits and were immediately placed in ice cold Krebs solution. Sections

of artery from different locations in the pulmonary vascular tree were then dissected free; these were the main intrapulmonary artery and pulmonary resistance arteries (see figure 2.2). The isolated arteries were cleaned of surrounding parenchymal tissue, placed in a vial containing the reaction solution for the particular experiment, (see section 2.3.3) and incubated for 15 minutes at 37°C. The tissues were then rapidly removed from the solutions and placed in pre-weighed eppendorf tubes. The eppendorfs were re-weighed and the tissues were then rapidly frozen in liquid nitrogen and stored at -80°C for the second part of the protocol. The weight of intrapulmonary arteries for each sample were typically 10-12 mg, whilst samples of resistance arteries tended to weigh approximately 4-6 mg.

The frozen tissue was homogenised in 200µl of 4% perchloric acid (PCA), briefly vortexed and left overnight at 4°C. The samples were sonicated on ice for 10 minutes and centrifuged for 10 minutes at 12000 rpm. For each sample, 150µl of the supernatant was removed into a fresh tube and 5µl of universal indicator was added. and This was neutralised by the addition of 1.5 M KOH and the final sample volume was noted. If the neutral pH point was passed, this was corrected by the addition of an appropriate volume of 0.04% PCA. All samples were then stored at -20°C for cAMP or cGMP determination.

2.3.3 Experimental conditions

Measurement of the intracellular cyclic nucleotides was made under various experimental conditions. Both cAMP and cGMP levels were determined under basal conditions. In this case, the arteries were incubated in Krebs solution containing 1mM isobutylmethylxanthine (IBMX). The effect of ET-1 was examined via incubating the isolated arteries in Krebs solution containing 0.1µM ET-1. For cAMP measurements, tissues were also incubated in a solution of 10µM forskolin, and the effect of ET-1 on forskolin-stimulated cAMP was investigated following incubation of tissues in Krebs containing 10µM forskolin plus 0.1µM ET-1. The general phosphodiesterase inhibitor IBMX (1mM) was present in all reaction solutions.

2.3.4 Protocol for cAMP assay

The assay procedure for the measurement of intracellular cAMP concentration is given below. Using assay buffer, serial dilutions of stock solution of unlabelled cAMP (16 pmol/ 50µl) were prepared giving corresponding values in the assay of 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8 and 16 pmol / 50µl. These were used to prepare a standard curve for unknown cAMP determination by incubating with a fixed concentration of labelled cAMP and binding protein, also used for unknown samples as shown below.

[5', 8-^{3}H]-cAMP was diluted in assay buffer to give approximately 500 000 c.p.m./ml. Binding protein was diluted 1:25 in assay buffer for use. Samples of unknowns were performed in duplicate and were set up as shown below:

	Sample	Buffer	^{3}H-cAMP	Binding protein
Background	-	200 µl	100µl	-
Total bound	-	100 µl	100 µl	100 µl
Standards	50 µl	50 µl	100 µl	100 µl
Unknowns	50 µl	50 µl	100 µl	100 µl

The assay tubes were set up in an ice slurry bath. The incubation process was initiated by the addition of binding protein, hence this was added last. Tubes were briefly vortexed, and incubated for 2 hours at 4 ^{0}C to allow the reaction mixture to reach equilibrium. Following this, unbound cAMP was precipitated by the addition of 250 µl of charcoal solution which was prepared 15 minutes prior to use and continuously stirred in an ice slurry bath. Tubes were rapidly vortexed then centrifuged at 12 000 g for 5 minutes at 4 ^{0}C, to sediment the charcoal containing cAMP which had not bound to binding protein during the incubation. A volume of 300 µl of each supernatant was taken to which 3 ml of scintillation fluid was added and vials were briefly vortexed. ^{3}H-cAMP concentration was assessed by liquid scintillation counting, incorporating a purpose-designed curve fitting programme.

The results are expressed as pmol cAMP / mg weight of tissue. This was calculated as follows:

([(cAMP average / sample volume) x total vol.] / 3) x 4) / mg weight tissue

where, cAMP average = mean value of the duplicate sample expressed in pmol

sample volume = 50 µl

total volume = 200 µl (PCA) + vol. added for neutralisation

This value is divided by 3 and multiplied by 4 since in preparing the samples, tissues are originally in 200 µl of PCA however only 150 µl is removed for further processing, i.e. only counting 3/4 of sample. This is then divided by the mg weight of tissue for that particular sample, to give pmol cAMP / mg weight of tissue.

2.3.5 Protocol for cGMP assay

The assay procedure for the measurement of intracellular cGMP concentration was similar in only some respects as that described above for cAMP thus, details of important differences are given below. Using the acetate assay buffer, various dilutions of 2 mM stock solution of unlabelled cGMP (0.69 mg/ml) were prepared to give a stock solution of 40mM which contained 2 pmol/50µl and serial dilutions of this were made, giving corresponding values in the assay of 0.008, 0.0156, 0.03125, 0.0625, 0.125, 0.25, 0.5, 1, 2 pmol / 50µl. These were used to prepare a standard curve for unknown cGMP determination by incubating with a fixed concentration of labelled cGMP and binding protein, also used for unknown samples as shown below.

[^{125}I]-cGMP was diluted in acetate buffer to give approximately 130 000 c.p.m. / ml (i.e. ~ 6500 cpm / assay tube). A 1:20 stock solution of anti-cGMP antibody was diluted in Tris buffer to give 1:5000 dilution per assay point. Samples of unknowns were performed in duplicate and were set up as shown below:

	Buffer	Sample	[^{125}I]cGMP	Antibody
Background	100 µl (acetate)	-	50 µl	-
	* 100 µl (Tris)			
Total bound	100 µl (acetate)	-	50 µl	100 µl
Standards	50 µl (acetate)	50 µl	50 µl	100 µl
Unknowns	50 µl (acetate)	50 µl	50 µl	100 µl

Note * The antibody dilutions were made using the Tris buffer and since the antibody is not added to the "background" tube, 100 µl of Tris buffer was added to this tube so that all tubes would contain Tris buffer.

The assay tubes were set up in an ice slurry bath. The various solutions were added to the tubes in the order shown, hence since the incubation process was initiated by the addition of antibody, this was added last. Tubes were briefly vortexed, and incubated overnight at 4 ^{0}C to allow the reaction mixture to reach equilibrium. Following this, unbound cGMP was precipitated by the addition of 1000 µl of charcoal-dextran solution which was continuously agitated to ensure complete mixing. Tubes were rapidly vortexed then centrifuged at 12 000 g for 15 minutes at 4 ^{0}C. This was to sediment the charcoal containing cGMP which had not bound to binding protein during the incubation. The entire volume of each clear supernatant was removed using a clean glass pasteur pipette and placed into gamma counter tubes. [^{125}I]cGMP concentration was assessed using a gamma counter, incorporating a purpose-designed curve fitting programme. The results are expressed as pmol cGMP / mg weight of tissue and this was calculated as described for cAMP in section 2.3.4..

2.4 Radioligand Binding

2.4.1 Introduction

Radioligand binding assay is a relatively simple but extremely powerful tool for studying receptors and therefore, is an important technique in many biological sciences. The principal of radioligand binding is incubation of a biologically active radioligand

with a receptor preparation until a steady state has been reached. Bound radiolabel is then separated from free, either by centrifugation or filtration, and the bound fraction counted.

In the studies of this thesis, ET-receptors of pulmonary arteries were primarily investigated using a functional pharmacological technique. In order to gain further information on the ET-receptor population of this preparation, competition binding studies were performed using rabbit intrapulmonary artery membrane preparations. For a competition experiment, the receptor concentration, the radioligand ([^{125}I]-ET-1) concentration and the time are all constant, with the variable being the concentration of unlabelled competition drug (ET-1). When the drug concentration is zero, only a small fraction of the receptors are bound as radioligand-receptor complex but as the concentration of drug increases, it competes with the radioligand for the receptor binding site. This decreases the concentration of free receptors and thus the concentration of radioligand-receptor complex is also reduced.

2.4.2 Membrane preparation

Lungs were obtained from 8 week sham-operated rabbits and placed immediately in ice cold Krebs solution. Intrapulmonary arteries were dissected free as described in section 2.2.4.1, except all arteries were isolated including the main (axial) artery located within the lung lobe. Isolated vessels were placed in an eppendorf, immediately frozen in liquid nitrogen, and stored at -80°C for preparation of membranes. The procedure for the membrane preparations was as follows. Frozen arteries were pulverised under liquid nitrogen by hand using a stainless steel percusson mortar followed by a pestle and mortar. Powdered frozen tissue (from 3-4 lungs) were homogenised in 2 ml of ice cold assay buffer (see section 2.6.2 for details) on ice, using a precooled polytron for 3 times 5 second bursts at setting 4. The homogenate was filtered through a 100 µm gauze mesh and centrifuged at 600 x g for 5 minutes at 4 °C. The supernatant was centrifuged at 100 000 x g for 30 minutes at 4 °C and the resulting pellet was resuspended in 530 µl of ice cold assay buffer. This suspension was then

homogenised by hand using a needle and syringe, and separated into aliquots for use in ligand binding studies and a 30 µl volume was taken for protein assay. Preparations were stored at -80°C until needed.

2.4.3 Protein assay

Protein content of the membrane preparation was determined using Coomassie Plus protein assay reagent and bovine serum albumin (BSA) as standards. Stock solution of 10 mg/ml BSA was diluted, using buffer for membrane assay, to give corresponding values in the assay of 1, 0.75, 0.5, 0.25 and 0.1 mg/ml; these were used to prepare a standard curve. The assay was carried in a well plate which was set up as follows:

	Sample	Buffer	assay reagent
Background	-	10µl	200µl
Standards	10µl	-	200µl
Sample	5µl	5µl	200µl

All standard and samples were done in triplicate. Thus in order to reduce the amount of membrane preparation used, thereby conserving a maximal amount for the ligand binding studies, a 5 µl volume was used in each well and the result obtained was multiplied by two. The plate was read immediately at 595 nm on a Dynatech plate reader; results were analysed using Protein Program on PC (DELL 425s/NP) and expressed as mg protein / ml.. In the competition assay, the membrane preparation was used at a concentration of approximately 10 µg protein per tube.

2.4.4 Competition study

The protocol for the competition assays between unlabelled ET-1 and [^{125}I]-ET-1 is described below. Stock solution of 50 nM [^{125}I]-ET-1 was diluted in assay buffer (see section 2.6.2 for details) to give a 20 pM stock, thus a 25 µl volume of this in each assay tube with had a final volume of 250 µl, gave a final concentration of 2 pM.

Various dilutions of unlabelled stock solution of 0.1mM ET-1 were made to give final concentrations in the assay within the range 0.01 pM - 30nM; details of this are as follows:

	Buffer	[ET-1] (µl)	=final [ET-1]	[^{125}I]-ET-1	Membrane
(*)	200 µl	-	-	25 µl	25 µl
	175 µl	0.1pM (25 µl)	0.01 pM	25 µl	25 µl
	175 µl	1pM (25 µl)	0.1 pM	25 µl	25 µl
	125 µl	1pM (75 µl)	0.3 pM	25 µl	25 µl
	175 µl	10 pM (25 µl)	1 pM	25 µl	25 µl
	125 µl	10 pM (75 µl)	3 pM	25 µl	25 µl
	175 µl	0.1 nM (25 µl)	10 pM	25 µl	25 µl
	125 µl	0.1 nM (75 µl)	30 pM	25 µl	25 µl
	175 µl	1 nM (25 µl)	0.1 nM	25 µl	25 µl
	125 µl	1 nM (75 µl)	0.3 nM	25 µl	25 µl
	175 µl	10 nM (25 µl)	1 nM	25 µl	25 µl
	125 µl	10 nM (75 µl)	3 nM	25 µl	25 µl
	175 µl	0.1 µM (25 µl)	10 nM	25 µl	25 µl
	125 µl	0.1 µM (75 µl)	30 nM	25 µl	25 µl

Non-specific binding was determined in the presence of 200 nM unlabelled ET-1 and specific binding was defined as total binding (*) minus non-specific binding. All samples were performed in duplicate or triplicate.

The binding reaction was initiated by the addition of the membrane preparation hence this was added last. After a 2 hour incubation at 22°C, separation of bound from free ligand was carried out by rapid vacuum filtration through Whatman GF-B filters which had been presoaked for 2 hours in 0.3% PEI to reduce non-specific binding. Filters were washed 4 times with 4 ml of assay buffer. Radioactivity associated with the

filters was determined with a gamma counter with a protocol for iodide cpm. Specific binding was defined as total binding minus non-specific binding.

2.5 Analysis of Data

2.5.1 Interpretation of results

For *in vitro* measurements of isometric tension, data from preparations undergoing the same protocol were grouped together and expressed as the mean value ± standard error of the mean (SEM). Functional data are expressed as absolute contraction (mg weight tension), percentage of the reference contraction to the second exposure to 50mM KCl (% 50mM KCl), or expressed as a percentage of the own maximum response in each preparation (% own maximum) depending on the agonist used. For relaxatory responses, data is expressed as a percentage of the response relative to the pre-induced tone in each tissue (see individual results chapters).

Data for intracellular cyclic nucleotide levels are expressed as absolute pmol / mg weight of tissue. The correct cpm per sample was calculated by subtracting the blank value in each case. Details of the calculation to express these values as pmol / mg weight is given in section 2.2.3. The n numbers for each group are given in parenthesis and are expressed number of animals or n/ n = number of ring preparations from number of animals (lungs).

For the radioligand binding studies, data was analysed by non-linear least-square curve fitting, for a one-site or two-site model, using Graphpad Prism Program. The IC_{50} defines the concentration of the competing drug that inhibits 50% of the specific binding. K_i is the inhibition constant and expresses the affinity of the inhibitor for the receptor. K_i values were calculated using IC_{50} values provided by the program and the Cheng Prussof relationship (Cheng & Prussof, 1973), defined as follows:

$$K_i = IC_{50} / (1 + F / K_D)$$

where F is the estimated free radioligand concentration. This calculation also requires a K_D value which is the affinity of the radioligand for the receptor. The K_D for this specific preparation was not available as this has to be determined by a saturation

experiment and unfortunately due to time and tissue constraints, these studies were not carried out. However, after referring to similar studies in other pulmonary artery preparations including human (Davenport, *et al.*, 1995), an estimated value of 0.2 nM was used.

2.5.2 Calculation of agonist potency

As a measurement of agonist potency in arterial preparations pEC_{50} values are given as standard; where EC_{50} is the concentration of an agonist that produces 50% of the maximal possible effect of that agonist, and pEC_{50} is equal to the -log of the EC_{50}. In order to compare potencies to lower concentrations of agonists, pEC_{10} and pEC_{25} values were also calculated. These values were calculated by computer interpolation from individual concentration response curves in each vessel.

2.5.3 Calculation of antagonist potency

To assess the effects of antagonists and give an estimation of affinity pK_B values (where appropriate) have been calculated according to the methods described by Arunlakshana and Schild (1959). The pK_B value is the -log of the K_B which is the dissociation equilibrium constant for an antagonist. This is defined as the molar concentration of ligand required to occupy 50 % of the receptor pool. An estimate of the pK_B value was calculated using the following direct fit equation,

$$pK_B = -\log K_B \text{ and } \qquad K_B = [\text{antagonist}] / r - 1$$

Where (1) [antagonist] = concentration of antagonist used and (2) r = concentration ratio [A*] / [A], that is the concentration of agonist required to elicit an equal effect in the presence ([A*]) and absence ([A]) of antagonist. The common value chosen here in the EC_{50}.

Several assumptions have to be taken into account when estimating pK_B values in the direct fit model. The equation used to calculate the pK_B value assumes that the antagonist interacts with the agonist for unoccupied receptors in a simple, reversible manner. As has been discussed in chapter 1, the nature of interaction of ET with

receptors is thought to be essentially irreversible, and this factor therefore questions the validity of the pK_B calculations. For calculation of pK_B values, agonist response curves are normally with and without antagonist in the same tissue preparation. The fact that only a single response curve to ET and related peptides can be constructed in isolated preparations will also increase the degree of error. However it was decided to include these values as estimates of antagonist potency, although these points must be taken into account when interpreting the data.

In some antagonists studies, a maximum response to the agonist was not attained within the concentration range of the peptide examined. Hence in these cases, pK_B values could not be calculated. The reason that the peptide concentration was not extended beyond the normal maximum concentration of 0.3 μM was two fold. Firstly, the relative solubility of the peptide yields a stock concentration of 100 μM therefore a large volume of stock solution must be added to a 5 ml bath to give a final concentration of 1 μM. Secondly, ET-1 and related peptides are extremely expensive, it therefore was not financially feasible to use large volumes of stock solution when considering the large number of studies in which these compounds were to be used.

2.5.4 Statistical analysis

Statistical comparisons between two groups of data were made using Students t-test. The nature of the studies were such that an unpaired t-test was appropriate. Comparisons between three or more groups of data were studied using one way analysis of variance (ANOVA) followed by the appropriate ad hoc post test to access which groups were statistically different. $P < 0.05$ was considered to be statistically significant. Where a significant difference was found, the test used is always stated. The statistic software package InStat P203 base on a Macintosh Classic computer was used.

2.6 Solutions

Unless otherwise stated all solutions were stored at 4°C and used only for the duration of the recommended periods.

2.6.1 Functional pharmacological and biochemical studies

The composition of the modified Krebs-Heinslet solution was as follows:
NaCl 118.4 mM, $NaHCO_3$ 25mM, KCl 4.7mM, KH_2PO_4 1.2mM, $MgSO_4$ 0.6mM, $CaCl_2$ 2.5mM, glucose 11mM.

Buffer for cAMP assay:
50mM Tris HCl, 4mM EDTA, pH 7.4 (with 1M HCl) at 4°C

Charcoal solution for cAMP assay:
1 g of 2% Norit GSX charcoal, 0.5 g of 1% (w/v) BSA, made up in 50ml assay buffer.

cAMP binding protein:
preparation was essentially as described by Brown *et al.* (1972). Briefly, cortical tissue obtained from bovine adrenal glands was homogenised in 1.5 volumes of ice-cold homogenisation buffer (5mM MgCl2, 25mM KCl, 0.25mM sucrose, 50mM Tris HCl, pH7.4). Homogenate was centrifuged at 2000 x g for 5 minutes at 4°C. Supernatant decanted then re-centrifuged at 6000 x g for 15 minutes at 4°C. Final supernatant was kept on ice and, while agitated, aliquoted into 1.5 ml eppendorf tubes and dropped into liquid nitrogen for rapid freezing. The binding protein was stored at -80°C until required.

Buffers for cGMP assay:
Tris HCl buffer- 50mM Tris-HCl, pH 7.4 (with 1M HCl) at 4°C
Acetate buffer- 50mM Na acetate, pH 6.2 (with 1M acetic acid) at 4°C.

Charcoal-dextran solution for cGMP assay:

(a) 50mM Tris, 5mM EDTA, pH 7.4. Dissolve 6.07g Tris in 500ml of autoclaved H_2O. Add 250ml, 100mM HCl and mix. Add 2g neomycin sulphate and 1.85g disodium EDTA and allow to dissolve. Sprinkle 5g BSA (fraction V) on the surface and leave to dissolve (do not stir). Adjust to pH 7.4 at 22°C with 1M HCl and make up to 1 L with autoclaved H_2O.

(b) To the above add 0.62g dextran T70 and dissolve. Add 6g Norit GSX charcoal and stir to ensure complete mixing.

2.6.2 Radioligand binding studies

Buffer for membrane preparation:

150mM NaCl, 50mM Tris-HCl, 5mM EDTA, 1μg/ml leupetin, 500μg/ml soyabean trypsin inhibitor (SBTI), 1mg benzamidine, 10mM $MgCl_2$, pH7.4 at 22°C.

Buffer for competition assay:

20mM HEPES, 135mM NaCl, 2.68mM KCl, 1.8mM $CaCl_2$, 2.05mM $MgCl_2$, 1μM bautracin, 1μg/ ml leupetin, 1μM SBTI, 10mM benzamidine, pH 7.4 at 22°C.

2.7 Drugs and chemical reagents

The following alphabetical list is of the drugs and reagents used in the following experimental studies; the supplier is given in parenthesis.

Acetylcholine (Sigma);

Anti-cGMP antibody (Gift);

Bovine Serum Albumin (Sigma);

BQ788 (Peptide International)

(N-cis-2,6-dimethylpiperidinocarboxyl-L-g-methylleucyl-D-I-methocarbonyltrypophanyl-D-norleucine);

Charcoal (Norit A) (Sigma);

Endothelin-1 (Thistle Peptides);

Endothelin-3 (Peninsula laboratories);

FR139317 (Neosystems)

((R)2-[(R)-2-[(S)-2-[[1-(hexahydro-1H-azepinyl)]carbinyl]amino-4-methylpertanoyl]amino-3-[3-(1-methyl-1H-indoyl)]propionyl]amino-3-(2-pyridyl)propionic acid);

L-NAME (N^{ω}-nitro-L-arginine methylester) (Sigma);

Millers staining solution (BDH);

Noradrenaline (Sigma);

Pentobarbitone Sodium (Uthetal) (Rhone);

Coomassie Plus Protein Assay Reagent (Pierce);

Sarafotoxin S6c (Sigma);

SB209670 (Gift)

(1S,,2R,,3S)-3-(2-carboxymethoxy-4-methoxyphenyl)-1-(3,4-methyleudioxy-phenyl)-5-(prop-1-yloxylindane-2-carboxylic acid);

Sodium nitroprusside (Sigma);

Sumatriptan (GR 43175) (Glaxo);

Superoxide dismutase (Sigma);

U46619 (Upjohn)

(9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin F2α);

5- carboximidotryptamine (Research Biochemicals Int.);

5-Hydroxytryptamine creatine sulphate (Sigma);

^{3}H cAMP (Amersham);

[^{125}I] cGMP (NEN, Life Science Products) ;

[^{125}I] ET-1 (Amersham).

Stock solutions of SXS6c were prepared in 0.1% acetic acid, those of BQ788 in 0.1% DMSO and those U46619 in absolute alcohol. All other drugs and all subsequent dilutions were prepared in distilled water.

Chapter 3

Rabbit coronary artery ligation model of left ventricular dysfunction: Vascular reactivity in pulmonary resistance arteries

3.1 Introduction

The reasons for the changes in the systemic vasculature secondary to left-sided heart disease are incompletely understood (Cleland & Oakley, 1991; Habib, *et al.*, 1994) and this is even more the case for the alterations in the pulmonary circulation. An initial change in the pulmonary circulation secondary to left-sided cardiac disease is a passive response to a rise in pulmonary venous (left atrial) pressure. The increase in pulmonary venous pressure is transmitted through the pulmonary vascular bed to the pulmonary artery and the pulmonary artery pressures (PAP) rise "passively". As pressure rises in the pulmonary artery, dilation of the proximal arteries occurs while the distal arteries become attenuated and tortuous. The pathological aspects of pulmonary hypertension in left heart disease are described in chapter 1 of this thesis. Briefly however, pulmonary hypertension (PHT) is generally characterised by increased thickening of the walls of pulmonary arteries, narrowing of the pulmonary artery lumen, increased pulmonary vascular resistance, and right-sided heart failure (Heath & Edwards, 1958). The changes in the pulmonary circulation in response to left heart disease may not only contribute to the production of symptoms, but may also be important determinants of the response to treatment, the risk of surgery and ultimately prognosis.

The rabbit coronary-ligation model of left ventricular dysfunction (LVD) has been reported previously (Pye, *et al.*, 1996; Denvir, *et al.*, 1996) and is briefly described in section 2.1.1 of this thesis. The presence of pulmonary hypertension in this model has also been reported (Douchar, *et al.*, 1997) and thus may act as an ideal model to examine pulmonary vascular changes occurring secondary to left ventricular dysfunction (LVD). In this chapter, I describe studies examining the morphology of the pulmonary vasculature in this model, and also alterations in ejection fraction and heart and lung weight from the cohort of animals used in my studies.

Various agents have been implicated in the condition of PHT, amongst these is the peptide endothelin-1 (ET-1) (Cody, *et al.*, 1992; Kiowski, *et al.*, 1995). The role of ET-1 in the pulmonary vasculature of the rabbit coronary ligation model is extensively

examined and discussed in chapters 4, 5, 6, and 7 of this thesis. 5-hydroxytryptamine (5-HT) (or serotonin) has also been implicated in the aetiology of PHT, both clinical and experimental. 5-HT is produced by activated platelets and pulmonary neuroendocrine cells, and has been implicated in PHT in the presence of pulmonary thromboemboli (Comroe, *et al.*, 1953) and may be involved in PHT related to hypoxia in newborns (Johnson & Georgieff, 1989). Platelet release of 5-HT may also be involved in PHT secondary to cardiac surgery and in primary pulmonary hypertension (Johnson & Georgieff, 1989; Reneman & Starre, 1990; Herve, *et al.*, 1990; 1995). Responses to 5-HT are potentiated in the rat monocrotoline-induced model of PHT (Wanstall & O'Donnell, 1990) and has also been associated with the development of PHT in these animals (Kanai, *et al.*, 1993). The vascular effect of 5-HT in the pulmonary circulation appears to be dependent on both the species under study and on the degree of initial vascular tone. For example, 5-HT has been shown to act as a pulmonary vasoconstrictor (Wanstall & O'Donnell, 1990), but in some species such as the cat or sheep, 5-HT can also mediate vasodilation of the pulmonary vascular bed when the vascular tone is raised (Neely, *et al.*, 1993; Cocks & Arnold, 1992). In bovine isolated large intrapulmonary arteries, responses to the 5-HT_{1D} receptor agonist sumatriptan were uncovered under conditions of raised vascular tone (MacLean, *et al.*, 1994b). In the pulmonary hypertensive state vessels are subjected to increased vascular tone which may therefore modulate responses to various spasmogens. Hence, in this study I examined 5-HT receptor mediated responses in isolated rabbit pulmonary arteries induced by the agonists 5-HT, 5-CT, a selective agonist for 5-HT_1 receptor subtypes, and sumatriptan (GR43175), a 5-HT_1-like agonist which has been classified as a selective agonist for the 5-HT_{1D} receptor subtype (Peroutka & McCarthy, 1989; Sumner & Humphrey, 1989; Humphrey, *et al.*, 1993). Muscular pulmonary arteries, referred to as pulmonary resistance arteries (PRAs), contribute most to the resistance to flow in PHT (Nagasaka, *et al.*, 1984). Indeed, previous reports indicate these arteries as being the most likely to have severe morphological abnormalities in patients with PHT (Heath & Edwards, 1958;

Heath, 1993). Hence I examined functional responses of PRAs from the rabbit coronary ligation model of LVD.

Functional changes in the endothelium are known to occur in both experimental and clinical PHT. However, discordant results have been reported on the effect of disease on endothelium-dependent relaxations. For example, studies on isolated pulmonary artery rings from patients of Elsenmenger's syndrome (Dinh-Xuan, *et al* 1990a) or chronic obstructive lung disease (Dinh-Xuan, *et al.*, 1991), showed diminished endothelium-dependent relaxations. In addition, the degree of impairment was shown to correlate well with intimal thickening. In vessels from chronic hypoxic rats, Adnot *et al.*, (1991) reported that the ability of ACh to relax preconstricted pulmonary arteries was reduced compared with those from control animals, whilst MacLean *et al.*. (1995) demonstrated an augmentation of ACh-induced relaxation in this model. Giaid and Saleh (1995) demonstrated that pulmonary hypertension in humans was associated with diminished expression of endothelial nitric oxide synthase. Whilst other studies in animal models of PHT have reported regional up-regulation of NOS (e.g. Xue, *et al*, 1994; MacLean *et al.*.,1995). Thus, in this chapter I also examined responses to various endothelium-dependent vasodilator agents and the endothelium-independent agent sodium nitroprusside (SNP) in isolated preconstricted PRAs from the rabbit coronary ligation model.

3.2 Methods

Measurement of ventricular and lung weight

The hearts from a proportion of the eight week sham-operated and coronary-ligated rabbits used in this study were dissected into right ventricle and left ventricle plus septum, blotted and weighed, according to the procedure described in chapter 2, section 2.1.1.3 (i).

A proportion of all the lungs obtained during this study were weighed, according to the procedure described in section 2.1.1.3 (iii), before vessels were dissected out for *in vitro* studies. The data from different experimental groups were compared.

Histological examination of lung sections

Light Microscopy

Preparation of lung sections from stock/sham-operated and coronary-ligated rabbits is described in the methods section 2.1.1.3 (ii). Separate sections from each lung were stained using haematoxilin and eosin stain and an elastic-Van gieson stain in order to examine the morphology and to detect the presence of increased muscularisation and changes in elastic tissue. Analysis of changes in the degree of muscularisation is also described in this section of chapter 2 of this thesis.

Electron and confocal microscopy

Small muscular pulmonary arteries of the same size and anatomical location as used in in vitro studies were dissected from sham-operated and coronary-ligated rabbits by the procedure described in section 2.1.1.3 (ii). Vessels were then processed and examined using electron or confocal microscopy to access any structural changes in these vessels, according to the procedures described in chapter 2.

Functional studies: rabbit pulmonary resistance arteries

Lungs were obtained from the rabbit coronary ligation model of left ventricular dysfunction. In the 8 week and 16 week procedure period groups, age matched animals underwent the same methodology as the experimental animals (referred to in results and discussion as "ligated") except the ligatures placed around the coronary artery were not secured. These are subsequently referred to as "sham-operated". Also, age-matched animals in which no operational procedures were performed were used as controls in the

16 week and 32 week procedure period groups These are subsequently referred to as "stock". Measurements of ejection fraction were made in all animals and animals were killed by sodium pentabarbitone 8, 16 or 32 weeks following the procedure. The lungs were promptly removed and small intralobar pulmonary resistance arteries (PRAs) of ~150 μm internal diameter (see table 3.3) were dissected out according to the methods stated in section 2.2.3.1. These were mounted as ring preparations (~2mm long) on a wire myograph, bathed in Krebs solution at 37°C. Using the normalisation process explained in section 2.2.5, vessels were tensioned to an equivalent transmural pressure of ~ 16 mmHg (see table 3.3). This pressure was chosen as it is similar to *in vivo* pressures of rabbit pulmonary arterioles. Preliminary studies in our laboratory showed that bubbling with 95% O_2 inhibits responses to vasoconstrictors in rabbit PRAs so we bubbled with 16% O_2/ 5% CO_2 balance N_2. These gas mixtures were chosen as they are similar to those which the vessels would be exposed to *in vivo*.

Experimental protocol

Vessels were allowed to equilibrate for 1 hour prior to the addition of any drugs. The response of the PRAs to 50mM KCl was determined twice; once the contractile response had reached a plateau, the vessels were washed out at least 6 times with fresh Krebs solution. After a further equilibration of at least 20 minutes, vessels were then subjected to one of the following protocols.

(1) Cumulative concentration-response curve (CCRC) to KCl (from 5mM-120mM)

(2) CCRC to 5-HT (1nM-30μM).

(3) CCRC to 5CT (1nM-30μM) and addition of 30μM 5-HT at the end of the response (8 week group animals only).

(4) CCRC to sumatriptan (1nM-0.1mM) and addition of 30μM 5-HT at the end of the response (8 week group animals only).

(5) Preconstriction with 30nM U46619 or 1μM 5-HT followed by CCRC to one of several endothelium-dependent relaxatory agents namely; ACh, substance P, bradykinin, histamine, and calcium ionophore A23177 (all at 1nM-10μM).

(6)Preconstriction with 30nM U46619, followed by CCRC to SNP (1nM-0.1mM).

Data analysis

Data for body and lung weight are expressed as g or kg, ventricular ratios were calculated for each individual heart, and lung to body weight ratios for each individual animal. Representative photographs from the histological studies are shown in the following results section. Morphological changes are expressed as percentage thick walled pulmonary vessels. For functional studies, pEC_{10}, pEC_{25} and pEC_{50} values (where appropriate) were calculated according to the methods stated in section 2.5.2, and expressed as -log M concentration. Responses to KCl are expressed graphically as percentage own maximum response or mg weight tension. CCRC to 5-HT receptor agonists are expressed as percentage reference contraction to second application of 50mM KCl in each preparation. Relaxatory responses to SNP are expressed graphically as percentage level of preconstriction to U46619 in each preparation. Statistical comparison of the means of groups of data were made by one way analysis of variance (ANOVA) or Student's unpaired t test; $P<0.05$ was considered statistically significant. Throughout, data are expressed as mean± SEM, n= number of animals and n /n = number of ring preparations / number of animals.

3.3 Results

3.3.1 Rabbit coronary ligation model of left ventricular dysfunction

Body and ventricular weights and ventricular ratios from 8 week sham-operated and coronary-ligated rabbits are shown in table 3.1. As a change in left ventricular

weight was evident in this model, ventricular ratios are expressed relative to body weight, rather than total ventricular weight.

	sham-operated (n=17)	coronary -ligated (n=28)
RV/ body weight	0.42±0.01	0.51±0.02***
LV+S/ body weight	1.36±0.04	1.48±0.04*
total ventricular weight (g)	6.14±0.11	6.99±0.16***
body weight (kg)	3.46±0.06	3.53±0.04

Table 3.1 Ventricular and body weights of 8 week sham-operated and coronary-ligated rabbits. Statistical comparisons were made by Students unpaired t test *P<0.05, ***P<0.001. Data presented as mean ±SEM. RV, right ventricle; LV+S, left ventricle plus septum; n, number of animals.

Eight weeks of permanent coronary artery ligation resulted in a marked increase in the ratio of right ventricular to body weight and also in the ratio of left ventricle plus septum to body weight. Ventricular hypertrophy was also evident from the comparatively greater total ventricular weight of hearts from coronary-ligated compared to sham-operated rabbits. Animals from both experimental groups had similar body weights.

The lung and body weights of animals from the various experimental groups are shown in table 3.2. Lungs from 8 week coronary-ligated rabbits were comparatively heavier than those from age matched sham-operated rabbits. This marked increase was also evident in the ratio of lung to body weight. However, no significant difference was noted between groups in the 16 week and 32 week procedure animals. All animals had similar body weights.

	n	Lung weight (g)	Body weight (g)	lung /body (x100)
8 week				
sham-operated	43	12.02±0.26	3670.8±48.7	0.328±0.01
coronary-ligated	64	13.13±0.32*	3565.4±32.1	0.370±0.01*
16 week				
stock	3	11.53±0.59	3737.7±74.3	0.308±0.01
sham-operated	8	11.48±0.54	3817.2±154.9	0.303±0.02
coronary-ligated	8	13.25±0.81	3634.6±124.8	0.367±0.02
32 week				
stock	6	13.81±0.96	3929.6±166.8	0.353±0.02
coronary-ligated	9	12.88±0.40	3996.6±171.1	0.327±0.02

Table 3.2 Lung and body weights of 8, 16 and 32 week stock/sham-operated and coronary-ligated rabbits. Statistical comparisons were made by one way analysis of variance (ANOVA), followed by Tukeys post test *$P<0.05$. Data presented as mean ±SEM. n, number of animals.

3.3.2 Morphological studies

Light microscopy

Figures 3.1 and 3.2 show photographs taken from stained sections of lung viewed under the light microscope. Figure 3.2B shows a typical example of small pulmonary arteries from a sham-operated rabbit which comprise of a single elastic lamina and sparse traces of vascular smooth muscle. An EVG section obtained from an 8 week coronary-ligated rabbit is shown in figure 3.2A. An arteriole of similar size to the sham-operated (control) examples has developed a relatively thick muscular media, bound by two distinct elastic laminae. Increased muscularisation of small muscular

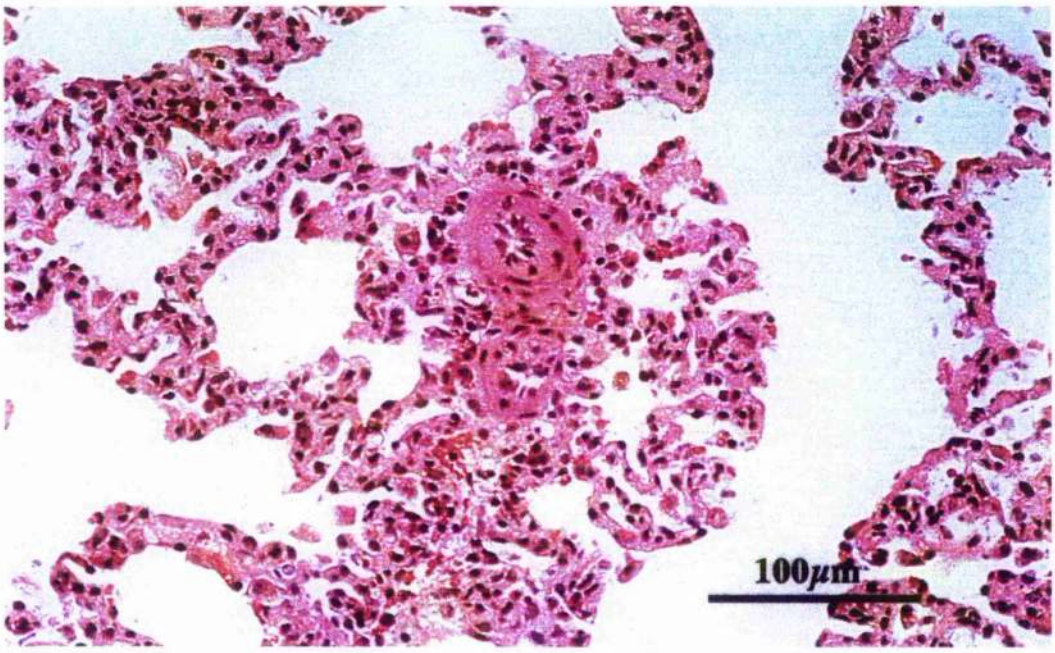


Figure 3.1 A

Lung section (H&E stain) from an 8 week coronary artery ligated rabbit. Section shows increased muscularisation of small pulmonary arteries. The prominent medial hypertrophy has decreased the pulmonary artery lumen.

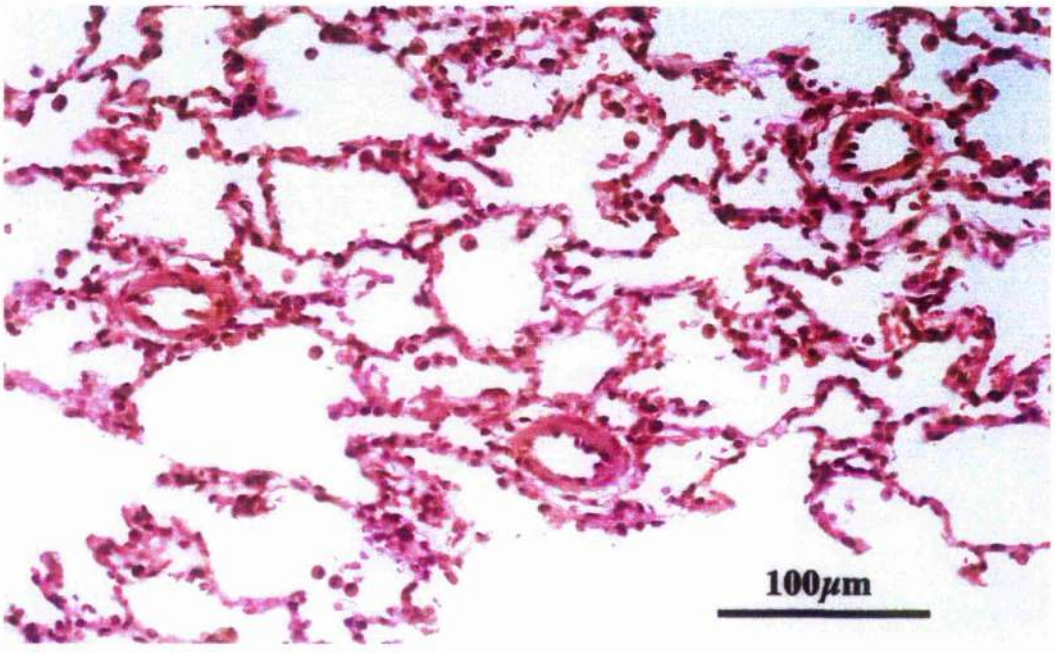


Figure 3.1 B

Lung section (H&E stain) from an 8 week sham-operated rabbit. Section shows normal appearance of small pulmonary arteries; these are thin-walled with relatively sparse vascular smooth muscle.

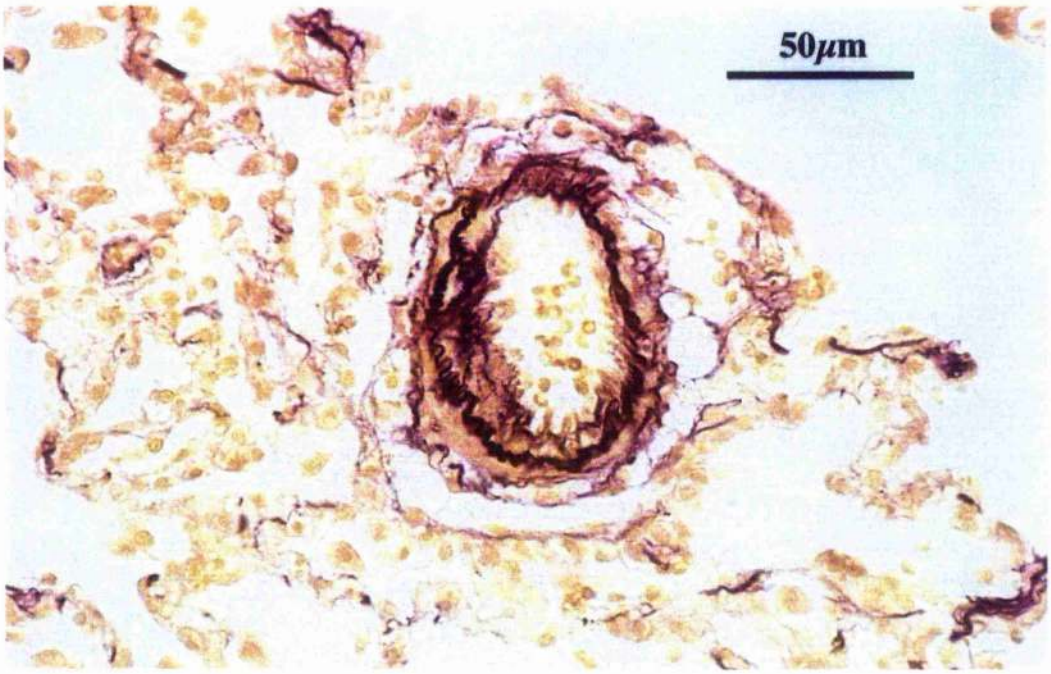


Figure 3.2 A

Lung section (E.V.G. stain) from an 8 week coronary artery ligated rabbit. Section shows a thick-walled small pulmonary artery, with double elastic laminae surrounding the newly developed smooth muscle layer.

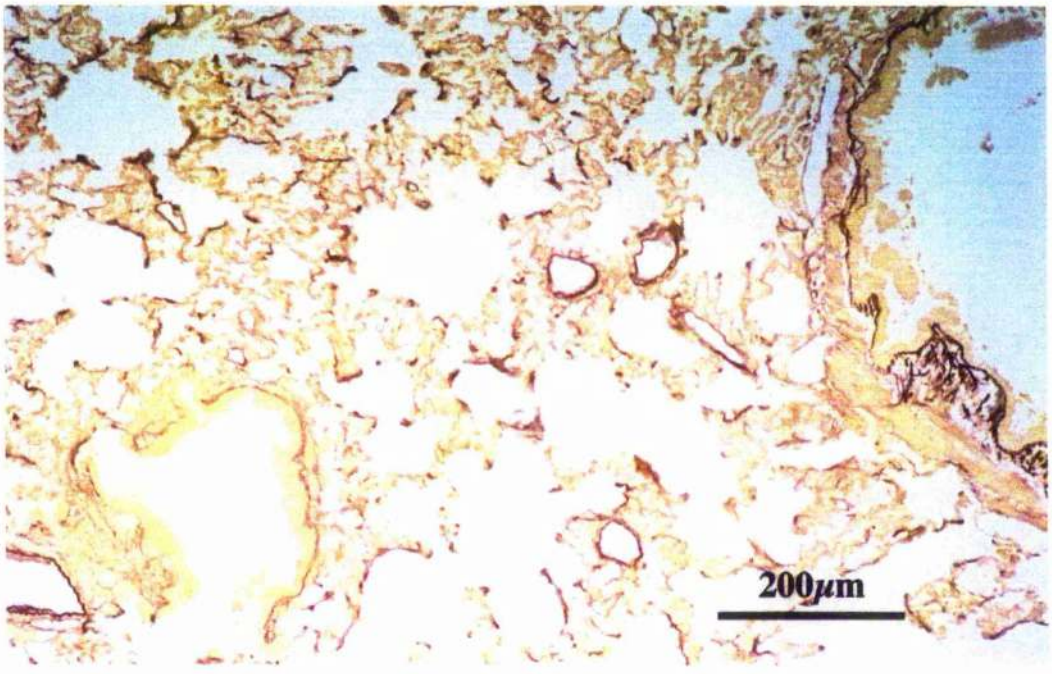


Figure 3.2 B

Lung section (E.V.G. stain) from an 8 week sham-operated rabbit. Section shows normal appearance of small pulmonary arteries, comprising of a single elastic laminae and sparse vascular smooth muscle.

pulmonary arteries from 8 week ligated animals is evident from sections of lung stained with haematoxylin and eison shown in figure 3.1A. This compares with section illustrated in figure 3.1B, showing the relatively little muscularisation of similar arteries in lung from sham-operated rabbits.

On viewing the sections obtained from rabbit lungs from both experimental groups, thick walled pulmonary vessels (TWPV) were abundant and easily detected in the ligated compared with the control preparations. Quantification of the percentage of TWPV in lungs from 8 week procedure animals, provided values of 8.5±0.4% (n=6 lungs) for sham-operated rabbits and 21.0±0.4% (n=7 lungs) for coronary-ligated rabbits ($P<0.001$). Similar percentages were noted in sections from 16 and 32 week procedure animals.

Electron microscopy

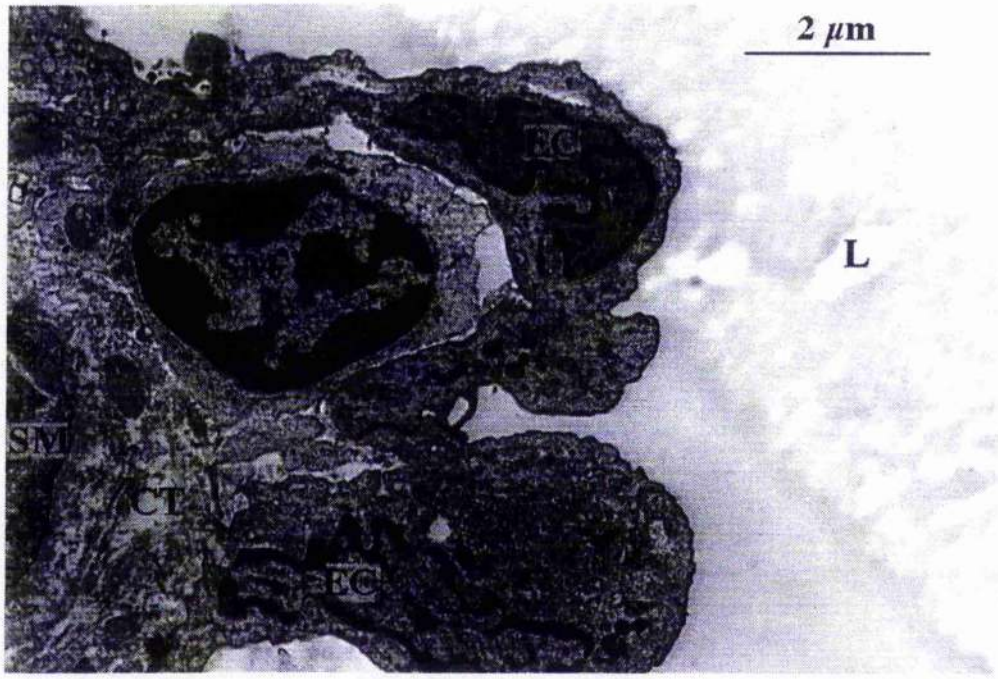
Figure 3.3A and B shows endothelial cells of pulmonary artery of ~200 μm internal diameter from 8 week sham-operated rabbit. These cells have a normal, intact appearance. In contrast, figure 3.4A and B is an example of an endothelium from 8 week coronary-ligated rabbit. In these sections the endothelium appears severely disrupted. Endothelial cells are in a defective state and debris from dying cells is evident in the lumen.

Confocal microscopy

Vessels of identical size and location to pulmonary resistance arteries used in *in vitro* studies were examined using confocal microscopy. An example of adventitia of sham-operated and coronary-ligated rabbit PRAs in shown in the picture of figure 3.5A and 3.5B, respectively. Adventitia from sham-operated preparation had a normal typical appearance, whilst that of pulmonary artery from coronary-ligated rabbit appeared disrupted.

Endothelial cells and underlying smooth cells in PRAs from 8 week sham-operated and coronary-ligated rabbits in shown in figure 3.5C and 3.5D, respectively.

A



B

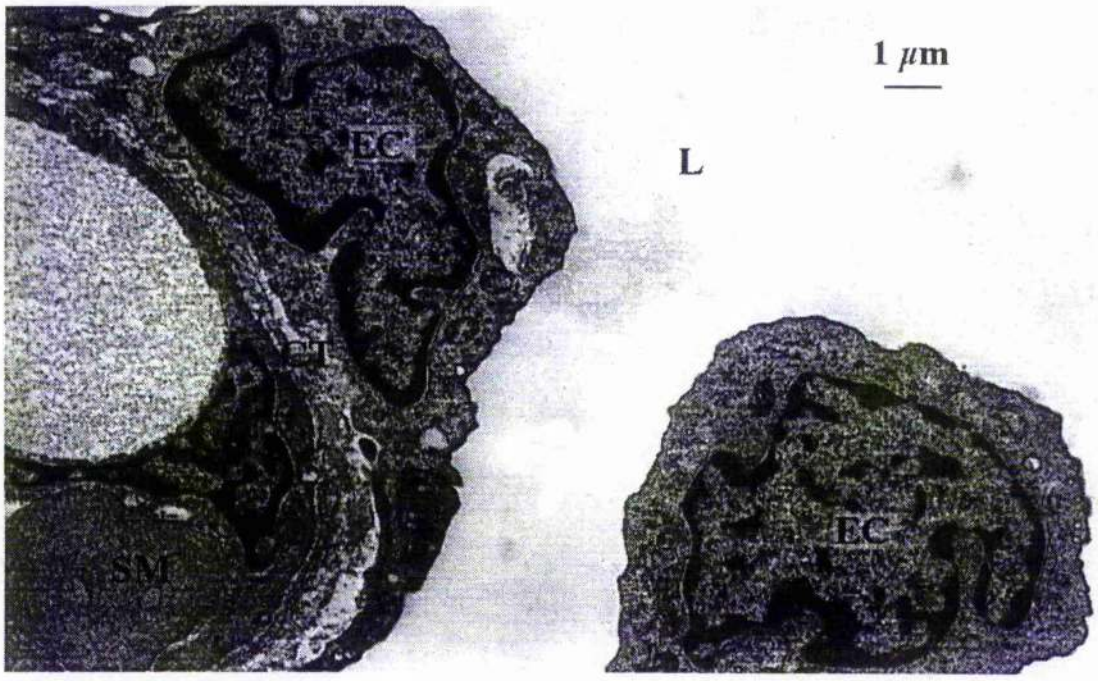
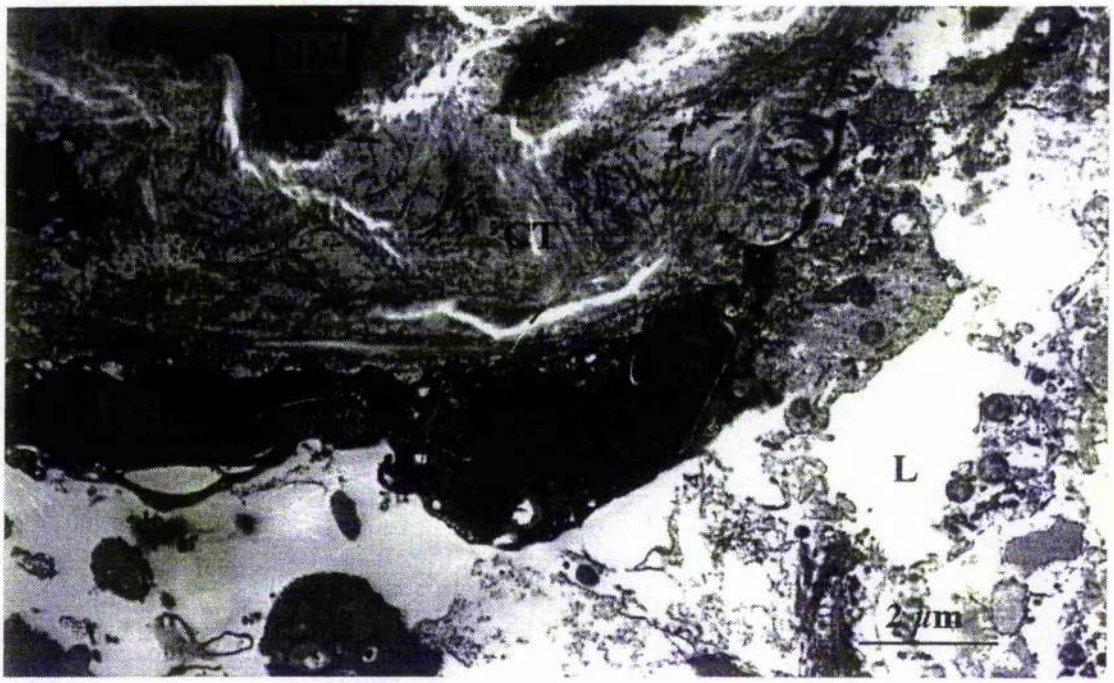


Figure 3.3 A and B Endothelium of isolated pulmonary resistance arteries from an eight week sham-operated rabbit. Electron micrographs shows distinct endothelial cells with typical appearance. EC = endothelial cell; SM = smooth muscle cell; CT = connective tissue; L=lumen.

A



B

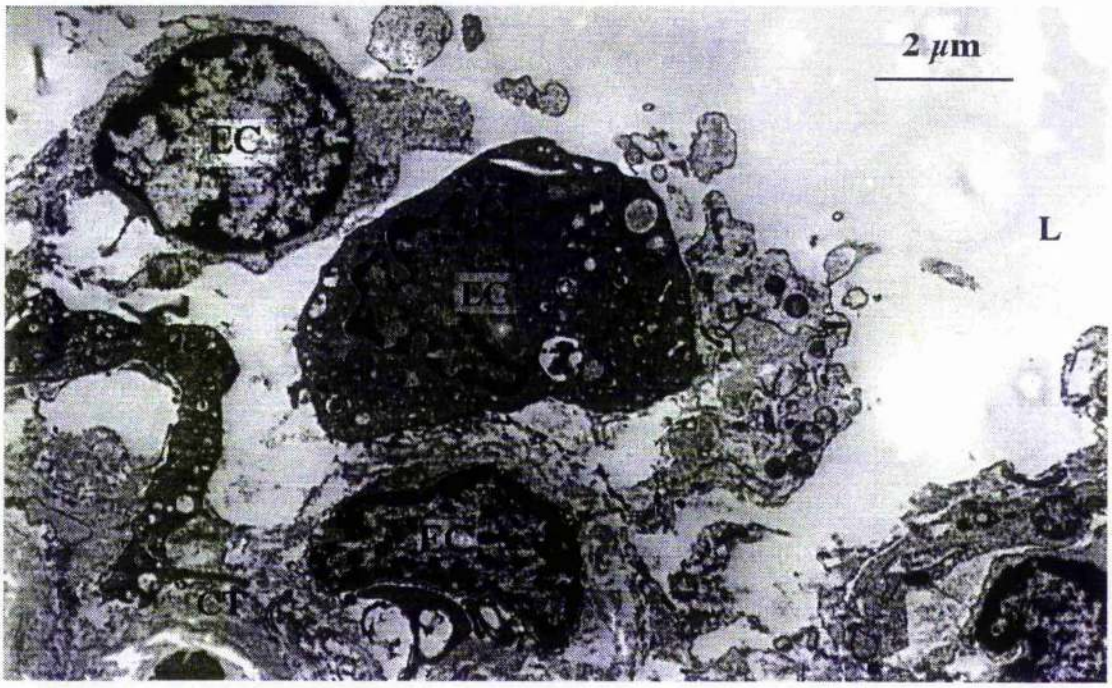


Figure 3.4 A and B Endothelium of isolated pulmonary resistance arteries from an eight week coronary artery-ligated rabbit. Electron micrographs shows disrupted appearance of endothelial cells and visible cellular debris in the vessel lumen. EC = endothelial cell; SM = smooth muscle cell; CT = connective tissue; L = lumen.

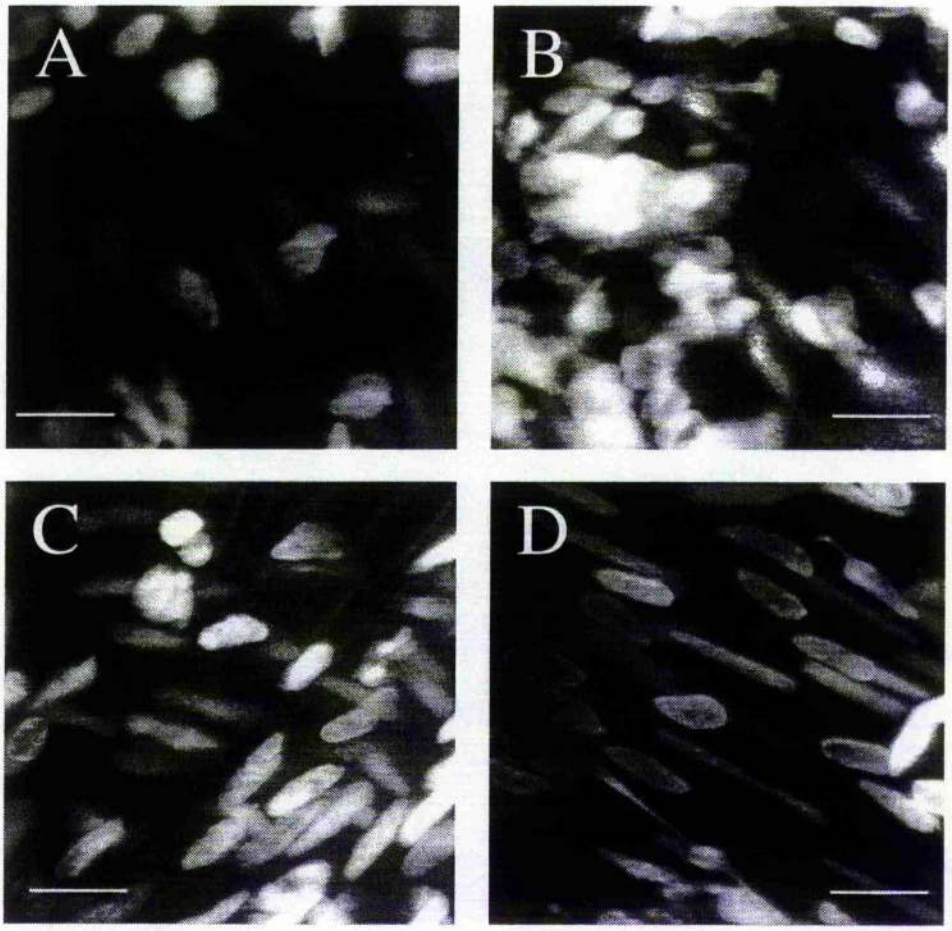


Figure 3.5

Example of the adventitia (A and B) and of endothelial cells and underlying smooth muscle cells (C and D) of pulmonary resistance arteries (~200µm intermal diameter) isolated from an eight week sham-operated (A and C) and coronary-ligated rabbit (B and D). Bar = 20 µm.

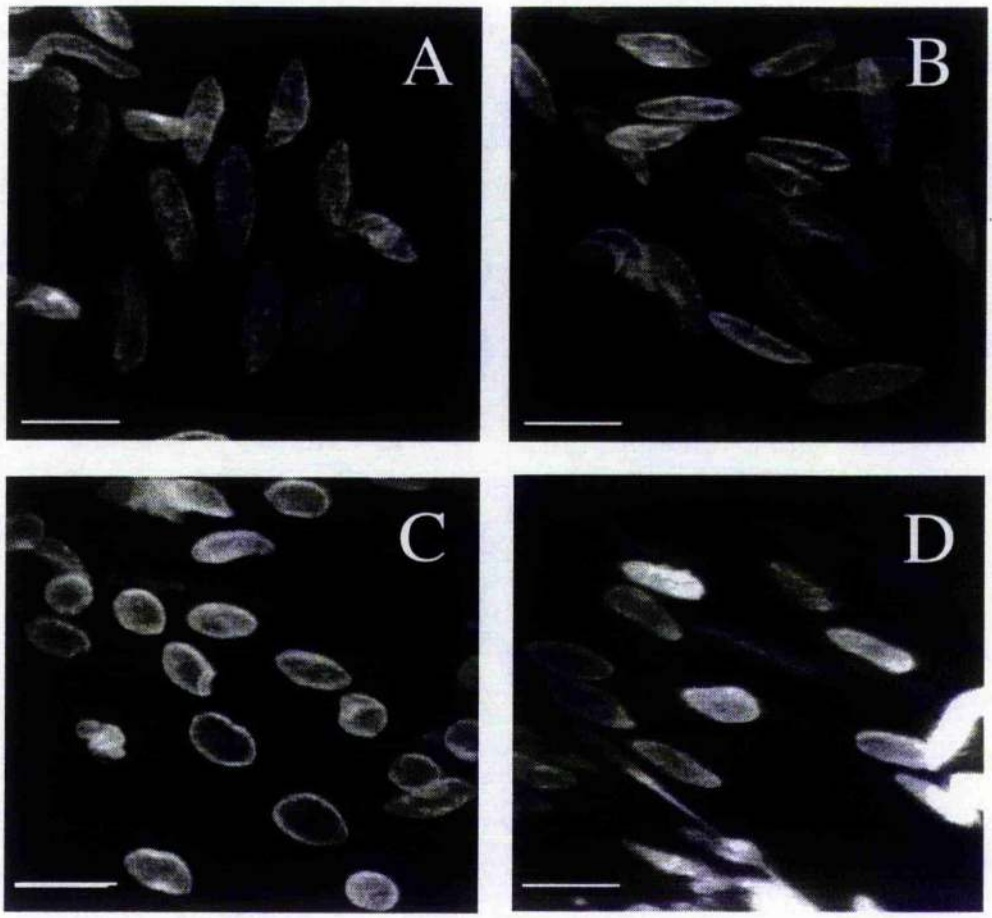


Figure 3.6

Example of endothelial cells of pulmonary resistance arteries (~200μm internal diameter) isolated from an 8 week sham - operated (A, B, and C) and coronary-ligated rabbit (D). Bar = 20 μm.

The smooth muscle cells of the example of a sham-operated preparation had a typical elongated appearance, running perpendicular to the longitudinally arranged endothelial cell layer. In some ligated rabbit artery preparations, smooth muscle cells were difficult to view and this may have been due to the thickness of the elastic lamina. In the example shown in figure 3.5B, the cells underlying the endothelial cells are presumed to be smooth muscle in nature due to their shape. However, these cells are running in the same direction as the endothelial layer and thus do not appear to have a typical circumferential orientation.

Figure 3.6 shows endothelial cells in PRAs from 8 week sham-operated (3.6A, B, C) and coronary -ligated (3.6D) rabbits. These cells had an unsmooth appearance, indicated by the differences in intensity of nuclei staining. Intensity was either uneven throughout cell, or towards the outside of the nuclei, or very dark staining was evident around the perimeter of the endothelial cells. This indicates differences in the DNA concentration at different locations throughout the nuclei and thus possibly different states of activity. This appearance was evident in vessels from both sham-operated and coronary-ligated rabbits.

3.3.3 Vascular reactivity of rabbit pulmonary resistance arteries

The ejection fraction of animals from the various experimental groups, from which lungs were obtained and PRAs dissected for the studies described in this chapter, are shown in table 3.1. The effective pressure to which these vessels were tensioned and the resulting estimate of internal diameter are also shown in table 3.1. The ejection fraction was markedly reduced in ligated animals compared with sham-operated/stock rabbits in 8, 16 and 32 week procedure groups. Also, the reduced ejection fraction was significantly lower in 16 week ligated compared to 32 week ligated rabbits. Vessels from all experimental groups were tensioned to a similar effective pressure and internal diameter.

	(n/n)	Internal diameter (μM)	Effective pressure (mmHg)	Ejection fraction (%)
8 week				
sham-operated	(32/20)	148.9±5.4	19.7±0.9	72.94±1.6
ligated	(33/22)	165.3±6.8	19.8±1.1	43.4±2.0***
16 week				
stock	(8/5)	135.8±3.3	17.7±2.0	78.2±2.4
sham-operated	(20/19)	146.7±6.0	19.6±0.8	74.4±1.0
ligated	(38/32)	161.2±7.5	18.4±0.7	38.8±1.2***[a]
32 week				
stock	(13/5)	152.4±8.5	16.5±1.2	75.0±1.3
ligated	(13/7)	150.7±10.8	18.3±1.6	48.9±1.2***

Table 3.3 (1) Ejection fraction of animals from which PRAs were dissected and studied in this chapter and (2) internal diameter and effective pressure of PRAs from this cohort of rabbits. Statistical comparisons were made by one way analysis of variance (ANOVA), followed by Tukeys post test: ligated vs. age matched sham-operated/stock, $***P<0.001$; 16 wk. ligated vs. 32 wk ligated, $^{a}P<0.01$. Values are mean±SEM. n/n, number of ring preparations/ number of animals.

The vasoconstrictor response to cumulative concentrations of KCl in PRAs from 8 week sham-operated and coronary-ligated rabbits is shown in figure 3.7A. No difference in sensitivity was seen between the two experimental groups and a similar response was noted in all vessels examined from 16 and 32 week group animals (not shown). For all PRAs, a maximal vasoconstriction was noted at 50mM KCl. This concentration was subsequently used to preconstrict vessels at the beginning of each protocol for all functional studies carried out in this thesis. The magnitude of the response to the second application of 50mM KCl was used as a reference contraction to compare subsequent vasoconstrictor responses to other agents, in the same preparation. The absolute magnitude of the contractile response to 50mM KCl was similar in all

Figure 3.7

Responses to KCl in rabbit PRAs.

A. CCRC's to KCl in PRAs from 8 week sham-operated (○, n=8/5) coronary ligated (●, n=12/6) rabbits. Data are expressed as a percentage of their own maximum contraction.

B. Vasoconstriction to 50mM KCl in PRAs from: 8 week sham-operated (open-columns; n=73/63) and coronary-ligated (hatched columns; n=67/58) rabbits; 16 week stock (stippled columns; n=8/5), sham-operated (open-columns; n=20/19) and coronary-ligated (hatched columns; n=38/32) rabbits; and 32 week stock (stippled columns; n=29/5) and coronary-ligated (hatched columns; n=37/7) rabbits. Data are expressed as mg weight tension.
Each point represents mean± SEM. n/n, number of vessels/ number of animals

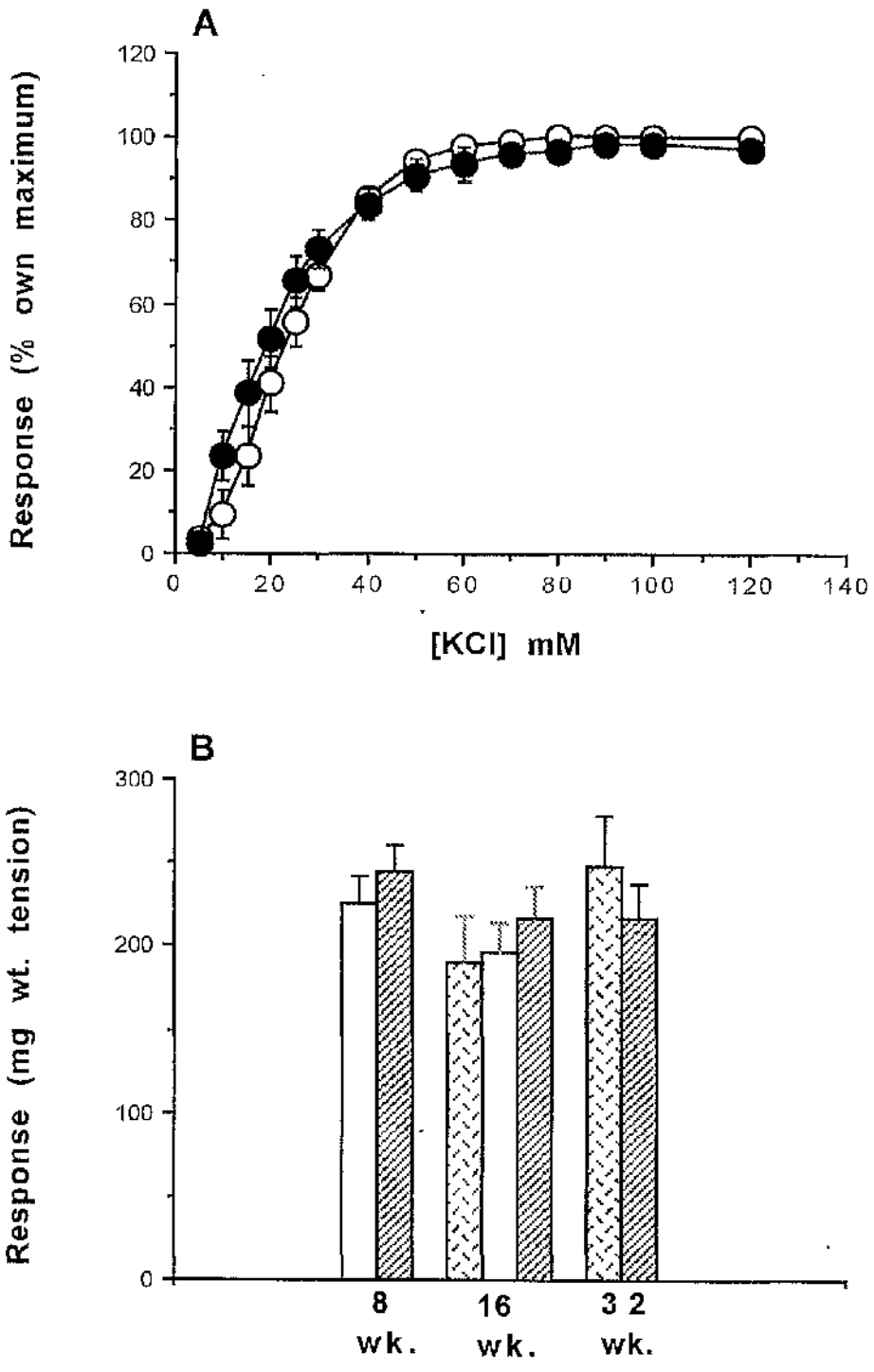


Figure 3.7

Responses to KCl in rabbit PRAs from 8, 16 and 32 week experimental group animals.

PRAs from both stock/sham-operated and coronary-ligated rabbits, regardless of the length of the procedure; this is shown in figure 3.7B.

Effect of 5-HT receptor agonists

The effect of 5-HT on PRAs from 8 week and 32 week procedure rabbits is shown in figure 3.8A and 3.8B, respectively. $p\mathrm{EC}_{10}$, $p\mathrm{EC}_{25}$ and $p\mathrm{EC}_{50}$ values are summarised in table 3.4. 5-HT had similar potency in all PRAs from 8 week group rabbits (table 3.4). However, the magnitude of the maximal vasoconstriction was markedly reduced in coronary-ligated compared to age-matched sham-operated preparations (50.5±6.6% vs. 73.3±3.2%; $P<0.05$; figure 3.8A). Since the response of PRAs from 32 week stock rabbits did not reach a maximum, *p*EC values could not be calculated for this group. However, if the final response obtained is taken as a maximum, the vessels from stock rabbits appeared to have similar sensitivity to 5-HT as the coronary-ligated rabbit preparations. Comparing sensitivity between 8 and 32 week experimental groups, the potency of 5-HT was markedly reduced in the 32 week rabbit PRAs than in the 8 week procedure group arteries ($p\mathrm{EC}_{25}$ and $p\mathrm{EC}_{50}$, $P<0.05$). The maximum response to 5HT tended to be smaller in the ligated preparation (47.5±10.1%) compared to age-matched stock rabbit vessels (76.2±12.6%) in the 32 week procedure group also, however, this was not statistically significant (figure 3.8B).

Responses to sumatriptan and 5-CT in PRAs from 8 week procedure group rabbits are shown in figures 3.9A and 3.9B, respectively. Vasoconstrictor response to sumatriptan were small and variable in magnitude. This was particularly the case in coronary-ligated rabbit PRAs where only a portion of vessels responded, whereas all sham-operated preparations examined showed a small response. The maximal contractile response to 30μM sumatriptan was markedly smaller in ligated rabbit PRAs compared to that of sham-operated rabbit vessels ($P<0.05$; figure 3.9A).

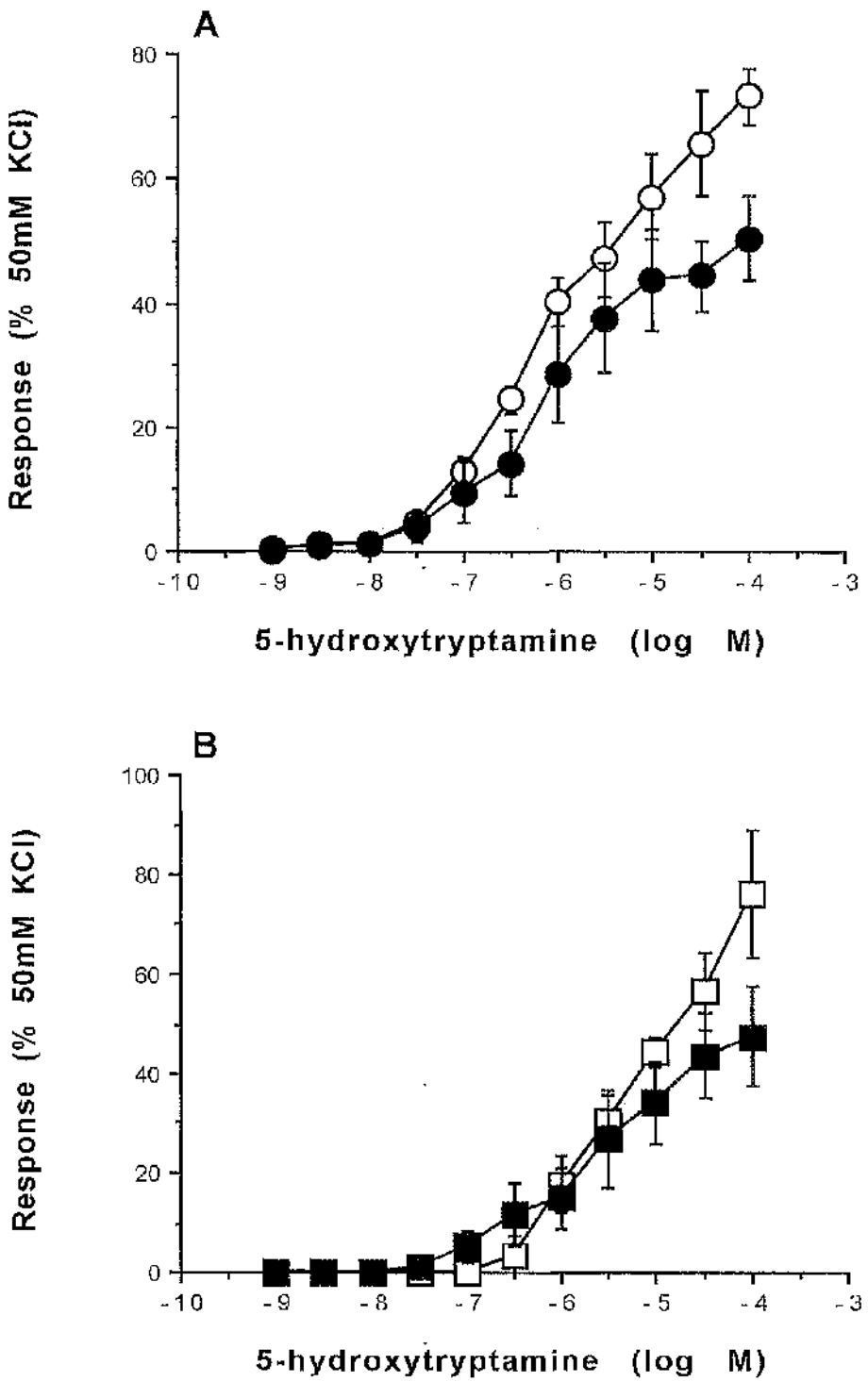


Figure 3.8 Effect of 5-HT on rabbit PRAs. Data are expressed as percentage reference contraction to 50mM KCl. **A**. PRAs from 8 week sham-operated (○, n=7/6) and coronary ligated rabbits (●, n=7/5). **B**. PRAs from 32 week stock (□, n=3/3) and coronary-ligated (■, n=6/6) rabbits. Each point represents mean ± SEM. n/n, number of vessels/ number of animals.

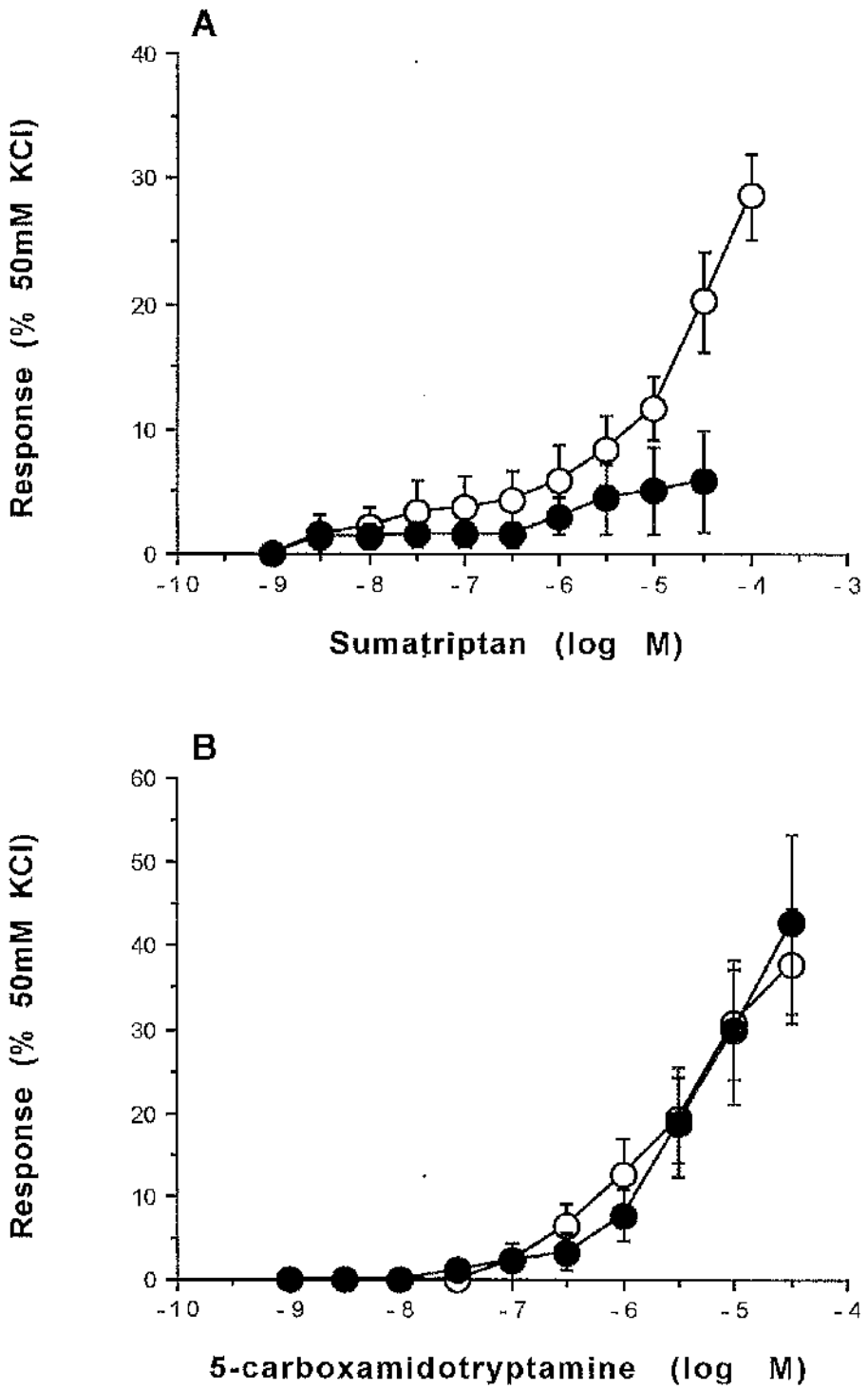


Figure 3.9 Responses to 5-HT receptor agonists in PRAs from 8 week sham-operated (○) and coronary-ligated (●) rabbits. Data are expressed as percentage reference contraction to 50mM KCl. **A**. Effect of sumatriptan (○, n=8/6; ●, n=10/8). **B**. Effect of 5-CT (○, n=10/6; ●, n=5/5). Each point represents mean ± SEM. n/n, number of vessels/ number of animals.

	pEC_{10}	pEC_{25}	pEC_{50}	n/n
8 week				
sham-operated	7.4±0.2	6.8±0.1	6.1±0.2	7/6
coronary-ligated	7.2±0.3	6.7±0.1	6.1±0.1	7/5
32 week				
stock	nc			
coronary-ligated	6.6±0.2	6.0±0.3^{+}	5.3±0.2^{+}	6/6

Table 3.4 Sensitivity to 5-HT in PRAs from 8 and 32 week procedure group animals. Results are expressed as mean±SEM. Statistical comparisons by ANOVA, $^{+}P<0.05$, 32 wk ligated vs. 8 wk ligated. n/n, number of ring preparations/ number of animals; nc, not calculated.

5-CT-induced responses were similar in PRAs from both experimental groups, eliciting a maximal response of ~40% KCl -induced vasoconstriction (figure 3.9B). Since a plateau was not attained in the CCRCs to sumatriptan or 5-CT, *p*EC values were not calculated. 30μM 5-HT was added at the end of each sumatriptan and 5-CT CCRC and the amplitude of the vasoconstrictions to these agonists were significantly smaller compared to 5-HT in the same preparations. For example, the maximal response to (30μM) sumatriptan, expressed as a percentage of the response to 30μM 5-HT in the same preparations, was 22.3±3.3% and 13.6±4.3% for 8 week sham-operated and coronary-ligated rabbit PRAs, respectively. Whilst, the maximal response to 5-CT was 62.2±5.1% and 58.6±6.6% of the response to 30μM 5-HT in the same PRAs from control and LVD animals, respectively.

Responses to 5-HT receptor agonists at 30μM were compared in 8 week group rabbit vessels. In PRAs from control animals, the vasoconstrictions to 5-HT (65.6±8.4% reference contraction to 50mM KCl) and 5-CT (42.6±10.7%) were of a similar magnitude, whereas the magnitude of the contractile response to sumatriptan was significantly smaller compared to that of 5-HT (20.2±4.0%, $P<0.001$). In arteries from

LVD animals, 5-HT and 5-CT responses did not significantly differ, however the vasoconstrictions to sumatriptan were markedly smaller compared to both these agonists (13.2±4.5%, vs. 44.3±5.8% (5-HT, $P<0.001$), vs. 37.9±6.9% (5-CT, $P<0.01$)).

Effect of endothelium-dependent vasodilatory agents

There was no evidence for endothelial dependent relaxations using any of the agents tried in either the 8 week sham rabbit vessels or the coronary artery-ligated rabbit PRAs. ACh evoked a transient concentration-dependent contractile response in this preparation.

Effect of endothelium-independent vasodilatory agent, SNP

The thromboxane mimetic U46619 (30nM) produced the same degree of preconstriction in the 8 week control group (54.0±7% of response to 50mM KCl) and in the LVD group (48.6±4.6%). However in PRAs from the 32 week group animals, 30nM U46619 induced a markedly greater preconstriction in the PRAs from the LVD group (121.6±17.8%) compared to vessels from age-matched stock animals (67.1±4.8%; $P<0.01$).

	pEC_{10}	pEC_{25}	pEC_{50}	n/n
8 week				
sham-operated	8.2±0.3	7.8±0.3	7.3±0.2	14/6
coronary-ligated	7.7±0.1	7.3±0.1	6.8±0.1*	14/7
32 week				
stock	8.3±0.2	7.8±0.2	7.0±0.2	9/5
coronary-ligated	7.9±0.1	7.4±0.2	6.7±0.2	9/5

Table 3.5 Sensitivity to SNP in PRAs from 8 and 32 week procedure group animals. Statistical comparisons were made by Students unpaired t test; ligated vs. age-matched control, *$P<0.05$. Results are expressed as mean±SEM. n/n, number of ring preparations/ number of animals.

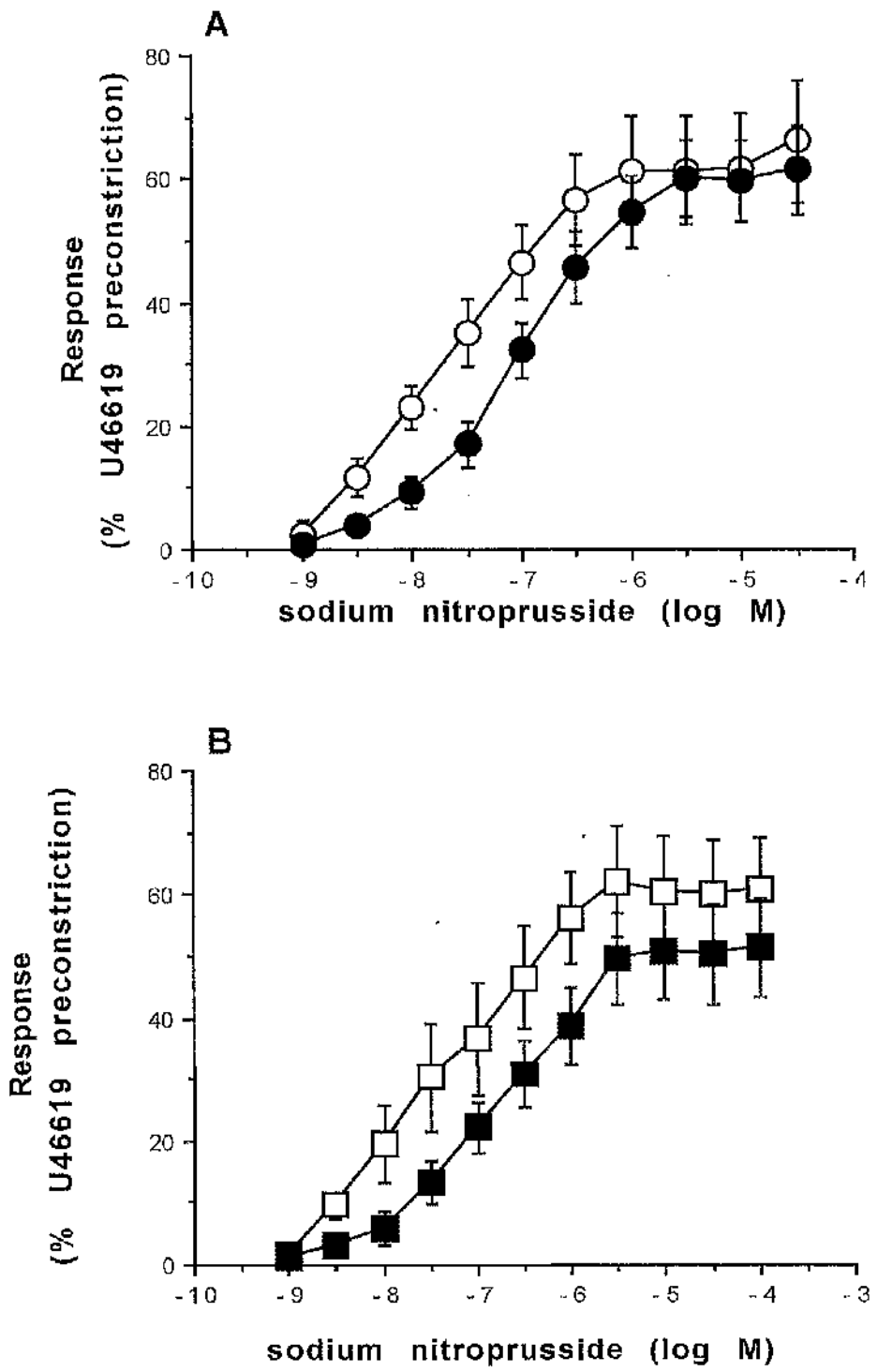


Figure 3.10 Effect of SNP on rabbit PRAs. Data are expressed as percentage 30nM U46619-induced tone. **A**. PRAs from 8 week sham-operated (○, n=14/6) and coronary ligated rabbits (●, n=14/7). **B**. PRAs from 32 week stock (□, n=9/5) and coronary-ligated (■, n=9/5) rabbits. Each point represents mean ± SEM. n/n, number of vessels/ number of animals.

Figure 3.10A and table 3.5 show that PRAs from the 8 week rabbits with LVD were less sensitive to the relaxant effects of SNP, although the maximum response to SNP was unaffected. In the 32 week group rabbits, no significant difference in sensitivity or maximum response was noted between stock and coronary-ligated rabbit vessels (figure 3.10B; table 3.5).

3.4 Discussion

Rabbit coronary artery ligation model of left ventricular dysfunction

The marked attenuation in the ejection fraction and the significant left ventricular hypertrophy of rabbits in which the left coronary artery was ligated indicated left ventricular dysfunction (LVD) in these animals. In this cohort of animals, the ejection fraction of animals ligated for 16 weeks was similar to that of animals ligated for 8 weeks. However, the ejection fraction of 32 week ligated animals was significantly greater than that of 16 week coronary ligated rabbits. Thus, animals ligated for a longer duration do not appear to represent a more severe LVD condition but are more likely to be survivors in these groups.

Moreover, following 8 weeks of permanent coronary artery ligation, the rabbits exhibited a significant right ventricular hypertrophy, as demonstrated by the ~21% increase in the right ventricular/body weight ratio, with no change occurring in body weight. Also, an ~16 % increase was evident in lung/body weight ratio in the LVD rabbits This right ventricular hypertrophy and lung congestion indicate the development of PHT in the animals used in this study. These findings are similar to previous investigations using this model, where the marked reduction in cardiac output and increase in right ventricular and lung weight were also reported (Deuchar, *et al.*, 1997). Furthermore, this previous *in vivo* study showed a 44% increase in pulmonary artery pressure in 8 week coronary-ligated rabbit, thus providing further evidence for the existence of PHT secondary to LVD in these animals.

The results of several histological studies in this chapter provide yet more evidence for the development of PHT secondary to LVD in this model. The sections taken from control and experimental rabbit lungs showed that pulmonary vascular remodelling had taken place. Pulmonary arterioles of ~50μm are normally nonmuscular, thin walled and comprise of a single elastic lamina; this was demonstrated in lung sections from the cohort of animals examined in this study. However in lung sections from coronary-ligated rabbits , vessels of the same size and location were now muscular and comprised of two elastic laminae, surrounding the newly developed smooth muscle layer. The apparent progression of muscularisation occurs due to the pericytes normally present in nonmuscular vessels differentiating into a smooth muscle cell via an intermediate cell (Meyrick & Reid, 1978). The marked increase in the degree of muscularisation of small pulmonary arterioles was demonstrated further by the significant increase in the percentage of thick walled pulmonary vessels, compared to stock/sham-operated preparations. This prominent medial hypertrophy is indicative of considerable increase in the pulmonary arterial pressure, and therefore is consistent with previous haemodynamic findings in this model (Deuchar, *et al.*, 1997). Common to many forms of PHT is the proliferation of smooth muscle cells in the vascular media and frequently in the intima (Wagenvoort, 1981). The alterations in the medial layer shown in this present study are similar to previous reports in other animal models and in humans with PHT. For example, Hunter *et al.* (1974) described similar findings in hypoxic rat model, and Heath *et al* (1987) and Heath & Edwards (1958) reported pulmonary vascular pathology in the condition of primary PHT and PHT, of various degrees of severity, secondary to congenital heart defects. Results of a limited confocal microscopy study of isolated coronary-ligated rabbit PRAs showed that cells underlying endothelial cells ,which were presumed to be smooth muscle in nature, were not arranged in the typical circumferential manner but rather, appeared to be running in the same direction of the endothelial cells, i.e. longitudinally. Consistent with this, Wagenvoort & Wagenvoort (1984) reported the appearance of longitudinally orientated bundles of vascular smooth muscle cells in the remodelling of muscular pulmonary

arteries. These were most common in the intima and in the adventia, but occasionally affected the media.

Another associated histological lesion occurring in severe pulmonary hypertension is concentric-laminar proliferation of cells ("onion-skin proliferation") in muscular pulmonary arteries and their branches; the cells which proliferated have been shown to be myofibroblasts (Smith & Heath, 1979). In PRAs (100-300μm internal diameter) substantial medial thickening has been reported (Rabinovitch, *et al.*, 1981). The morphological studies of my own investigations are too limited to allow quantitative conclusions as to which components of the medial wall are altered secondary to left ventricular dysfunction. Previous studies in large diameter hilar pulmonary arteries of the chronic hypoxic rat demonstrated hypertrophy, rather than hyperplasia as being responsible for the medial thickening (Meyrick & Reid, 1978) but this remains controversial. Similarly, the factor(s) involved in pulmonary vascular remodelling are as yet unclear. Several growth factors are thought to play important roles in the proliferation of VSMCs. Numerous studies indicate ET-1 and/or NO in the vascular hypertrophy associated with the pathogenesis of hypertension (e.g. Janakidevi, *et al.*, 1992, Ferro & Webb, 1997).

In PHT, remodelling occurs in all three vascular layers but the pattern of thickening varies depending on the size of the artery. Electron microscopy of random samples from 8 week procedure groups showed that the endothelium was severely disrupted in sections from coronary-ligated animals. Rabinovitch *et al.* (1986) reported abnormalities in the pulmonary artery endothelium in patients with congenital heart defects and PHT. Nitric oxide (NO) also plays an important role in the *in vitro* destruction of endothelial cells by neutrophils (Fligel, *et al.*, 1984, Varani, *et al*, 1985; Warren & Ward, 1986). Activated neutrophils can generate superoxide anion that, together with NO, leads to hydroxyl radical formation. This highly reactive free radical appears to be ultimately responsible for the death of endothelial cells (Warren & Ward, 1986). Moreover, the results of chapter 6 indicate an increase in NO in PRAs from coronary-ligated rabbits. In addition, it is known that in response to endothelial injury,

the migration of vascular smooth muscle cells (VSMCs) from the medial layer to intima triggers abnormal VSMC proliferation. Thus, this may play a role in the medial hypertrophy evident in these vessels. Disruption of the adventitia of isolated PRAs from ligated rabbits was evident from limited confocal microscopy studies. Increased matrix deposition is also well documented in PHT. Previous studies show a significant increase in the arterial adventitia due to increase in collagen deposition in the hypoxic rat model of PHT (Meyrick & Reid, 1978; Hislop & Reid, 1976). Alterations in the adventitia have also been reported in various grades of severity of PHT induced by congenital cardiac defects (Heath & Edwards, 1958).

Many previous studies use increased right ventricular hypertrophy, vessel muscularisation, combined with pulmonary pressures as markers to confirm PHT in animal models (Wanstall & O'Donnell, 1990; Sakai, *et al.*, 1996; Enriquez-Sarano, *et al.*, 1997). Thus, the results of this chapter indicate that the animals with LVD induced by permanent coronary artery ligation, exhibit similar alterations previously observed in other models of PHT.

5-HT receptor -mediated responses: effect of left ventricular dysfunction

The comparable magnitudes of vasoconstrictions to KCl showed that the contractile ability was similar in vessels from both control and LVD rabbits, regardless of the duration of the coronary artery ligation. The response to KCl, which is mediated through it's effect on the membrane potential (Burnstock, *et al.*, 1963) and is therefore receptor independent, indicates the integrity of the smooth muscle present in the preparations examined from the various experimental groups.

5-HT, 5-CT and sumatriptan evoked cumulative concentration dependent vasoconstrictor responses in all rabbit PRAs. 5-HT had a similar potency in stock/sham-operated and coronary-ligated preparations from both 8 and 32 week procedure groups. However, various studies on other animal models have reported increased pulmonary artery sensitivity to 5-HT. For example, 5-HT has been associated with the development of PHT in the rat monocrotoline-induced model and responses to 5-HT were potentiated

in these animals (Wanstall & O'Donnell, 1990; Kanai, *et al.*, 1993). In chronic hypoxic rat model, the sensitivity to, and the maximum response of, 5-HT was increased in the main, first branch and pulmonary resistance arteries removed from chronic hypoxic rats (MacLean, *et al.*, 1996b). From the effects of various selective antagonists, the authors concluded that the augmentation in the 5-HT-induced response was due to the enhanced effect of 5-HT_{2A} receptor stimulation combined with the increased influence of r5-HT_{1B}-like receptor stimulation. In this previous report, rat PRAs had a similar sensitivity to 5-HT (pEC_{50} of ~5.9) as was noted in rabbit PRAs (~6.1) in this present study. Similar pulmonary arterial hyperreactivity to 5-HT has been observed secondary to platelet 5-HT storage disorder in rats (Ashmore, *et al.*, 1991) and in isolated pulmonary arteries from patients with primary PHT (Brink, *et al.*, 1988).

In this present study, the magnitude of the maximal 5-HT-induced vasoconstriction was reduced in ligated preparations compared with the age-matched controls; this attenuation was significant in the 8 week experimental group. Hence, this finding is in contrast to the other aforementioned studies, where an increase in magnitude of 5-HT responses were reported. Moreover, a previous *in vivo* study using this rabbit coronary ligation model demonstrated an increased pulmonary pressor response to i.v. administered 5-HT in 8 week ligated rabbits when compared to sham-operated animals (Deuchar, *et al.*, 1997). Differences in findings between *in vivo* and *in vitro* studies have also been reported in humans; 5-HT was shown to contract human pulmonary arteries and veins *in vitro* (Raffestin, *et al.*, 1985), yet was shown to have no effect on pulmonary artery pressure *in vivo* (Harris, *et al.*, 1960). This may be caused by the balancing between the vasoconstrictor and vasodilator effects of 5-HT. Also, when analysing the results of *in vivo* studies, a role of parenchymal and/or vascular agents cannot distinguished.

In humans and fawn hooded rat, hypersensitivity to 5-HT may be associated with abnormalities in platelet 5-HT storage (Herve, *et al.*, 1995; Ashmore, *et al.*, 1991). However, there is no evidence for abnormalities in platelet 5-HT storage in animal models or in humans with heart failure. In addition, in small systemic arteries dissected

from gluteal skin biopsies, responses to 5-HT were similar in preparations obtained from normal and congestive heart failure patients (Angus, *et al.*, 1993).The difference in findings between this present study and previous reports in other models may be due to differences in species or preparations and/or in the aetiology of the PHT state.

In pulmonary arteries and systemic arteries 5-HT is known to cause endothelium -dependent and endothelium-independent vasodilation via 5-HT_{1D}-like and 5-HT_7-like receptors, respectively (Schoeffter & Hoyer, 1990; Terron, 1996). Hence the overall effect of 5-HT will be determined by the balance between direct smooth muscle vasoconstriction and vasorelaxation. Since all 5-HT receptor agonist examined evoked a vasoconstrictor response in rabbit PRAs, it appears that the direct contractile effect overrides any vasodilator effect. This finding is consistent with the overall pressor response of the pulmonary circulation *in vivo* to 5-HT, which has previously been demonstrated in this rabbit model (Deuchar, *et al.*, 1997). The decreased vasoconstrictor 5-HT-induced response of isolated PRAs from LVD rabbits may therefore be due to decreased vasoconstriction and/or increased vasodilation via the 5-HT receptors.

The presence of contractile 5-HT_{1D} receptors in this preparation was indicated by the cumulative concentration dependent contractile response evoked to sumatriptan. However, the magnitude of the vasoconstriction to sumatriptan was markedly smaller compared to 5-HT in sham-operated rabbit PRAs, and both 5-CT and 5-HT in the coronary-ligated rabbit PRAs. The comparatively smaller response to sumatriptan could be due to the fact that both 5-HT_1 (MacIntyre, *et al.*, 1993) and 5-HT_2 receptors (Raffestin, *et al.*, 1985; McMahon, *et al.*, 1993) are involved in the contractile response, whereas the relaxant response to 5-HT is mediated via 5-HT_1 receptors (Schoeffter & Hoyer, 1990; Terron, 1996). Thus activation of both contractile receptor subtypes by 5-HT would thereby elicit a comparatively greater response than the selective 5-HT_{1D} agonist sumatriptan. However, 5-CT evoked a similar response in PRAs from sham-operated and coronary-ligated rabbits and the magnitude of the response to this selective 5-HT_1 receptor agonist was not significantly different from that of 5-HT. This finding suggests the predominance of 5-HT_1-like receptors mediating vasoconstriction in this

preparation, however further studies using selective antagonists are required to confirm this. Since a maximum response was not reached in the 5-CT- and sumatriptan-evoked responses, EC_{50} values could not be calculated, however both agonists appeared less potent compared to 5-HT. These findings compare with a previous report in isolated larger pulmonary arteries from the adult rabbit. Morecroft & MacLean (1995b) demonstrated pEC_{50} values for 5-HT of ~7.4 and ~7.1 in the extralobal and intralobal pulmonary arteries, respectively. Thus the value of ~6.1 calculated for PRAs in this study suggests a lower potency of 5-HT in these smaller arteries. The disparity in sensitivities may be due to different anatomical location of the vessels studied. These finding are at odds with those of MacLean *et al* (1996b) in the chronic hypoxic rat model, who demonstrated that 5-HT was most potent in the PRAs compared with pulmonary branch and main pulmonary artery. This indicates yet again either species or model variation. In the rabbit larger pulmonary arteries 5-HT was shown to be more potent than 5-CT however sumatriptan was inactive (Morecroft and MacLean, 1995b). These findings suggested that a "5-HT_2 type" receptor is mainly involved in the 5-HT-induced vasoconstriction in larger pulmonary arteries of the adult rabbit but 5-HT_{1D} receptors appear not to be involved. 5-HT is also thought to constrict larger pulmonary arteries of the calf due to an action at 5-HT_{2A} receptors (Frenkan & Kaumann, 1984). These findings are in contrast to the apparent role of contractile 5-HT_{1D} receptors in rabbit PRAs which is indicated by the contractile effect of sumatriptan in this preparation; and provides further evidence for regional differences in 5-HT receptor mediated responses.

As was noted with 5-HT responses, the maximal response to sumatriptan was markedly attenuated in 8 week LVD animals compared to control animals. This finding may indicate an alteration in 5-HT_{1D} receptor subtype in the LVD animals. The reduced vasoconstriction to 5-HT and sumatriptan in PRAs from 8 week ligated rabbits may also be due to an increase in the production of endothelium-derived nitric oxide secondary to LVD; indeed this is indicated by the results shown in chapter 6 of this thesis.

Other previous studies suggest the role of 5-HT_1-like receptors in the contractile response to 5-HT in the pulmonary circulation. Templeton *et al* (1993; 1994) have previously shown that 5-CT is more potent than 5-HT and sumatriptan is equipotent to 5-HT in human pulmonary arteries, suggesting 5-HT_{1D}-like activation in these vessels. Further evidence for this was reported more recently; GR55562 (5-HT_{1D} receptor antagonist) was shown to inhibit 5-HT and sumatriptan responses in a true competitive fashion, whereas ketanserin (selective 5-HT_{2A} receptor antagonist) and methiothepin (non-selective 5-HT_{1+2} receptor antagonist) had no effect on sensitivity to these agonists (MacLean, *et al.*, 1996a). Furthermore, Bard *et al* (1996) reported that rabbit and human recombinant 5-HT_{1D} receptors (α andβ) showed significant intraspecies and interspecies similarities in their ligand binding profiles, suggesting that 5-HT_{1D}-mediated responses in rabbit preparations may provide information relevant to the pharmacology of the 5-HT_{1D} receptor subtypes in humans. The species homologues of the human 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors have recently been reclassified as the r5-HT_{1D} and r5-HT_{1B} receptors, respectively (Hartig, *et al.*, 1996). In addition, McIntyre *et al* (1992) on studying the effect of and duration of action of sumatriptan on central haemodymnamics in humans, found that this selective 5-HT_{1D} receptor agonist produced a more pronounced effect on the pulmonary pressure when compared with the overall systemic pressure. Indeed, the effect of 5-HT on systemic arteries is mainly due to activation of 5-HT_2 receptors, more recently classified as 5-HT_{2A} receptors, which are antagonised by ketanserin (Humphrey, *et al*, 1993).

Although no difference in sensitivity was noted following coronary artery ligation, PRAs from 32 week procedure group were significantly less sensitive to 5-HT than the 8 week procedure group arteries. Since no change was noted with the presence of LVD, this difference may reflect an age-related reduction in potency. Such age-related alterations in agonist sensitivity have been reported previously. In rat mesenteric resistance arteries, Dohi and Luscher (1990) demonstrated that the sensitivity to ET-1-induced vasoconstrictions decreased with advancing age. Nakashima and Vanhoutte (1993) showed that in perfused larger mesenteric arteries of the rat, ageing decreased

endothelium-dependent hyperpolarisations evoked by both ET-1 and ACh. The authors suggested that with ageing, a constant exposure to the ETs may result in the decreased sensitivity to ET-1 and ET-3. One can only speculate, however given the important role of the lungs in the metabolism of 5-HT which escapes being taken up by the platelets (Said, 1982), a possible overexposure may be related to the reduced 5-HT sensitivity observed in the 32 week procedure rabbit PRAs compared to those from 8 week animals.

In conclusion, this study suggests that the rabbit PRA has a functional population of 5-HT_1-like receptors, possibly r5-HT_{1B}-like, which may play a role in the contractile response to 5-HT. In particular, the attenuation of 5-HT- and sumatriptan-induced responses in coronary ligated rabbit PRAs suggests that an alteration in 5-HT_{1D}-like receptors may occur in PHT state secondary to LVD in these animals. These results suggest that further examination of the role of 5-HT in this model is warranted. Studies with selective antagonists and further selective agonists would provide more information on receptor subtypes involved and also in the possible alteration in 5-HT receptor subtypes, particularly 5-HT_{1D}-like, in PRAs from rabbits exhibiting PHT secondary to LVD.

Endothelium dependent and independent relaxatory responses: effect of left ventricular dysfunction

The PRAs from eight week procedure group animals were examined for endothelium dependent relaxations. Surprisingly, I could find no evidence for this despite trying a range of agonists known to induce endothelium dependent relaxations in pulmonary arteries (see chapter 1). In vessels from both sham-operated and coronary-ligated rabbits ACh evoked similar concentration-dependent, transient contractile responses. ACh-mediated vasoconstriction of rabbit small pulmonary vessels has previously been reported (Sada, *et al.*, 1987). Catravas *et al* (1984) also observed vasoconstrictor responses to ACh in the rabbit pulmonary circulation. The inability of ACh to induce a vasorelaxation is in contrast to the results of the studies in fetal and

neonatal rabbit PRAs in chapter 8 of this thesis. In this latter study, ACh did induce a relaxatory response, however, in vessels from 7 day old rabbits, a contractile response was noted to higher concentrations of this agonist, thus similar to the responses observed in the adult vessels of this present study. These findings indicate an alteration in ACh-induced responses with age and this possibility is discussed in chapter 8 of this thesis.

However, endothelium-dependent relaxations have been reported in the larger pulmonary arteries of the rabbit (Morecroft & MacLean, 1995a). Such relaxations have also been demonstrated in isolated conduit pulmonary arteries from other animals (Chand & Altura, 1981) and man (Greenberg, *et al.*, 1987; Dinh-Xuan, *et al*, 1990b), as well as in more distal muscular arteries and PRAs of the rat (Leach, *et al.*, 1992; MacLean, *et al.*., 1994a). Similar results have been shown in isolated rabbit lung preparations (Hyman & Kadowitz, 1989) and *in vivo* in man (Fritts, *et al.*, 1958). The differences between my own observations and those of other groups may lie in the preparations studied, i.e. pulmonary resistance arteries as opposed to isolated extrapulmonary arteries or isolated perfused lungs, and may also be species related. Alternatively, the endothelium derived factor(s) released from rabbit PRAs in response ACh may be contractile in nature, e.g. ET, superoxide anions (Vanhoutte & Kataustic, 1988) . Another possibility is that contractile muscarinic receptors predominate in this preparation. Consistent with this is a previous study using autoradiographic mapping and *in situ* hybridisation which showed the predominance of muscarinic M_4-receptors in pulmonary vessels of the rabbit lung (Mak, *et al.*, 1993).

In addition, I also examined these adult preparation for relaxatory responses to ET-receptor agonists in chapter 4 of this thesis, as ET-1 itself is known to induce endothelium-dependent relaxations in the pulmonary circulation via the release of NO, and possibly adrenomedullin, following activation of endothelial ET_B receptors. For example, previous studies have demonstrated this in the pig pulmonary artery (Namiki, *et al.*, 1992), in the isolated perfused rat lung (Lal, *et al.*, 1995) and the pulmonary vascular bed of the intact cat where it acts, in part, by activation of potassium channels and may act as an endothelium-derived hyperpolarising factor (Lippton, *et al.*, 1991).

The lack of relaxatory responses may be due to the absence of NO production by the endothelium or absence of the appropriate channels or receptors for the agents tested. However, the studies of chapter 6 of this thesis provides evidence for NOS in the endothelium of the PRAs, particularly in those vessels removed from the rabbits with LVD as L-NAME, evoked a marked potentiation of ET-1- and SXS6c-induced responses in these vessels. Other reports in animal models of PHT also demonstrate an up-regulation of NOS (e.g. Xue, *et al,* 1994).

SNP evoked a concentration-dependent relaxation in all preconstricted PRAs examined. The PRAs removed from 8 week procedure animals with LVD demonstrated a decreased sensitivity to SNP, whereas maximum responses were unaffected. Whilst in PRAs from 32 week procedure animals, SNP evoked a similar response in vessels from stock and coronary-ligated rabbits. In contrast, Dinh-Xuan *et al* (1993) reported that relaxation with SNP was significantly greater, whereas endothelium-dependent relaxations to ACh were markedly reduced in isolated pulmonary arteries from patients with chronic obstructive lung disease compared to those from normal subjects. However this condition is somewhat different from alterations in the pulmonary circulation which occur secondary to left-heart disease. For example, all the patients in this latter study were hypoxaemic whereas this condition is unlikely to exist in this model of LVD. In preconstricted small arteries (<300μM internal diameter) from gluteal skin biopsy specimens from patients with congestive heart failure, Angus *et al* (1993) reported that relaxatory responses to SNP were similar whereas those to ACh were reduced, compared to those in vessels from normal controls. The authors suggested that the release of EDRF (NO) is limited in congestive heart failure. Again, it is difficult to compare these findings with my own as the systemic and pulmonary vasculatures differ in many respects both functionally and structurally. Several possibilities may account for my own observation. Firstly, this may reflect an increase in local levels of ET-1, indeed numerous studies show increased plasma levels in PHT of several etiologies including heart failure (Kiowski, *et al.*, 1995; Cody, *et al.*, 1992). This possible explanation extends from the findings of Yang *et al* (1989), who demonstrated that ET

inhibited SNP relaxations in human mammary veins. Also, ET-induced vasoconstrictions of rabbit pulmonary veins were shown to be resistant to isoproterenol or forskolin-induced relaxations (Russell & Roberts, 1991). These results reflect that the potent contractile endothelium derived substance ET-1 may modulate the actions of vasodilators in vascular preparations. Thus the reduced potency of SNP in the 8 week LVD animals may reflect increased local ET-1 levels. Unfortunately local ET-1 in pulmonary vasculature of this model are unknown. Nevertheless, a previous report demonstrated that thrombin stimulates production of ET in cultured endothelial cells (Schini, *et al.*, 1989), suggesting that activation of the coagulation cascade, which occurs upon endothelial damage, can ultimately result in ET-1 formation. This information, taken together with the endothelial damage in coronary-ligated rabbit pulmonary arteries and the pulmonary congestion indicated by the significant increase in lung weight in 8 week animals, which were demonstrated in this chapter, provides further evidence for the possible role of local ET-1. Alternatively, from the findings of the studies of chapter 6, vessels from this same group of animals appeared to have increased NOS compared to age-matched sham-operated. Neddleman & Johnson (1973) reported that in man, a tolerance to the effects of nitrates was noted following an increase in stimulation by NO by long term nitrate administration. In the same line, another previous study reported that in rats chronically treated with L-NAME such that eNOS was depleted, the pulmonary arteries were hypersensitive to SNP (MacLean & MacMillan, 1993). These findings taken together suggest that when there is increased stimulation by NO either by upregulation of eNOS or administration of nitrates, the vascular smooth muscle compensates by a reduction in the sensitivity to NO. Whereas when eNOS is inhibited, the reverse case holds, with the smooth muscle becoming hypersensitive to NO. Furthermore, the findings of the studies in 32 week procedure group animals provide more evidence for this latter possibility. In PRAs from these animals, SNP evoked a similar response in vessels from coronary-ligated and age matched stock rabbits. In the studies of chapter 6, L-NAME had no significant effect on responses to ET-1 and SXS6c

in PRAs from 32 week procedure rabbits. Thus these findings taken together are in keeping with the possible role of NOS in SNP-induced responses.

The thromboxane A_2 (TXA_2) mimetic U46619 was used to preconstrict the PRAs. The contractile response to this agent was similar in all vessels from the 8 week procedure group, however was markedly augmented in PRAs from 32 week ligated rabbits compared to age-matched stock and 8 week preparations. Increased activity of the pulmonary vasoconstrictor TXA_2 has been implicated in several forms of PHT. TXA_2 has been shown to be responsible for the early phase of sepsis-induced PHT (Weitzberg, *et al.*, 1995). It has also been implicated in other experimental models of PHT induced by heparin/ protamine (Montalescot, *et al.*, 1990), leukotriene D_4 (Noonan & Malik, 1986), microembolism (Garcia-Szabo, *et al.*, 1988) and ischaemia-reperfusion (Zamora, *et al.*, 1993). Furthermore, elevated levels of thromboxane B_2, the metabolite of TXA_2, has been found in neonatal PHT (Dodyns, *et al.*, 1994). The augmented contractile response to U46619 in 32 week ligated rabbit PRAs may indicate an alteration in receptors and /or intracellular pathway in this preparation. G-protein linked membrane TP receptors for TXA_2 are coupled with phospholipase C (PLC) (Strader, *et al.*, 1995). Activated PLC catalyses the hydrolysis of PIP_2 into IP_3, which releases Ca_{2+} from intracellular stores, and DAG, which activates PKC (see chapter 1). In addition, Sornik and Toro (1992) demonstrated that in pig coronary artery, U46619 inhibited Ca^{2+} activated-K^+ channel activity; thus an alteration in K^+ channel activity is another possibility for the augmented contractile response. Further investigations using agents which interfere which the underlying intracellular mechanism, such as PKC activators (e.g. phorbol myristate acetate (PMA)) or inhibitors (e.g. staurosporine), or selective K^+ channel blockers would provide more insight into this observation.

To summarise, following coronary artery ligation, rabbits exhibited a marked decrease in ejection fraction, left ventricular hypertrophy, right ventricular hypertrophy, augmentation in lung weight and pulmonary vascular remodelling. No functional differences to KCl in isolated PRAs were found between age-matched control rabbits

and those which were ligated for 8, 16 or 32 weeks. However, impaired contraction to sumatriptan and 5-HT, and relaxation to SNP was noted in PRAs from 8 week coronary artery ligated animals compared to sham-operated rabbit vessels. These results demonstrate the existence of PHT secondary to LVD in this model and show alterations in functional responsiveness of isolated PRAs which does not appear to be related to changes the integrity of the vascular smooth muscle.

Chapter 4

ET-receptor mediated responses in rabbit pulmonary resistance arteries: Effect of pulmonary hypertension secondary to left ventricular dysfunction

4.1 Introduction

The role of endothelin-1 (ET-1) in the pulmonary circulation, including biosynthesis, clearance, and the marked effects on the vasculature, both structurally and functionally, has been introduced in chapter 1 of this thesis. Increases in the plasma concentration of ET-1 has been described in patients with acute myocardial infarction (Miyauchi, *et al.*, 1989) and in patients with pulmonary hypertension associated with congenital heart defects (Yoshibayashi, *et al.*, 1991). In this latter study the authors indicated that this elevation is mainly due to increased production of ET-1 in the pulmonary circulation. Kiowski *et al* (1995) and Cody *et al* (1992) reported that plasma levels of ET-1 are significantly elevated in patients with chronic heart failure and this correlated directly with several variables including the extent of pulmonary hypertension and pulmonary vascular resistance. After administration of ET-1 in man, pulmonary vascular resistance increased more than systemic vascular resistance (Weitzberg, *et al.*, 1993). Sorensen *et al* (1994) also reported pronounced effects of exogenous ET-1 in human pulmonary circulation. Intravenous infusion of the non-selective ET-receptor antagonist bosentan was reported to decrease pulmonary vascular resistance in patients with chronic heart failure (Kiowski, *et al.*, 1995) and was reported to be effective in a rat model of chronic heart failure (Teerlink, *et al.*, 1994). ET antagonist have also been shown to reduce the PHT associated with coronary artery ligation in the rat (Sakai, *et al.*, 1996). These observations provide more support for the notion that ET-1 is important in the regulation of pulmonary vascular tone and suggest the possible involvement of ET in the pathophysiology of PHT associated with heart abnormalities.

Measurement of haemodynamic indices in these previous *in vivo* studies however reflects only the sum of responses. Furthermore, the control of PVR is not uniform throughout the pulmonary vasculature and arteries with a diameter greater than 500μm are thought to relatively unimportant (Andersson, *et al.*, 1985). In addition PHT has been shown to be associated with increased expression of ET-1 in vascular endothelial cells and the greatest degree of immunostaining occurred in the endothelium

of elastic and muscular pulmonary arteries (which also displayed severe medial thickening an intimal proliferation) compared with other pulmonary vessels (Giaid, *et al.*, 1993). The majority of previous *in vitro* studies of ET-1 in the pulmonary vasculature were conducted in large pulmonary arteries with internal diameters ranging from 2-5mm, *in situ* location of these arteries were either main or branch extrapulmonary arteries or intrapulmonary arteries, depending on the species (Panek *et al*, 1992; LaDouceur *et al*, 1993; Watanabe, *et al.*, 1991). Thus a greater understanding of the role of ET-1 in influencing the pulmonary vascular tone would be gained by examining the response of isolated small muscular arteries. I pursued this possibility by examining the functional responses in rabbit small muscular intrapulmonary arteries (referred to as pulmonary resistance arteries (PRAs)) throughout this study.

In this chapter I examined the vascular reactivity to ET-1 in PRAs obtained from rabbit coronary-ligation model of left ventricular dysfunction. The results in chapter 3 showed evidence for the condition of PHT in these animals. ET-1 and ET-3 are isopeptides, encoded by distinct genes (Inoue *et al*, 1989). The ET_A receptor shows selectivity of ET-1 over ET-3 (Arai, *et al.*, 1990; Masaki, *et al.*, 1991) whereas, the ET_B receptor is non-isopeptide selective (Sakuri, *et al.*, 1990; Masaki, *et al.*, 1991). I used ET-1 as a non-selective agonist at ET_A and ET_B receptors, and the peptides ET-3 and sarafotoxin S6c (SXS6c) as a selective ligands for the ET_B receptor subtype; these peptides have ~2000- and 300000 fold selectivity, respectively, for the ET_B over the ET_A receptor (Williams, *et al.*, 1991a;b). The responses in coronary ligated preparations were compared with those in vessels from stock and sham-operated rabbits to examine the possible influence of PHT secondary to left ventricular dysfunction on ET-receptor mediated responses in PRAs. Furthermore, I investigated the effect of different durations of permanent coronary artery ligation using preparations from rabbits studied 8, 16 and 32 weeks after coronary ligation.

4.2 Methods

Rabbit pulmonary resistance arteries

Lungs were obtained from the rabbit coronary ligation model of left ventricular dysfunction which has been extensively characterised (Pye *et al*, 1996) and is described in detail in section 2.1.1 of this thesis. In the 8 week and 16 week procedure period groups, age matched animals underwent the same methodology as the experimental animals (referred to in results and discussion as "ligated") except the ligatures placed around the coronary artery were not secured. These are subsequently referred to as "sham-operated". Also, age matched animals in which no operational procedures were performed were used as controls in the 16 week and 32 week procedure period groups These are subsequently referred to as "stock". Measurements of ejection fraction were made in all animals and animals were killed by sodium pentabarbitone 8, 16 or 32 weeks following the procedure. The lungs were promptly removed and small intralobar pulmonary resistance arteries (PRAs ~150 μm i.d.) were dissected out according to the methods stated in section 2.2.4.1. These were mounted as ring preparations (~2mm long) on a wire myograph, bathed in Krebs solution at 37°C. Using the normalisation process explained in section 2.2.6, vessels were tensioned to an equivalent transmural pressure of ~ 16 mmHg. This pressure was chosen as it is similar to *in vivo* pressures of rabbit pulmonary arterioles. Preliminary studies in our laboratory showed that bubbling with 95% O_2 inhibits responses to vasoconstrictors in rabbit PRAs so we bubbled with 16% O_2/ 5% CO_2 balance N_2. These gas mixtures were chosen as they are similar to those which the vessels would be exposed to *in vivo*.

Experimental protocol

Vessels were allowed to equilibrate for 1 hour prior to the addition of any drugs. The response of the PRAs to 50mM KCl was determined twice; once the contractile

response had reached a plateau, the vessels were washed out at least 6 times with fresh Krebs solution. Cumulative concentration-response curves (CCRCs) were then constructed to ET-1 or SXS6c (1pM-0.3μM) in PRAs; ET-3 (1pM-0.3μM) responses were examined in 8 week procedure group only. In a proportion of SXS6c experiments in vessels from 8 and 16 week procedure groups, 0.1μM ET-1 was added to the bath at the end of the SXS6c CCRC, before washing, so as to estimate the SXS6c response as a percentage of response to a maximal ET-1 concentration.

The possibility of SXS6c-induced vasorelaxations was studied in 8 week preparations only. After the second KCl response, following washout and appropriate rest period, vessels were preconstricted with 1μM 5-HT or 30nM U46619 and SXS6c (0.01pM-0.1nM) was added cumulatively to the bath.

Note

(1) ET-receptor agonists are known to be slow acting in terms of production of functional responses (see chapter 1). Thus, in all studies involving these peptides, I left intervals of at least 5 minutes between each subsequent concentration in order to ensure that a plateau in the response had been reached.

(2) Due to the expense of ET-1 and the vast number of experiments in which it was to be utilised, cumulative responses were examined in the PRAs up to a concentration of 0.3μM. In some results shown in this chapter it is not always evident if indeed a maximum response had been attained by 0.3μM ET-1. However in several studies, I constructed CCRC's up to 1μM ET-1 in order to investigate this. At this financially feasible top concentration the contractile response was very similar to that evoked by 0.3μM ET-1. Hence I was satisfied that terminating the CCRC's at this concentration gave viable maximal responses of the vessels to this peptide.

(3) In order to examine the possibility that the actual procedures undertaken on the sham-operated rabbits had any affect on the functional responses of isolated tissues eventually obtained, age-matched stock animals were included in the 16 week procedure

experimental group. This allowed comparisons to be made with age-matched sham-operated rabbit tissue.

Data analysis

Results are expressed graphically as percentage of their own maximum contraction, or percentage of reference contraction to second application of 50mM KCl., or percentage of contraction to 0.1μM ET-1. pEC_{10}, pEC_{25} and pEC_{50} values (where appropriate) were calculated according to the methods stated in section 2.5.2, and expressed as -log M concentration. Statistical analysis of the means of groups of data were made by Student's unpaired t-test when two groups of data were compared, and one-way analysis of variance (ANOVA) followed by Tukeys post test when three groups or more were compared; $P<0.05$ was considered statistically significant. Throughout, data are expressed as mean ± SEM and n/n = number of ring preparations / number of animals.

4.3 Results

The ejection fraction of animals from the various experimental groups, from which lungs were obtained and PRAs dissected for the studies described in this chapter, are shown in table 4.1. The effective pressure to which these vessels were tensioned and the resulting estimate of internal diameter are also shown in table 4.1. The ejection fraction was markedly reduced in ligated animals compared with sham-operated/stock rabbits in 8, 16 and 32 week procedure groups. Also, the reduced ejection fraction was significantly lower in 16 week ligated compared to 8 and 32 week ligated rabbits. Vessels from all experimental groups were tensioned to a similar effective pressure and internal diameter.

	(n)	Internal diameter (μM)	Effective pressure (mmHg)	Ejection fraction (%)
8 week				
sham-operated	(29)	162.2±7.2	20.8±1.2	72.8±1.0
ligated	(34)	169.1±8.6	20.7±0.8	45.2±0.9 ***
16 week				
stock	(12)	151.1±13.6	20.2±1.8	75.1±1.5
sham-operated	(9)	135.8±3.3	17.7±1.8	77.8±1.9
ligated	(36)	163.2±8.2	18.9±0.9	39.3±1.0***+
32 week				
sham-operated	(10)	151.7±9.5	15.7±1.2	75.0±1.3
ligated	(12)	146.4±9.1	19.5±1.5	47.7±1.3***

Table 4.1 (1) Ejection fraction of animals from which PRAs were dissected and studied in this chapter and (2) internal diameter and effective pressure of PRAs from this cohort of rabbits. Statistical comparisons were made by Student's unpaired t-test, ligated vs. sham-operated/stock, ***P<0.001; ANOVA, 16 week ligated vs. 8 and 32 week ligated, ^{+}P<0.001. Values are mean±SEM. n, number of ring preparations.

8 week experimental group

Figure 4.1 demonstrates CCRC's to ET-1, SXS6c and ET-3 in 8 week sham-operated and coronary-ligated rabbits. These peptides evoked phasic responses. For ET-1 and ET-3, the first component showed a gradual slope up to ~1nM and a markedly steeper incline was evident at higher peptide concentrations. SXS6c-evoked CCRC was of a different shape, reaching a maximum at 10nM and "dropping off" at higher concentrations (figure 4.1A; 4.1B). All three ET-receptor agonists were potent vasoconstrictors of rabbit PRAs from both experimental groups and $p\mathrm{EC}_{10}$, $p\mathrm{EC}_{25}$ and $p\mathrm{EC}_{50}$ values are summarised in table 4.2. Considering the $p\mathrm{EC}_{50}$ values, the potency order for these peptides were SXS6c>ET-3=ET-1 in vessels from both groups of animals. No difference in sensitivity were noted between sham-operated and coronary ligated rabbit vessels.

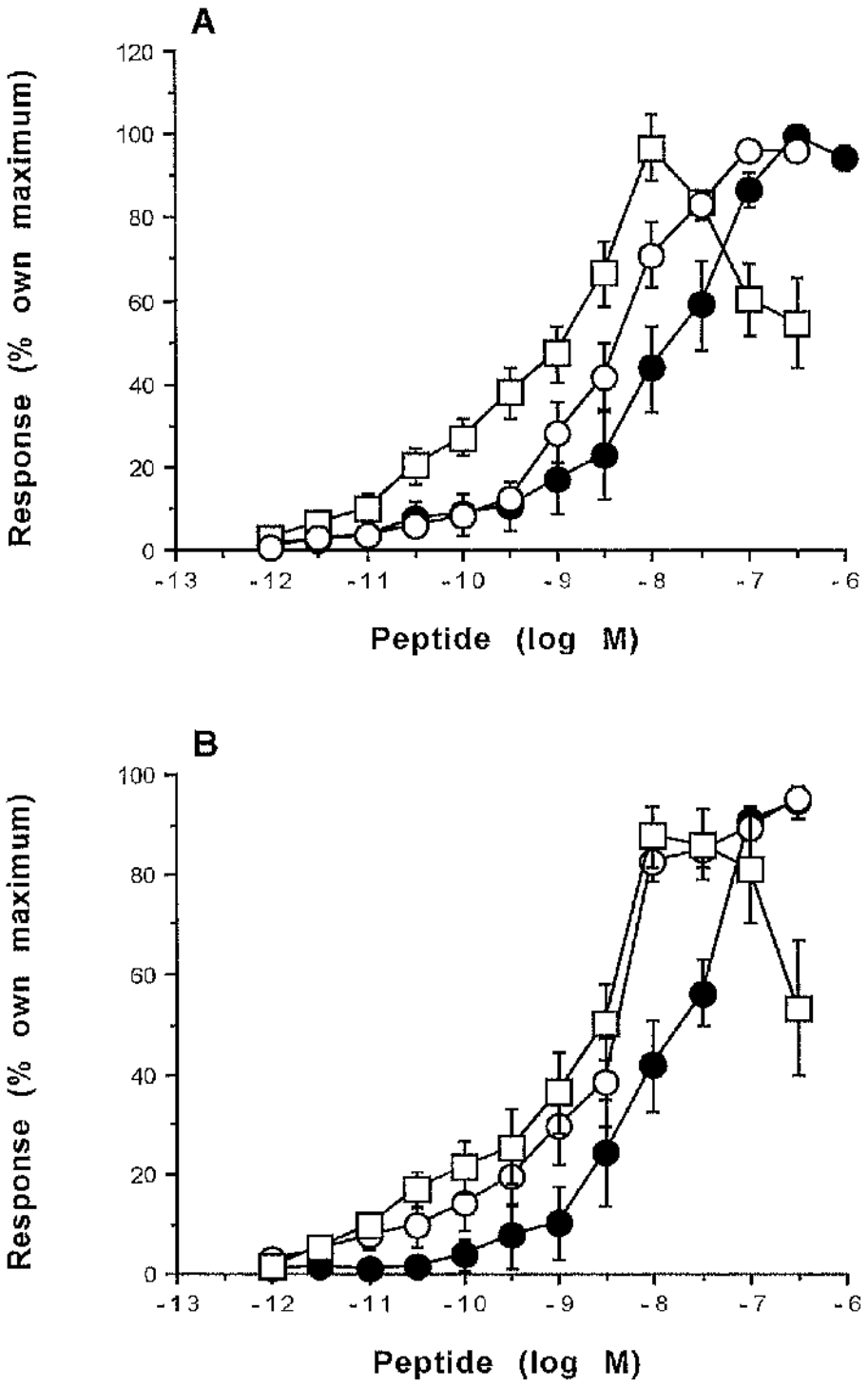


Figure 4.1 Responses to ET-receptor agonists in PRAs from 8 week experimental group animals. Data are expressed as a percentage of their own maximum. **A**. CCRC's to ET-1 (●, n=8/6), SXS6c (❑, n=10/9) and ET-3 (○, n=10/9) in PRAs from sham-operated rabbits. **B**. CCRC's to ET-1 (n=12/7), SXS6c (n=11/7) and ET-3 (n=9/6) in PRAs from coronary ligated rabbits. Each point represents mean± s.e. mean.

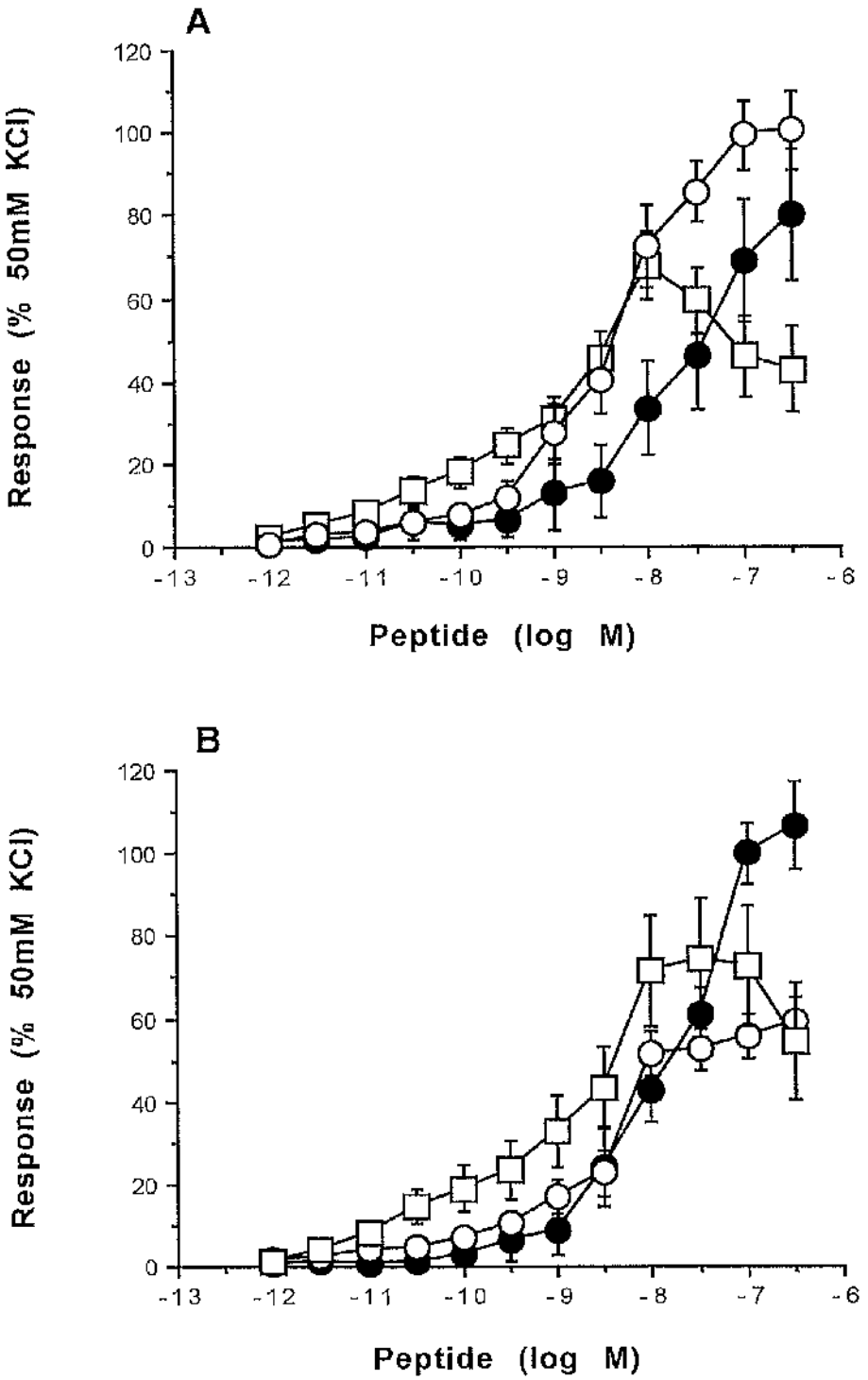


Figure 4.2 Responses to ET-receptor agonists in PRAs from 8 week experimental group animals. Data are expressed as a percentage reference contraction to 50mM KCl. **A**. CCRC's to ET-1 (●, n=8/6), SXS6c (□, n=10/9) and ET-3 (○, n=10/9) in PRAs from sham-operated rabbits. **B**. CCRC's to ET-1 (●, n=12/7), SXS6c (□, n=11/7) and ET-3 (○, n=9/6) in PRAs from coronary ligated rabbits.

Figure 4.3

A. ET-receptor mediated vasoconstriction in PRAs from sham-operated (open columns) and coronary-ligated (hatched columns) rabbits. Statistical comparisons by ANOVA: coronary-ligated vs. sham-operated response, $^{**}P<0.01$; SXS6c or ET-3 response vs. ET-1 response in same group, $^{+}P<0.05$, $^{+++}P<0.001$. Data are expressed as absolute contraction mg weight tension.

B. ET-3 response in PRAs from 8 week sham-operated (○, n=10/9) and coronary-ligated (●, n=9/6) rabbits. Data are expressed as a percentage reference contraction to 50mM KCl. Each point represents mean±s.e. mean.

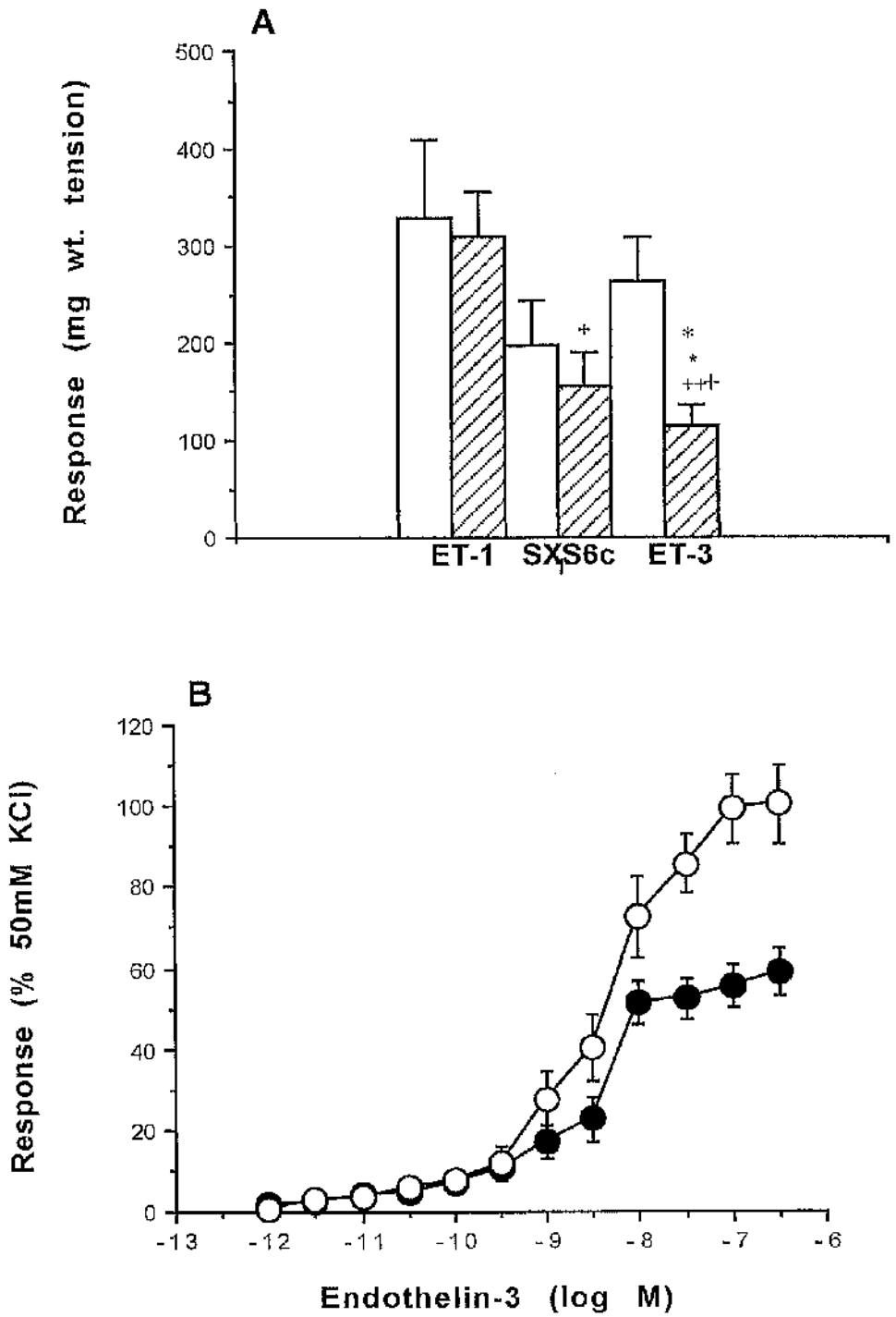


Figure 4.3 Magnitude of ET-receptor mediated vasoconstriction in PRAs from 8 week sham-operated and coronary-ligated rabbits.

The magnitude of responses to these ET-receptor agonists in PRAs from sham-operated and coronary ligated rabbits are shown in figures 4.2 and 4.3. The maximal responses to SXS6c and ET-3 were markedly less than that to ET-1 in the coronary-ligated rabbit vessels, both in terms of percentage reference contraction to 50mM KCl (110±8.3% (ET-1) vs. 78.4±12.1% (SXS6c, $P<0.05$); vs. 5±9.2±5.9% (ET-3, $P<0.001$) ; figure 4.2B), and absolute contraction mg weight tension (308.9±47.6 (ET-1) vs. 156.7±34.2 (SXS6c, $P<0.05$); vs. 113.5±22.6 (ET-3, $P<0.01$) ; figure 4.3A). In PRAs from sham-operated animals, ET-1- and ET-3-induced maximal vasoconstrictions were similar (~90% / 300 mg wt. tension); SXS6c responses tended to be comparatively smaller however this was not significant (figures 4.2A and 4.3A). The amplitude of responses to ET-1 and SXS6c were similar in the two experimental groups however, the ET-3-evoked maximal vasoconstriction was significantly reduced in PRAs from coronary-ligated rabbits compared to the response from sham-operated vessels ($P<0.01$, figure 4.3B). ET-1 (0.1μM) was added at the top of the SXS6c CCRC to examine the amplitude of the response compared to ET-1 in the same tissue; this is illustrated in figure 4.4A. No difference was evident between the sham-operated and coronary ligated preparations, with both displaying SXS6c maximum of ~65% response to 0.1μM ET-1.

Other vessels from this group were preconstricted with either 1μM 5-HT or 30nM U46619, prior to the addition of SXS6c (0.01pM-0.1nM). In both sham-operated and coronary-ligated preparations no vasorelaxations were observed in either 5-HT or U46619 preconstricted vessels. However, significant constrictor responses were seen from 0.1pM onwards, compared with time controls (results are not shown; n/n=4/4).

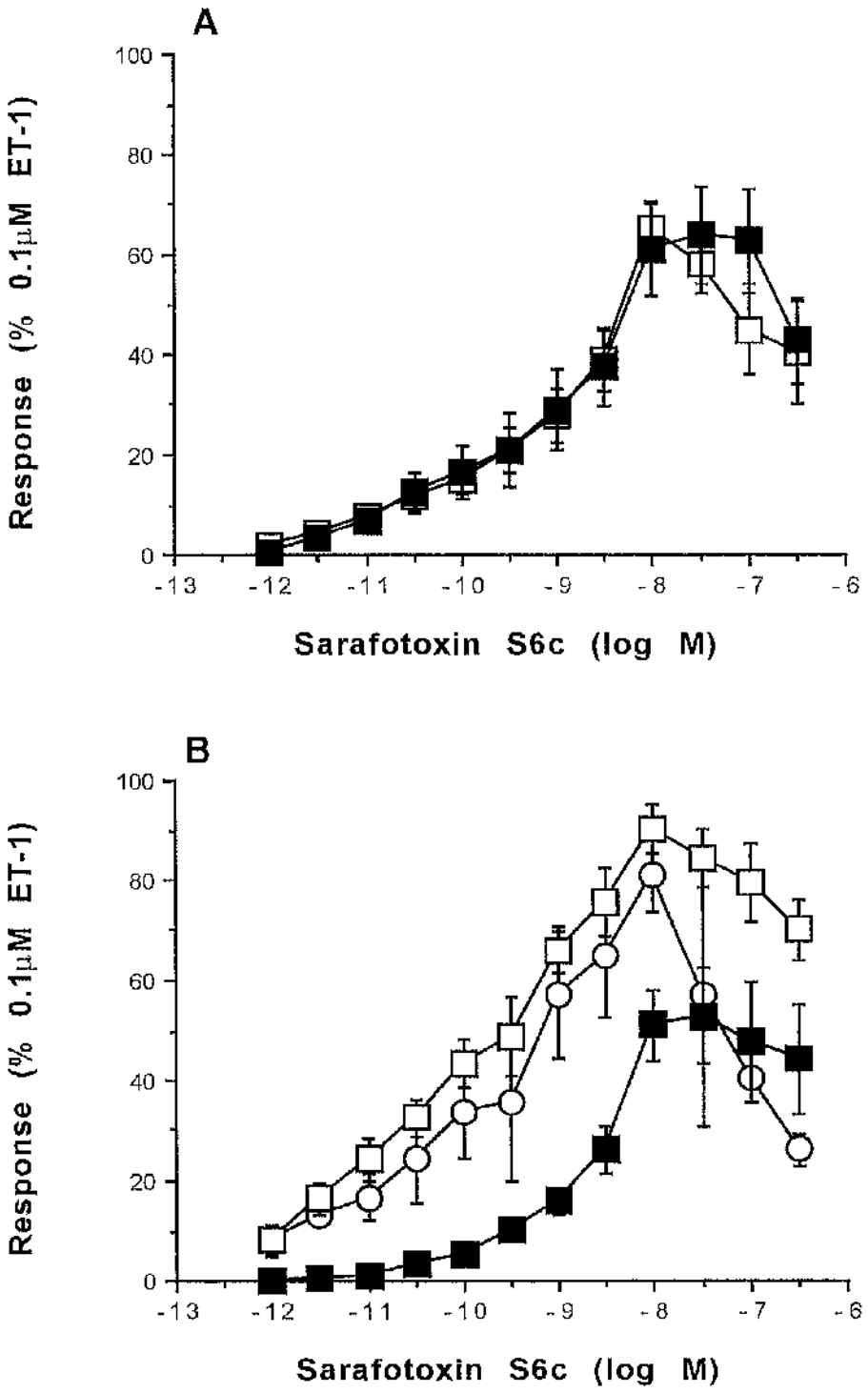


Figure 4.4 SXS6c-evoked vasoconstrictions in rabbit PRAs. Data are expressed as a percentage contraction to 0.1μM ET-1. **A**. CCRC's in vessels from sham-operated (❑, n=10/9) and coronary-ligated (■, n=11/7) 8 week group animals. **B**. CCRC's in vessels from stock (○, n=3/3), sham-operated (❑, n=6/6) and coronary-ligated (■, n=9/6) 16 week group animals. Each point represents mean± s.e. mean.

16 week experimental group

Vasoconstrictor responses in 16 week stock, sham-operated and coronary ligated rabbit PRAs to ET-1 and SXS6c are displayed in figures 4.5A and 4.5B, respectively.

	pEC_{10}	pEC_{25}	pEC_{50}	n/n
sham-operated				
ET-1	9.1±0.4	8.6±0.4	7.9±0.3	8/6
SXS6c	10.6±0.26**	9.9±0.3*	9.0±0.2**	10/9
ET-3	9.6±0.3^{+}	8.9±0.2^{++}	8.4±0.2^{+}	10/9
ligated				
ET-1	8.9±0.4	8.3±0.2	7.9±0.2	12/7
SXS6c	10.4±0.3**	9.4±0.3**	8.6±0.2*	11/7
ET-3	9.7±0.5	9.1±0.3*	8.5±0.2	9/6

Table 4.2 Sensitivity to ET-receptor agonists in PRAs from 8 week procedure rabbits. Statistical comparisons were made by ANOVA followed by Tukey's post test: SXS6c vs. ET-1, *$p<0.05$, **$p<0.01$; ET-3 vs. SXS6c, $^{+}p<0.05$, $^{++}p<0.01$. Values are mean ± SEM. ET-1, endothelin-1; ET-3, endothelin-3; SXS6c, sarafotoxin S6c; n/n, number of ring preparations/number of animals.

ET-1 CCRCs were again seen to contain a relatively shallow component at the lower concentrations. Vessels from sham-operated rabbits appeared more sensitive in this lower portion of the ET-1 curve however this was not significant (figure 4.5A). The nature of the SXS6c response was also similar to that exhibited in 8 week preparations (figure 4.5B). pEC_{10}, pEC_{25} and pEC_{50} values are summarised in table 4.3. SXS6c was markedly more potent than ET-1 in vessels from all 16 week animals, as was noted in all 8 week rabbits. However, in contrast to findings between different groups after 8 weeks on procedure, both ET-1 and SXS6c differed in sensitivity in 16 week ligated preparations compared to stock/sham-operated rabbit vessels. ET-1 was significantly

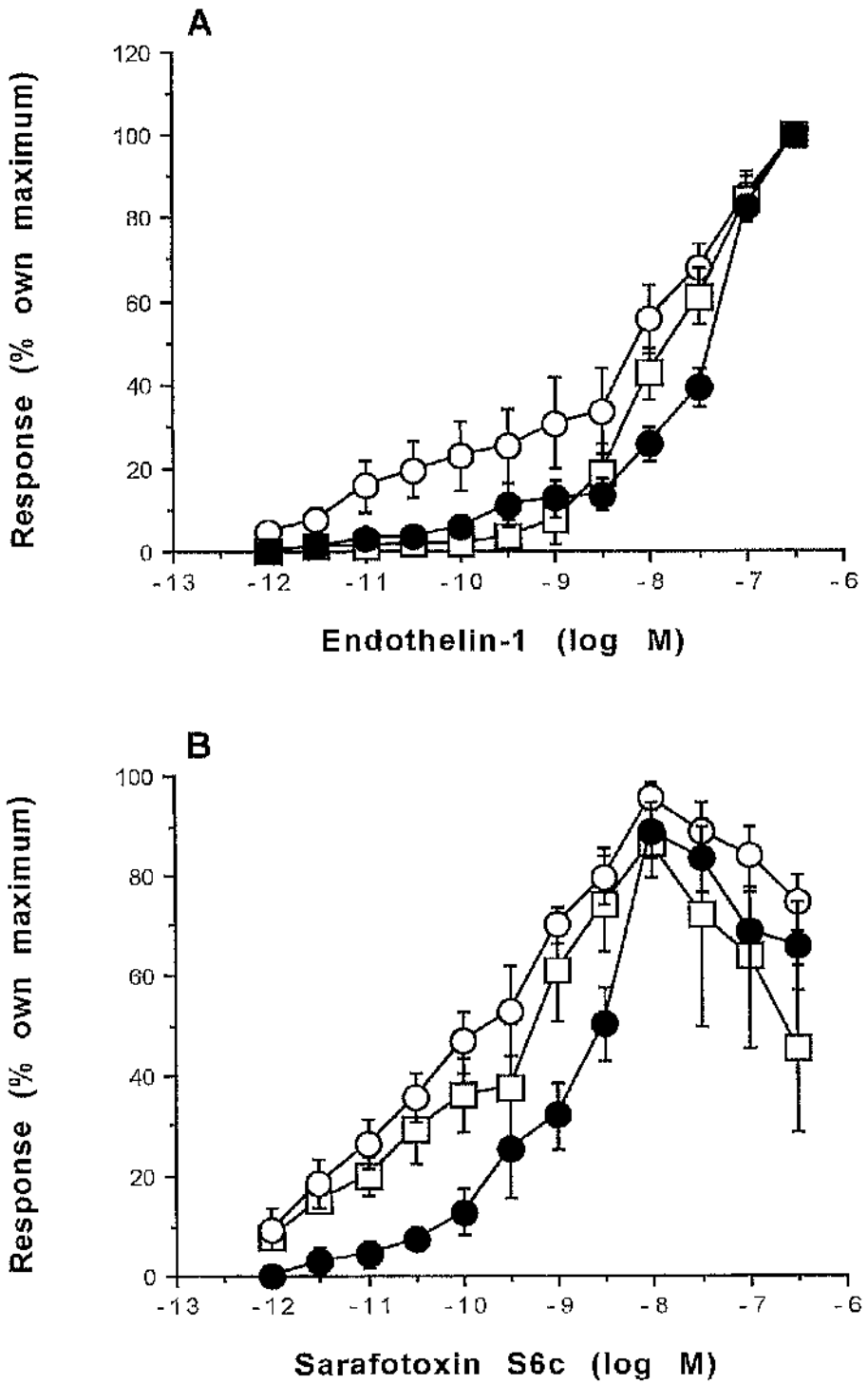


Figure 4.5 Responses to ET-receptor agonists in PRAs from 16 week experimental group animals. Data are expressed as a percentage of their own maximum. **A** ET-1 CCRC's in stock (❑, n=6/5), sham-operated (○, n=5/5) and coronary-ligated (●, n=13/7) rabbit PRAs. **B** SXS6c CCRC's in stock (❑, n=3/3), sham-operated (○, n=6/6) and coronary-ligated (●, n=9/6) rabbit PRAs. Each point represents mean± s.e. mean.

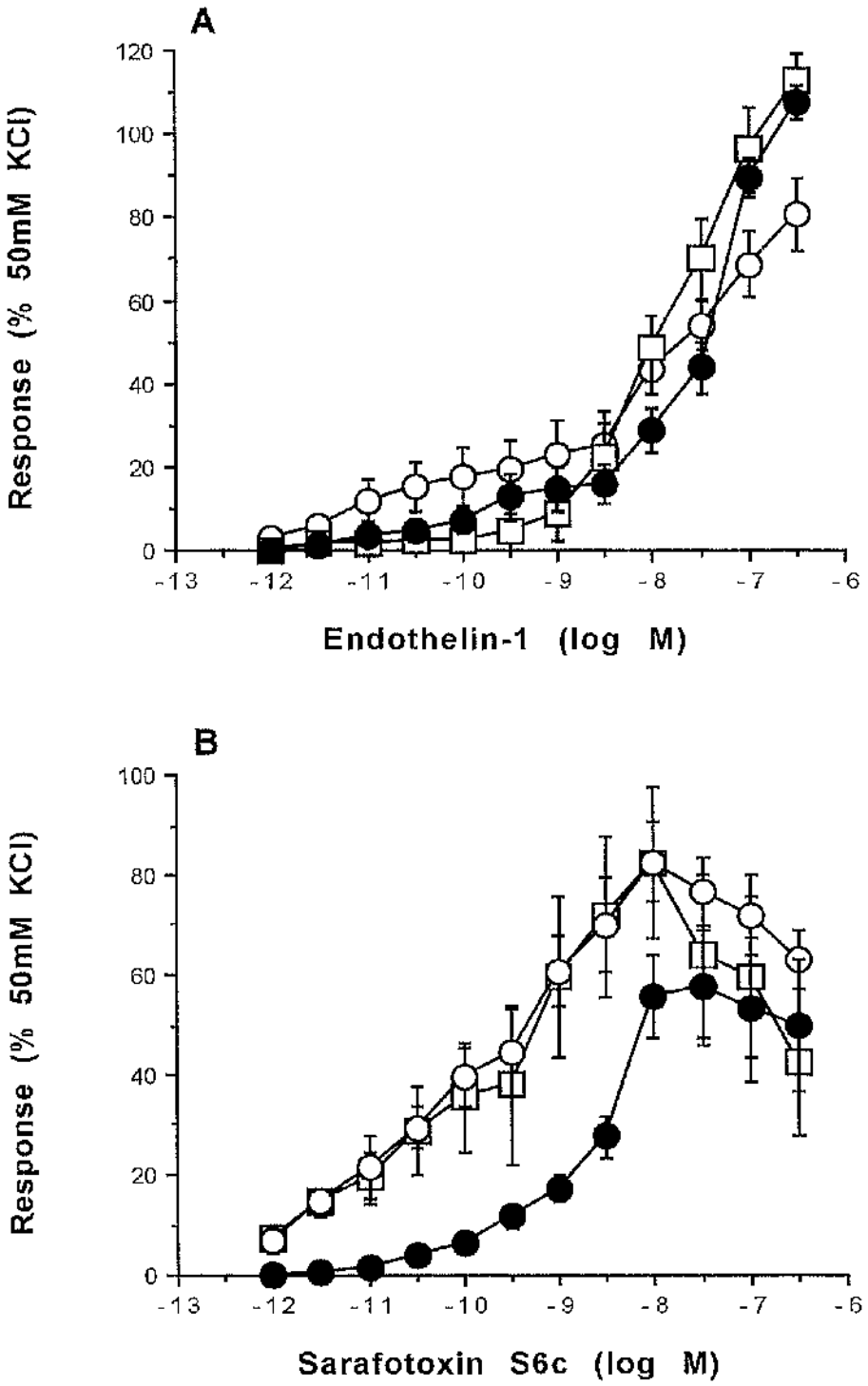


Figure 4.6 Responses to ET-receptor agonists in PRAs from 16 week experimental group animals. Data are expressed as a percentage reference contraction to 50mM KCl.. **A** ET-1 CCRC's in stock (□, n=6/5), sham-operated (○, n=5/5) and coronary-ligated (●, n=13/7) rabbit PRAs. **B** SXS6c CCRC's in stock (□, n=3/3), sham-operated (○, n=6/6) and coronary-ligated (●, n=9/6) rabbit PRAs. Each point represents mean±SEM.

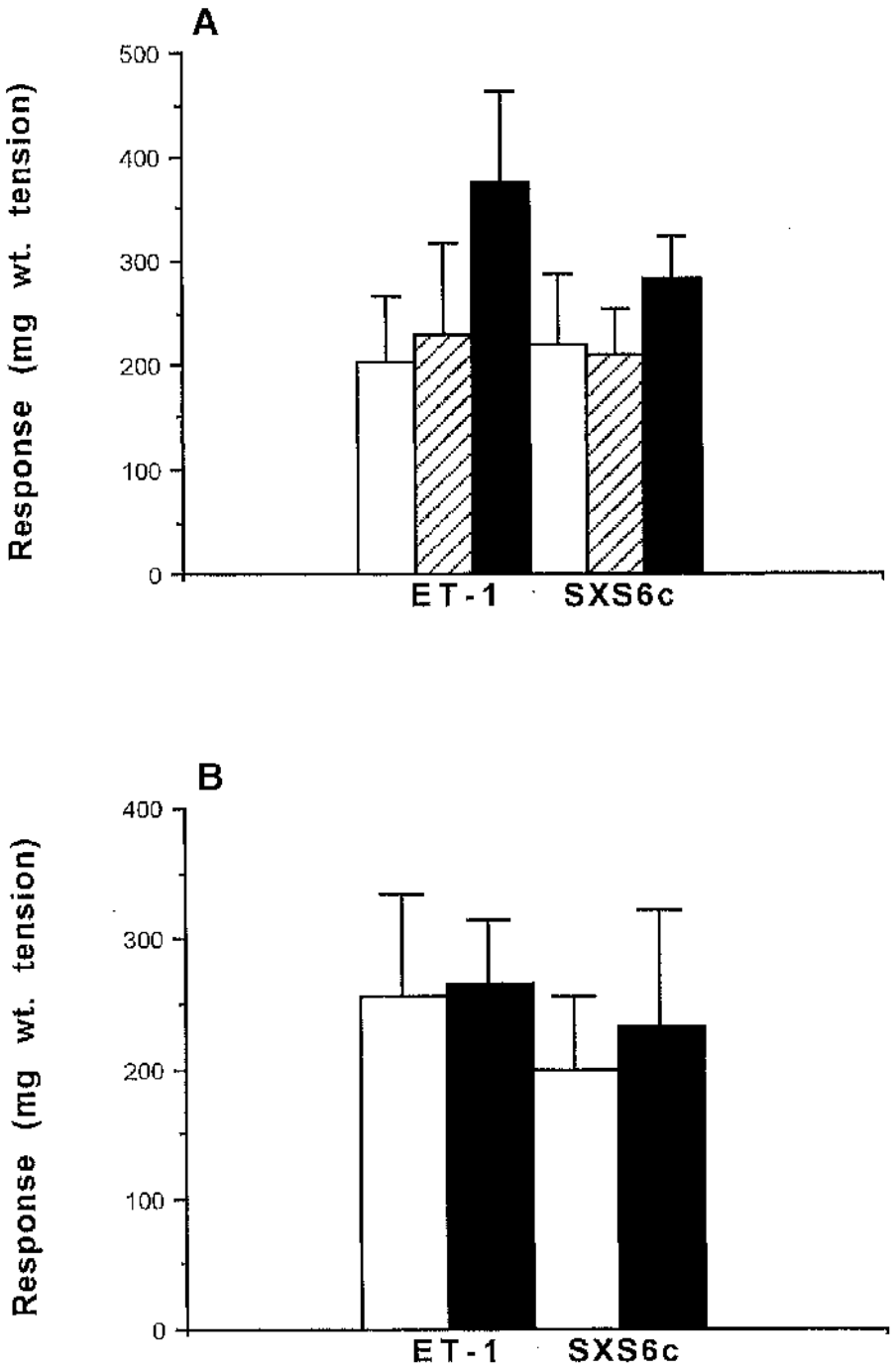


Figure 4.7 ET-1- and SXS6c-induced vasoconstrictions in PRAs from 16 and 32 week procedure rabbits. **A**. 16 week stock (open columns), sham-operated (hatched columns) and coronary-ligated rabbits (filled columns); n/n as figure 4.6. **B**. 32 week stock (open columns) and coronary-ligated rabbits (filled columns); n/n as figure 4.8. Data are expressed as absolute contraction mg weight tension. Each point represents mean±SEM.

less potent in PRAs from coronary-ligated compared to sham-operated animals (table 4.3, figure 4.5A). The sensitivity to SXS6c was also markedly reduced in coronary-ligated rabbit PRAs compared to vessels from stock and sham-operated animals (table 4.3, figure 4.5B). For both ET-1 and SXS6c, no difference in sensitivity existed between the stock and sham-operated preparations.

The magnitude of responses to these peptides in PRAs from 16 week procedure rabbits are shown in figures 4.6A, 4.6B and 4.7A. In PRAs from stock and sham-operated animals, both peptides evoked similar maximal vasoconstrictions, in terms of percentage reference contraction to KCl and absolute response (figure 4.7A). However, maximal responses to SXS6c were markedly less than that to ET-1 in the coronary-ligated rabbit vessels; this attenuation was significant when analysed as percentage KCl response (89.6±4.8% vs. 57.5±11.5%, $P<0.01$). This is similar to situation noted following 8 weeks on procedure. No significant difference was noted in the absolute response to these peptides between the different experimental groups (figure 4.7A). Whereas on examining the vasoconstrictions relative to KCl response, the following was noted. ET-1 maximum was similar in stock and coronary ligated tissues (112.8±6.5% and 107.8±3.9%, respectively) but that noted in sham-operated animal PRAs was significantly lower (80.9±8.7%, $P<0.01$; figure 4.6A). In the case of SXS6c, the maximum vasoconstriction was significantly attenuated in PRAs from coronary ligated rabbits (57.5±11.5%) compared to those from stock (82.5±15.1%) and sham-operated (82.6±8.0%) animals ($P<0.01$). This marked difference in amplitude of response was evident at all SXS6c concentrations up to 10nM (figure 4.6B). Furthermore, on examining the responses relative to the vasoconstriction to 0.1μM ET-1 in the same preparation, the magnitude was again evidently reduced in coronary-ligated rabbit vessels compared to those from sham-operated animals (52.8±9.5% vs. 90.3±8.8%, $P<0.001$, figure 4.4B). This is in contrast to the 8 week group where no difference existed (figure 4.4A). However, the response was significantly lower in sham-operated preparations from 8 week procedure compared to 16 week procedure animals ($P<0.01$).

	pEC_{10}	pEC_{25}	pEC_{50}	n/n
stock				
ET-1	8.9±0.3	8.4±0.2	7.8±0.2	6/5
SXS6c	$11.7\pm0.04^{+++}$	$10.2\pm0.4^{++}$	$9.3\pm0.4^{++}$	3/3
sham-operated				
ET-1	9.7±0.6	10.1±1.0	8.2±0.2	5/5
SXS6c	$11.5\pm0.3^{+}$	10.8±0.3	$9.8\pm0.2^{+++}$	6/6
ligated				
ET-1	9.0±0.3	8.2±0.2*	7.5±0.1**	13/7
SXS6c	$9.9\pm0.2^{***b}$	$9.1\pm0.2^{***+++a}$	$8.6\pm0.2^{**+++}$	9/6

Table 4.3 Sensitivity to ET-1 and SXS6c in PRAs from 16 week procedure rabbits. Statistical comparisons were made by ANOVA: ligated vs. sham-operated, *p<0.05, **p<0.01, ***p<0.001; ligated vs. stock, ^{a}p<0.05, ^{b}p<0.001; SXS6c vs. ET-1, $^{+}$p<0.05, $^{++}$p<0.01, $^{+++}$p<0.001. Values are mean±SEM. ET-1, endothelin-1; SXS6c, sarafotoxin S6c; n/n, number of ring preparations/number of animals.

	pEC_{10}	pEC_{25}	pEC_{50}	n/n
stock				
ET-1	9.2±0.2	8.5±0.1	7.9±0.1	5/5
SXS6c	10.0±0.3	$9.4\pm0.2^{++}$	$8.7\pm0.15^{++}$	5/5
ligated				
ET-1	9.3±0.5	8.7±0.4	7.7±0.15	5/5
SXS6c	$10.8\pm0.2^{*+}$	$10.3\pm0.2^{**++}$	$9.4\pm0.2^{*+++}$	6/6

Table 4.4 Sensitivity to ET-1 and SXS6c in PRAs from 32 week procedure rabbits. Statistical comparisons were made by Student's unpaired t-test: ligated vs. stock, *p<0.05, **p<0.01; SXS6c vs. ET-1, $^{+}$p<0.05, $^{++}$p<0.01, $^{+++}$p<0.001. Values are mean±SEM. ET-1, endothelin-1; SXS6c, sarafotoxin S6c; n/n, number of ring preparations/number of animals.

32 week experimental group

The only controls available for this group were 32 week stock animals. The vasoconstrictor responses to ET-1 and SXS6c in PRAs from this group are displayed in figures 4.7B and 4.8. As described for the previous two experimental groups, a biphasic response was evident to ET-1 and the SXS6c-evoked vasoconstriction reached a maximum by 10nM, with further increasing concentrations causing a "fall off" in tension. pEC_{10}, pEC_{25} and pEC_{50} values are summarised in table 4.4. PRAs from both 32 week experimental groups were markedly more sensitive to the selective ET_B-receptor agonist SXS6c compared ET-1 (figure 4.8A), as was noted in all 8 and 16 week rabbit tissues. 32 week stock and coronary-ligated rabbit PRAs had similar sensitivity to ET-1 (figure 4.8A; table 4.4). Hence, this is the same as findings in the 8 week experimental group but differs from the 16 week group, where coronary-ligated preparations were comparatively less sensitive than the corresponding control vessels. Coronary-ligated vascular preparations were significantly more sensitive to SXS6c, over the entire rising component of the CCRC, compared to vessels from stock animals in this group (figure 4.8A; table 4.4). This again differs from the previously described experimental groups since no difference was noted after 8 weeks, whereas reduced sensitivity was evident following coronary ligation after 16 weeks.

The magnitude of responses in PRAs from 32 week procedure rabbits to ET-1 and SXS6c are shown in figures 4.7B and 4.8B. Maximal vasoconstrictions to SXS6c were markedly less than that to ET-1 in the coronary-ligated rabbit vessels in terms of percentage KCl (57.9±10.9% vs. 102.1±7.0%, $P<0.01$; figure 4.8B) but not absolute response (figure 4.8B). SXS6c maximal responses tended to be relatively smaller than those to ET-1 in stock animal vessels but this was not significant. This is the same situation noted in preparations from 8 and 16 week experimental groups. The magnitude of the responses to both ET-1 and SXS6c in the PRAs did not differ significantly between the stock and coronary ligated groups.

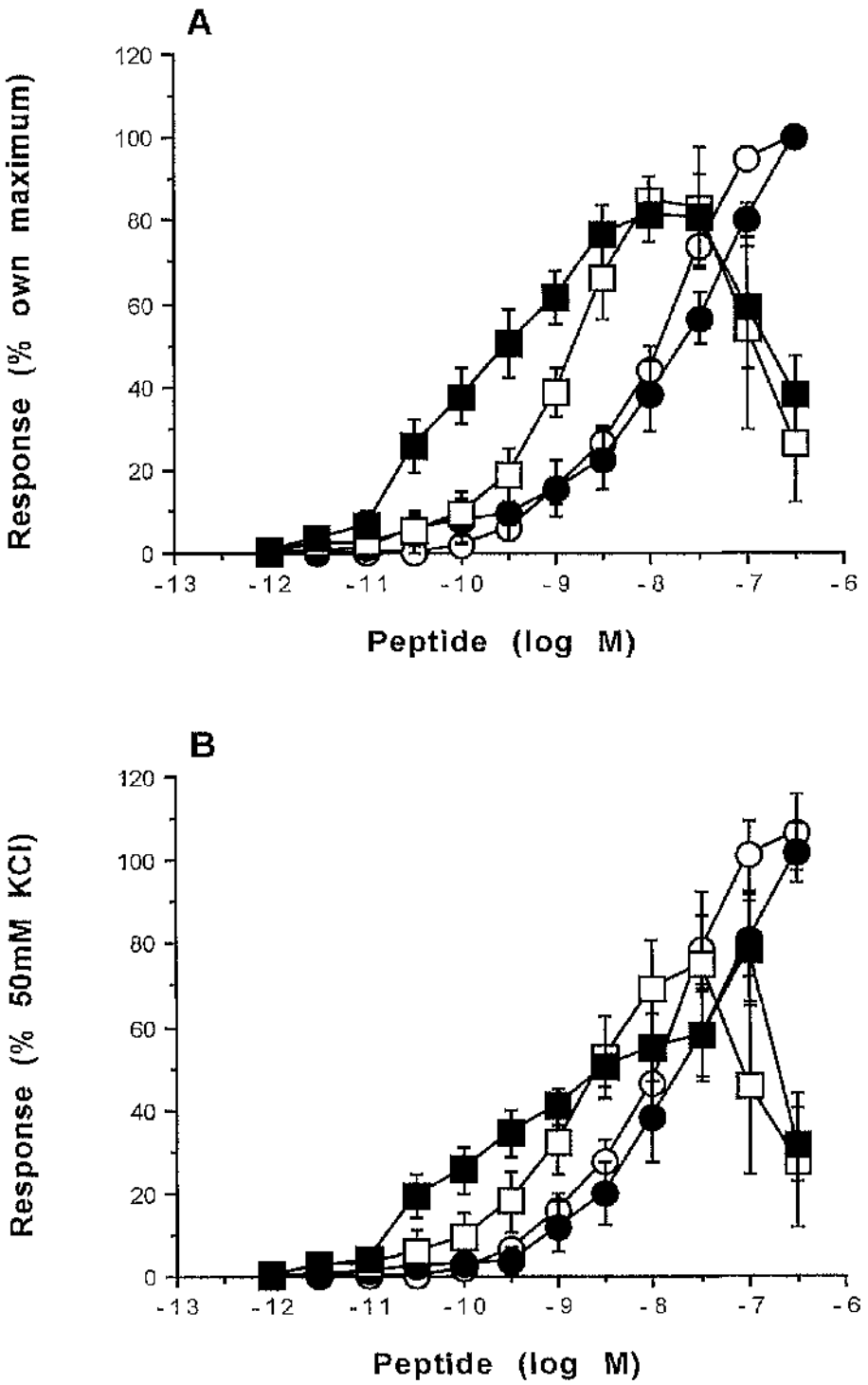


Figure 4.8 Responses to ET-receptor agonists in PRAs from 32 week experimental group animals. CCRC's to ET-1 / SXS6c in stock (○ / □, n=5/5 / n=5/5) and coronary-ligated (● / ■, n=5/5 / n=6/6) rabbit PRAs. **A** Data are expressed as a percentage of their own maximum. **B** Data are expressed as a percentage reference contraction to 50mM. Each point represents mean± s.e. mean.

When the results were analysed to compare the responses to ET-1 and SXS6c in vessels from animals at different ages the following was evident. ET-1 had a similar potency in vessels from 8, 16 and 32 week procedure period animals in both stock/sham-operated and coronary-ligated groups. However, PRAs from 16 week sham-operated rabbits were more sensitive to SXS6c compared to those from 8 week sham-operated (pEC$_{10}$ and pEC$_{50}$, P<0.05) and 32 week stock (pEC$_{25}$ and pEC$_{50}$ P<0.01) animals. In addition, tissue from 32 week coronary-ligated rabbits had greater sensitivity to SXS6c than vessels from 8 week (pEC$_{50}$ P<0.05) and 16 week (pEC$_{10}$ and pEC$_{25}$ P<0.01; pEC$_{50}$, P<0.05) coronary-ligated animals.

4,4 Discussion

In the groups of animals from which PRAs were studied in this chapter, the ejection fraction was markedly reduced in rabbits which had undergone coronary artery ligation compared to corresponding control animals. This provides evidence that coronary artery ligation in these rabbits produced left ventricular dysfunction (LVD); the depressed ejection fraction has also been shown in previous studies in this model (Pye, *et al.*, 1996; Denvir, *et al.*, 1996; Deuchar, *et al.*, 1997). In addition, the results of chapter 3 provide evidence for the concomitant pulmonary hypertension in this model.

On examining the effect of different durations of permanent coronary artery ligation, in the cohort of animals used in this study, the ejection fraction of 16 week ligated animals was markedly lower than that of 8 week ligated rabbits. However, this progression of attenuation was not extended to the 32 week LVD group. Hence the rabbits ligated for a longer period do not appear to represent a more severe form of LVD but rather are "survivors" in this group.

ET-receptor mediated responses

In this chapter ET-1 and SXS6c were potent vasoconstrictors of rabbit PRAs

from both control and experimental animals regardless of the duration of the procedure period. Previous studies have shown ETs to be potent spasmogens of pulmonary artery isolated from humans (Brink, *et al.*, 1992; McKay, *et al.*, 1991; Hay *et al.*, 1993; Buchan *et al,* 1994) and rabbits (Panek, *et al.*, 1992; White, *et al.*, 1993). In all vessels ET-1-evoked responses of a biphasic nature, comprising of an initial '"shallow component" at lower ET-1 concentrations and a steep rising incline at the higher concentration range. CCRC's to ET-1 of a biphasic nature have previously been demonstrated in rat (McCulloch & MacLean, 1995) and human PRAs (McCulloch *et al,* 1996; chapter 5 this thesis). These findings suggest a heterogeneous population of ET-receptors mediating the response. Indeed, radioligand binding studies of ET-1, described in chapter 7 of this thesis, supports this view. McCulloch & MacLean (1995) suggest that the biphasic nature of the rat PRA responses may be due to a heterogeneous population of ET_B receptors or due to the presence of inhibitory ET_A receptors. In this study, SXS6c induced potent vasoconstrictions which reached a maximum at ~10nM in all vessels from all three procedure period groups and then "dropped off" at higher concentrations. This was not observed in the CCRC's to ET-1. I also showed a similar '"drop off" in tension to high SXS6c concentration in PRAs from fetal and neonatal rabbits (Chapter 8 and 9, this thesis) and this has previously been shown in rat (McCulloch & MacLean, 1995). The most likely reason for this is receptor desensitisation. I investigated the possible involvement of nitric oxide (NO) in this phenomenon by studying the effect of the nitric oxide synthase inhibitor L-NAME on SXS6c-induced responses in preparations from both paradigms of the pulmonary circulation studied in this thesis; (1) PHT secondary to heart failure (chapter 6) and (2) the transitional perinatal pulmonary circulation (chapter 8). In both cases, this component of the SXS6c curve did not appear to be due to NO production as it persisted in the presence of L-NAME. Similar findings have been shown in separate studies in rat PRAs in our laboratory. Thus the most likely reason for this drop off in tension is receptor desensitisation. Indeed, La Douceur *et al* (1993) demonstrated in rabbit pulmonary branches that the contractile activity mediated by the ET_B receptor develops tachyphylaxis with prolonged exposure to SXS6c.

Given that both SXS6c and ET-3 caused vasoconstriction of rabbit PRAs, these results suggest the presence of vasoconstrictor ET_B receptors. SXS6c was markedly more potent than ET-1 in vessels from all groups. Warner *et al* (1993) reported that ET-1 and SXS6c were approximately equipotent in constricting rabbit pulmonary artery branch (~3mm ID). However in agreement with my findings in PRAs, La Douceur *et al* (1993) reported that rabbit pulmonary artery branches also showed a sevenfold greater sensitivity to SXS6c compared to ET-1; Panek et al (1992) reported similar results with SXS6c in this preparation. Furthermore, both these studies used endothelium denuded preparations thus making the vascular smooth muscle, as opposed to the endothelium, the likely site of action of SXS6c. Responses to ET-3 were studied in 8 week group animals only. In these vessels the potency order for the ET receptor agonists were SXS6c>ET-1=ET-3. Equipotency of ET-1 and ET-3 has also been shown in the larger pulmonary artery of the rabbit (White, *et al.*, 1994). Sakurai *et al* (1990) reported that the ET_B receptor was non-isopeptide selective, thus the equipotency of ET-1 and ET-3 provides further evidence for vasoconstriction being mediated ET_{B2}-like receptors and is indicative of the predominance of this receptor in this vascular preparation. In chapter 8 and 9, I also showed the predominance of vasoconstrictor ET_B receptors in neonatal rabbit PRAs and this receptor subtype has also been reported to evoke vasoconstrictions in the larger PA of the rabbit (Fukuroda *et al,* 1994a; Hay *et al*, 1996) and human PRAs (McCulloch *et al*, 1996). The role of ET_B receptors in vasoconstriction of the rabbit pulmonary arterial system has not only been demonstrated by functional pharmacological experiments; for example, Fukuroda and co-workers (1994b) reported that binding assays of membrane preparations from rabbit pulmonary arteries (1-5mm outer diameter) contained ET_A and ET_B receptors in the ratio 23:77. I investigated ET-receptor subtypes in rabbit PRAs from the coronary artery- ligation model and in human PRAs using selective antagonists; the results are shown and discussed in chapter 5 of this thesis.

Effects of left ventricular dysfunction secondary to coronary artery ligation

The results of this chapter show that the responsiveness of PRAs to ET-receptor agonists is altered in vessels from rabbits exhibiting pulmonary hypertension secondary to left ventricular dysfunction (LVD). In PRAs from animals in the 8 week procedure group no difference in the sensitivity or magnitude of responses to either ET-1 or SXS6c was evident between corresponding control and experimental vessels. The period of coronary-artery ligation had differential effects on the resulting ET-1-induced responses in rabbit PRAs. Following 16 weeks of coronary artery ligation, PRAs were ~6 fold less sensitive to ET-1 than vessels from sham-operated rabbits and the magnitude of vasoconstriction was attenuated compared to age-matched stock animals. Whereas in the 32 week procedure group, the ET-1 mediated response was similar in stock and "experimental" preparations, as was seen following ligation period of 8 weeks. The pEC_{50} for ET-1 was ~7.9 for all vessels from both these groups. This is similar to ET-1 sensitivity previously reported in rat PRAs (McCulloch & MacLean, 1996) however, is comparatively less sensitive to previous reports in rabbit pulmonary artery branches where $pEC_{50}$of ~8.5-9.2 was reported (LaDouceur, *et al.*, 1993; Warner, *et al.*, 1993). This difference may be due to variation in vessel size as ET-receptor mediated responses have been shown to differ in pulmonary of different sizes in the rat (MacLean, *et al*, 1994).

LVD also effected responses to SXS6c in small muscular pulmonary arteries in this model. The sensitivity to SXS6c was not altered in PRAs from 8 week procedure group however, the potency of this selective ET_B-receptor agonist was significantly reduced as a result of ligation for a 16 week period. This was indicated by the ~ eight fold reduction in sensitivity in these "experimental" vessels compared to corresponding sham-operated PRAs. Whilst following coronary artery ligation for 32 weeks, isolated PRAs were significantly more sensitive to SXS6c than vessels from age-matched stock rabbits. The magnitude of the SXS6c-induced vasoconstriction was not significantly altered in coronary-ligated rabbit PRAs from any of the three procedure groups

examined. Thus the period of permanent coronary artery ligation effects the resulting responsiveness of PRAs to SXS6c also. The finding that LVD had differential influences in ET-1- and SXS6c-evoked responses in PRAs suggests that ET_A and ET_B receptors may demonstrate different activity in pathological conditions. Differences in the response to SXS6c and ET-1 following LVD have also been shown in patients with chronic heart failure. Infusion of peptides into the non dominant brachial artery with simultaneous measurement of forearm blood flow, using the technique of venous occlusion plethysmography, revealed that forearm vasoconstriction to ET-1 was significantly blunted whereas vasoconstriction to SXS6c was markedly enhanced in CHF patients compared with control subjects (Love, *et al.*, 1996). Similar results have also been reported in a dog model of experimental congestive heart failure (CHF). Thoracic inferior vena cava constriction is an experimental model of CHF which is characterised by a decrease in cardiac output and an increase in plasma ET-1. In this model intracoronary administration of peptides showed an attenuated coronary vasoconstrictor response to ET-1 with an enhanced vasoconstrictor response to SXS6c in CHF dogs compared with controls (Cannan, *et al.*, 1996). In this present study the sensitivity to selective ET_B receptor stimulation was also increased in PRAs from 32 week LVD group compared with control vessels. Another investigation using pithed rats with congestive heart failure, induced by coronary artery ligation, showed a reduced pressor response to a bolus injection of ET-1, while the vasodilator response was unaltered as compared with sham-operated controls. This investigation also demonstrated a marked reduction in ET-receptor density in mesenteric arteries from this model compared with controls. These authors concluded that there is a decreased vascular ET-receptor function due to a down-regulated ET-receptor and this may be impaired ET_A but not ET_B receptor function (Fu, *et al.*, 1993). In another rat model of experimental congestive heart failure (CHF) which was produced by aortocaval fistula, the effects intravenous infusion of big ET-1 on mean arterial pressure and renal haemodynamics were blunted in CHF rats. This suggested that rats with CHF have reduced sensitivity to the vascular and renal action of ET (Gurbanov, *et al.*, 1995).

Hence these previous studies in the systemic vasculature indicate reduced sensitivity to ET-1 secondary to CHF. The results of this chapter also indicate an attenuation in ET-receptor mediated responsiveness in pulmonary vasculature from 16 week ligated rabbits. An attenuation in ET-1 responses has not been found in all investigations; for example, Noll *et al* (1994) demonstrated that sensitivity to low concentrations of ET-1 was also enhanced in aorta from cardiomyopathic hamsters with pulmonary congestion compared with control preparations.

Given that alterations are also evident in SXS6c-evoked response, as shown in this chapter and also in the studies of Love *et al* (1996) and Cannan *et al* (1996), implies an upregulation of ET_B-receptor subtype. However, from the results of their *in vivo* study, Fu and co-workers (1993) concluded that the impairment appeared to be in the ET_A- rather than the ET_B-receptor subtype. However this was inferred since the vasodilator response of the coronary vasculature to ET-1 was unaltered in CHF. The predominance of the ET_A receptor subtype, with ET_B receptors accounting for only 5% of the total ET-receptor population, has previously been demonstrated by binding studies of human coronary artery (Russell *et al*, 1997). In contrast, the results of this chapter demonstrate the predominance of vasoconstrictor ET_B receptors in rabbit PRAs. Thus the differences in findings may be explained by heterogeneity in the ET-receptor subtypes of the different arterial preparations. Previous studies and the investigations of this chapter vary not only in terms of species but also in the manner in which ventricular dysfunction was created. The severity of the resulting alterations in the vasculature and thus the functional responsiveness of isolated arterial preparations may differ greatly and therefore cannot be realistically compared. Indeed even in non-pathophysiological conditions varying responses to ET-1 are evident in different arterial preparations. As already stated in chapter 1 of this thesis, the systemic and pulmonary circulation differ in many aspects therefore it is difficult to compare ET-receptor mediated effects in vascular preparation from both these circulations. Raffestin *et al* (1991) reported that circulating ET is a more potent constrictor of the systemic circulation than of the pulmonary vascular bed in the rat. Differences in ET-receptor mediated responses in

various arterial preparations from six different species was demonstrated by Moreland *et al* (1994). From this extensive study, the authors concluded that ET_A receptors were primarily located on the high pressure side of the circulation, whilst the vasoconstrictor ET_B-like receptor appeared to be concentrated on the low pressure side. Nevertheless, these previous reports together with the results of this chapter, suggest that alterations of ET-receptor mediated vascular function in heart failure may depend on the experimental model, the duration and severity of the disease, and the type of preparation studied.

Numerous investigation have been undertaken to examine systemic vascular alterations in response to heart failure; this is exemplified above. However, few reports exist examining the pulmonary circulation *in vitro* and the effects of experimental heart failure. This makes knowledge of changes in the pulmonary circulation in response to left heart disease very limited. Furthermore, the importance of the pulmonary circulation in the role of ET-1 in this condition should not be overlooked. Yang and co-workers (1994) reported that the elevated plasma ET levels in patients with CHF were positively correlated with several pulmonary haemodynamic variables including pulmonary vascular resistance and also the severity of the impairment of cardiac function. However no such correlation was noted with systemic vascular resistance. This suggests that elevated circulating ET may participate in the pathophysiology of CHF, especially closely related to occurrence and development of PHT. Furthermore, associated PHT with heart failure, following various forms of LVD, is a common occurrence (Rabinovitch, *et al.*, 1978; Trell, 1973). Hence the investigations of this chapter were an attempt to shed more light on pulmonary arterial alterations in this condition.

The maximum vasoconstrictions to SXS6c and ET-3 was significantly lower than that to ET-1 in 8, 16 and 32 week coronary ligated preparations. Smaller responses to ET-related peptides compared to ET-1 responses has also been demonstrated *in vivo* (Clozel, *et al.*, 1992). Thus, although the relative potencies of these peptides indicate the predominance of vasoconstrictor ET_B receptors, since the constriction to ET_B agonists were less than that to the non-selective $ET_{A/B}$ receptor agonist ET-1, this implies that ET_A receptors also contribute to vasoconstriction. In comparison no significant

ET_A receptors also contribute to vasoconstriction. In comparison no significant difference in the magnitude of the responses to the peptides was evident in control vessels; these differences were regardless of the duration of coronary ligation. This may indicate a greater involvement of ET_A receptors in the pulmonary vasculature following left ventricular dysfunction. On the other hand, it may also imply a reduced contribution from ET_B vasoconstrictor receptors in this model, as is also suggested by the marked reduction in sensitivity to ET-1 and SXS6c in PRAs from 16 week coronary ligated rabbits compared with control vessels. Indeed, the maximum vasoconstrictions to ET-3 was markedly reduced in 8 week coronary-ligated preparations compared with sham-operated preparations, and SXS6c-induced constrictions were significantly smaller following 16 weeks of coronary artery ligation compared to responses of both stock and sham-operated rabbit PRAs.

The observation that responses to ET-3 were reduced in PRAs from the LVD group may also be explained by an increase in endothelial ET_B-mediated vasodilation as ET-3 is extremely potent at this receptor (Douglas, *et al.*, 1995). Endothelial ET_B receptors have been shown to mediate pulmonary vasodilation in rats and lambs (Eddahibi, *et al.*, 1991; Wong, *et al.*, 1995a). However, in this chapter I also examined possible vasodilator responses to the ET_B receptor agonist SXS6c in 8 week preconstricted preparations but no relaxatory response was evident and a concentration-dependent increase in tone was again produced by this peptide. I used two different agonists, 5-HT or U46619, to preconstrict vessels as previous studies have shown that relaxatory responses can be influenced by the agent used to increase tone (Perezvizcaino, *et al.*, 1996); I also showed this in ACh-induced relaxations of newborn rabbit PRAs (chapter 8 this thesis). However, no relaxatory response was evident when either agent was used. Thus the presence of endothelial ET_B receptors in not indicated by the results of this study. A lack of endothelium-dependent relaxation to SXS6c has also been reported in rat PRAs; a preparation in which the involvement of vasoconstrictor ET_B receptors have also been shown (MacLean, *et al.*, 1994a). However, recently Higashi *et al.* (1997) demonstrated ET-3- and ET-1-induved transient

vasodilations in rat larger pulmonary arteries. In this study the ET_B receptor mediated vasodilations were shown to vary depending on the pulmonary arterial region; the relaxatory response was more potent in extrapulmonary artery compared with the intrapulmonary artery. One possible explanation is that the population of functional endothelial ET_B receptors is greater in extrapulmonary artery compared to the intrapulmonary artery. This hypothesis could be extended to explain the lack of ET_B receptor mediated vasorelaxations in the PRAs of this species (MacLean, *et al.*, 1994a), i.e. functional ET_B receptors on the endothelium might gradually decrease in number on approaching the distal segment. In addition, it should be noted that some of the effects of ET-3 could have been mediated by a putative ET_C receptor (ET-3-selective) receptor situated on endothelial cells. However, although there is evidence from binding (Yokokawa, *et al.*, 1991) and functional studies (Harrison, *et al.*, 1992) to support the existence of an ET_C receptor in the vasculature, and a potential candidate has been identified in Xenopus laevis melanphores (Karne, *et al.*, 1993), it remains to be established if this amphibian ET_C receptor has a mammalian counterpart.

Another possible explanation for altered ET-receptor mediated responses noted in PRAs from coronary-ligated rabbits and also in other vascular preparations from other models, may be an alteration in the clearance of ET-1. The pulmonary circulation has been shown to be a major site of ET-1 clearance in some animal models including the guinea-pig, rat, and rabbit (De Nucci, *et al.*, 1988; Sirvio, *et al.*, 1990; Shiba, *et al.*, 1989; Rimmar & Gillis, 1989). Infusion of the selective ET_B receptor antagonist BQ788, reduced ET-1 removal in the rat and completely abolished its removal by the lungs in the dog, whereas selective ET_A-receptor antagonists were without effect (Fukuroda, *et al.*, 1994b; Dupuis, *et al.*, 1996). Hence the ET_B-receptor appears to have a role as a clearance receptor in the pulmonary circulation and indeed appears completely and exclusively responsible for pulmonary ET-1 removal in the dog *in vivo*. There is normally no net arteriovenous differences in circulating ET-1 levels across the lung (Dupius, *et al.*, 1994; Stewart, *et al*, 1991); the lung consequently produces an amount of ET-1 equal to the amount to be extracted (Dupius, *et al.*, 1994). As already stated

plasma ET-1 levels are elevated in heart failure and pulmonary hypertension of various etiologies. Thus it is possible that reduced clearance of this peptide contributes to the hyperendothelinemia of these pathologies. ET_B clearance receptors may have a protective role by modulating the levels of this circulating potent vasoconstrictor. The expression of ET_B receptor mRNA was shown to be reduced in the in the lungs of rats with monocrotaline-induced pulmonary hypertension and elevated plasma ET-1 levels (Yorikane, *et al.*, 1993). Hence it is plausible that alterations in the expression or function of this ET_B receptor subtype may be involved in changes in responses ET-receptor agonists in the pulmonary vasculature.

The possibility that surgery *per se* may affect the responses of isolated vessels was examined by including age-matched stock animals in the 16 week procedure group. Sham-operation did not effect the sensitivity of PRAs to either ET-1 or SXS6c as this was similar to that noted in PRAs from age-matched stock animals. However, the magnitude of the maximal ET-1-induced vasoconstriction was reduced in the sham-operated compared with the stock preparations. Onizuka *et al* (1991) investigated the involvement of ET-1 in the physiological response to surgical stress in patients undergoing open-chest surgery. Plasma ET-1 levels from various sampling sites were reported to increase during pulmonary surgery however these values returned to baseline within 72 hours after surgery. ET-1 levels are unfortunately not available for during and shortly after surgery in the animals in this model. Sakurai *et al* (1992) and Yorikane *et al* (1993) suggested that the density of ET_B receptors is down-regulated by circulating ET-1. Thus if ET-1-levels were augmented in the pulmonary vasculature in this model, the possible down-regulation of ET_B receptors may account for the reduction in vasoconstriction to ET-receptor agonists.

The results of this chapter show that LVD induced by coronary artery ligation has differential effects on ET-1 and SXS6c-mediated vasoconstriction of PRAs and furthermore, these effects are altered by the duration of coronary artery ligation. LVD produced by 8 week of coronary artery ligation produced no significant effect on responsiveness of rabbit PRAs to either ET-1 or SXS6c. Whereas sensitivity to both

these peptides and the vasoconstriction to ET-1 was significantly reduced in preparations from the 16 week LVD group. The effect on ET-1-induced responses was no longer evident in PRAs from 32 week coronary artery ligated rabbits, whilst the alteration in SXS6c sensitivity now appeared as an augmentation rather than an attenuation. The finding that these alterations occurred particularly at low peptide concentrations suggests that it may be important in *in vivo* conditions. I can only speculate from the results of this study however, these findings may be due to alterations in ET-receptor subtypes, and/or the influence of other vasoactive factors, and/or mechanism of action of receptors. These possibilities are investigated further in chapters 5, 6 and 7, respectively, of this thesis.

Chapter 5

Endothelin-receptor subtypes in rabbit and human pulmonary resistance arteries: Effect of left ventricular dysfunction

5.1 Introduction

ET-1 has been shown to mediate both vasoconstriction and vasodilation of the pulmonary vasculature depending on the species, preparation and degree of initial vascular tone. In the rat, dog and guinea-pig isolated pulmonary arteries the vasoconstrictor response to ET-1 appears to be mediated solely via ET_A receptors, due to relative potency of ET isopeptides and/or the ability of selective ET_A receptor antagonists to inhibit the responses (Watanabae, *et al.*, 1991a; Douglas, *et al.*, 1993, Cardell, *et al.*, 1993). Previous studies in the rabbit pulmonary vasculature show vasoconstrictions to ET-1 to relatively resistant to the actions of BQ123 (selective ET_A receptor antagonist) and potent vasoconstrictor responses are observed to selective ET_B-receptor agonists. These findings suggest the role of ET_B receptors in mediating vasoconstriction in this preparation (Panek, *et al.*, 1992; LaDouceur, *et al.*, 1993). The results of chapter 4 of this thesis, showing the vasoconstrictor action of SXS6c and the greater potency of this selective ET_B receptor antagonist over the non-selective peptide ET-1, indicates that vasoconstrictor ET_B receptors predominate in rabbit PRAs. Thus the aim of this chapter was to investigate further the ET receptor subtypes in small muscular pulmonary arteries of the rabbit with the use of several ET receptor antagonists.

Endothelins have been implicated in many pathophysiologic conditions including pulmonary hypertension (PHT). Elevated circulating ET-1 levels have been reported in patients with both primary and secondary PHT (Stewart, *et al.*, 1991). Increased plasma levels also occur secondary to left heart dysfunction, congenital heart defects and cardiac surgery, and are positively correlated with the degree of PHT and negatively correlated with prognosis (Cody, *et al.*, 1992; Yoshibayashi, *et al.*, 1991). The results of chapter 3, including those of the morphological study showing the muscularisation of pre-alveolar pulmonary arterioles, demonstrated the presence of PHT in the rabbit coronary ligation model of left ventricular dysfunction (LVD). A previous report in this model demonstrated a 44% increase in pulmonary arterial pressure (PAP) and a 64% increase in lung weight and right ventricular weight in rabbits after 8 weeks

of coronary ligation (Deuchar, *et al.*, 1997). Therefore this is an ideal model to use to examine pulmonary vascular changes occurring in LVD. Recently there has been much interest in ET receptor antagonists as possible therapeutic agents for the treatment of cardiovascular disease including PHT. Kiowski *et al* (1995) reported that the non-selective $ET_{A/B}$ receptor antagonist bosentan may decrease pulmonary vascular resistance in patients with heart failure. In a rabbit arterial-occlusion model of acute myocardial infarction treatment with the selective ET_A receptor antagonist FR139317 before coronary artery occlusion, significantly reduced infarct size (Burke & Nelson, 1997). Similarly, Watanabe *et al.* (1991b) reported a reduction in infarct size on infusion of an antibody to ET-1 in a rat model of myocardial infarction. Furthermore, the development of PHT secondary to chronic heart failure, induced by coronary ligation in the rat, can be ameliorated by the long term treatment with an ET_A receptor antagonist (Sakai, *et al.*, 1996). In the search for therapeutics for PHT that are based on antagonising the effects of ET-1, it will be important to determine what ET receptor subtypes are responsible for the diverse effects of this proposed mediator in the pulmonary vasculature. A secondary aim of this study was, therefore, to examine if there were any changes in the effect of selective ET receptor antagonist on ET-receptor mediated responses in the pulmonary vasculature in this model of LVD.

Previous investigations have used isolated rabbit main pulmonary artery in their a studies of ET-1 (La Douceur, *et al.*, 1993; Fukuroda, *et al.*, 1994a; Hay, *et al.*., 1996) As it is the pulmonary resistance arteries (PRAs) which are thought to be functionally important in the resistance changes which occur in PHT (Marshall & Marshall, 1983; Staub, 1985), I examined the functional responses to ET-receptor stimulation in PRAs from the rabbit model of LVD, induced by eight weeks of coronary artery ligation, using ET-1, ET-3 ($ET_{A/B}$ non-selective agonists) and sarafotoxin S6c (SXS6c; an ET_B-selective agonist). The ET receptor antagonists used to characterise the endothelin receptors were the ET_A -selective antagonist FR139317 (Sogabe, *et al.*, 1992), BQ788, a potent and selective ET_B receptor antagonist (Ishikawa, *et al,* 1994) and the non-peptide non-selective ET-receptor antagonist SB209670 (Ohlstein, *et al.*, 1994a).

A previous investigation by Fukuroda *et al.* (1994a) suggested interspecies differences in the ET receptor subtypes that mediate ET-1-induced vasoconstrictions.. ET_A receptors appeared to be dominant in the human pulmonary artery whereas, as already stated, the predominance of ET_B receptors was indicated in the rabbit pulmonary artery; arterial preparations from both species were ~1mm outer diameter. Hence, in addition, in this chapter, I also examined ET-receptor mediated responses in human PRAs with the use of the peptides ET-1 and ET-3 and the antagonist compounds BQ788 and SB209670, and compared these with those of arterial segments of similar internal diameter from the rabbit.

Thus the main aims of this chapter was that the results of my own studies in small pulmonary arteries from both rabbit and human lung (along with the information available on larger pulmonary arteries, and with what others investigators have demonstrated *in vivo* and in the isolated perfused lung *in vitro*) would provide a more complete picture of ET-1-mediated vasoconstriction in the human and rabbit pulmonary arterial circulation and how such vasoconstriction may change in pulmonary hypertension.

5.2 Methods

Rabbit pulmonary resistance arteries

Lungs were obtained from the rabbit coronary ligation model of left ventricular dysfunction which has been extensively characterised (Pye, *et al*,. 1996) and is descried in detail in section 2.1.1 of this thesis. In the 8 week procedure period groups, age matched animals underwent the same methodology as the experimental animals (referred to in results and discussion as "coronary-ligated") except the ligatures placed around the coronary artery were not secured. These are subsequently referred to as "sham-operated". Measurements of ejection fraction were made in all animals and animals were killed by sodium pentabarbitone 8 weeks following the procedure. The

lungs were promptly removed and small intralobar pulmonary resistance arteries (PRAs ~170 μm internal diameter, see table 5.1) were dissected out according to the methods stated in section 2.2.3.1. These were mounted as ring preparations (~2mm long) on a wire myograph, bathed in Krebs solution at 37°C. Using the normalisation process explained in section 2.2.5, vessels were tensioned to an equivalent transmural pressure of ~ 16 mmHg. This pressure was chosen as it is similar to *in vivo* pressures of rabbit pulmonary arterioles. Preliminary studies in our laboratory showed that bubbling with 95% O_2 inhibits responses to vasoconstrictors in rabbit PRAs so we bubbled with 16% O_2/ 5% CO_2 balance N_2. These gas mixtures were chosen as they are similar to those which the vessels would be exposed to *in vivo*.

Human pulmonary resistance arteries

Human pulmonary arteries (~ 240 μM internal diameter, see table 5.1) were dissected from grossly normal sections of human lung removed from postoperative bronchial carcinoma tissue (as described in section 2.2.3.3). Samples were refrigerated in fresh Krebs' solution and were collected and studied no longer than 12 hours postoperative. As for the rabbit preparations, the human PRAs were mounted as ring preparations (~2mm long) on a wire myograph, bathed in Krebs solution at 37°C which was bubbled with gas mixture containing 16% O_2/ 5% CO_2 balance N_2. Using the normalisation process explained in chapter 2, vessels were tensioned to an equivalent transmural pressure of ~ 16 mmHg, thus similar to *in vivo* pulmonary arterial pressures.

Experimental protocol

Vessels were allowed to equilibrate for 1 hour prior to the addition of any drugs. The response of the PRAs to 50mM KCl was determined twice; once the contractile response had reached a plateau, the vessels were washed out at least 6 times with fresh Krebs solution. Cumulative concentration-response curves (CCRCs) were then

constructed in rabbit PRAs to ET-1, SXS6c or ET-3 (1pM-0.3μM) following either a 45 minute "rest period" or 45 minute incubation period with the selected concentration of an ET receptor antagonist. The antagonists used were FR139317, BQ788 and SB209670

The following protocols were carried out in PRAs from the rabbit coronary artery ligation model. The effect of FR139317 (at 1μM) was examined on ET-1-evoked responses only. Preincubation with BQ788 (at 1μM) was performed preceding ET-1, SXS6c and ET-3 CCRCs whilst preincubation with FR139317 plus BQ788 (both at 1μM) was performed preceding ET-1 responses only. The effect of SB209670 (at 0.1 and 1μM)was examined in both ET-1- and SXS6c-evoked responses.

In human PRAs, the effects of preincubation with BQ788 (1μM) was studied in ET-1- and ET-3 CCRC's (0.1pM-0.3μM). Whereas effects of SB209670 (10nM, 0.1μM and 1μM) was examined in ET-1-responses only.

Note

In human PRAs, control responses to ET-1 were performed in vessels from the same lung sample when the effects of BQ788 and SB209670 were being examined; hence these antagonist results are displayed with their own control data. In the rabbit vessels, control responses to ET-1, ET-3 and SXS6c were carried out whenever possible in each lung preparation. However, due to limitation in equipment and time it was not always possible to run a control CCRC to the peptide whilst studying the different antagonists. However the control curves in this study have been updated with each group of experiments . No differences were found in the control ET-1, ET-3 or SXS6c responses when tested throughout the period of investigation, thus the data for the control curves which are shown have been pooled over many protocols. Due to the inhibitory effect of SB209670 which became apparent during this study, I attempted to examine the effects of this antagonist at several different concentrations. Unfortunately due to time constraints it was not possible to study a range of concentrations for all the

antagonists in each tissue. The concentrations which were studied, were chosen due to their pA_2/pK_B values in other vascular preparations.

Data analysis

Results are expressed graphically as percentage of their own maximum contraction, or percentage of reference contraction to second application of 50mM KCl. pEC_{10}, pEC_{25} and pEC_{50} values were calculated according to the methods stated in chapter 2, and expressed as -log M concentration. Where ever possible, pK_B values were estimated for single stated concentration of antagonist according to the methods stated in chapter 2. Statistical comparison of the means of groups of data were made by Student's unpaired *t* test or where appropriate, ANOVA followed by Tukeys post test ; $P<0.05$ was considered statistically significant. Throughout, data are expressed as mean±SEM and *n* /*n* = number of ring preparations / number of animals.

5.3 Results

The ejection fraction of animals from the 8 week procedure group, from which lungs were obtained and PRAs dissected for the antagonist studies described in this chapter, are shown in table 5.1. The effective pressure to which these vessels and human PRAs were tensioned and the resulting estimate of internal diameter are also shown in table 5.1. Table 4.1 in chapter 4 shows these parameters for 8 week preparations in which control responses to ET-receptor agonists were examined. In the antagonist studies, vessels from both experimental groups were tensioned to a similar effective pressure and internal diameter and these values were similar to those in preparations in which control curves were constructed (table 4.1). The ejection fraction was markedly reduced in ligated animals compared with sham-operated in the 8 week procedure group (table 5.1).

	(n)	Internal diameter (μm)	Effective pressure (mmHg)	Ejection fraction (%)
Rabbit PRAs				
sham-operated	(72)	165.0±5.9	19.2±0.6	72.7±0.8
ligated	(62)	175.7±6.6	19.3±0.7	44.0±0.9 ***
Human PRAs	(37)	241.6±14.4	16.4±0.6	-

Table 5.1 (1) Ejection fraction of 8 week procedure animals from which PRAs were dissected and studied in this chapter and (2) internal diameter and effective pressure of PRAs from this cohort of rabbits and also for human vessels studied in this chapter Statistical comparisons were made by Student's unpaired t-test: ligated vs. sham-operated, ***P<0.001. Values are mean±SEM. n, number of ring preparations.

Effect of antagonists on ET-receptor mediated contraction of rabbit pulmonary resistance arteries

The effect of FR139317, BQ788 and a combination of both these antagonists on ET-1-induced vasoconstrictions in PRAs from 8 week sham-operated and coronary-ligated rabbits are shown in figures 5.1 and 5.2, respectively. pEC$_{10}$, pEC$_{25}$ and pEC$_{50}$ values are summarised in table 5.2.

FR139317 (vs ET-1)

The selective ET$_A$ receptor antagonist FR139317 failed to inhibit the ET-1-evoked responses in PRAs from either sham-operated of coronary-ligated rabbits. In fact, there was a tendency for the response noted above 1nM ET-1 to be greater in the presence of FR139317 in control vessels (figure 5.1A). A potentiation was also seen in preparations from the LVD group but at ET-1 concentrations below 1nM (figure 5.2A).

	n/n	pEC_{10}	pEC_{25}	pEC_{50}
Sham-operated rabbit PRAs				
ET-1 control	8/6	9.1±0.4	8.6±0.4	7.9±0.3
+1μM FR139317	7/6	10.0±0.3	9.1±0.1	8.2±0.1
+1μM BQ788	6/5	8.6±0.1	8.2±0.1	7.8±0.1
+1μM FR139317 plus 1μM BQ788	7/6	8.2±0.1	8.0±0.1	7.7±0.1
+0.1μM SB209670	6/6	9.0±0.3	8.4±0.2	7.9±0.1
+1μM SB209670	8/7	nc		
Coronary-ligated rabbit PRAs				
ET-1 control	12./7	8.9±0.4	8.3±0.2	7.9±0.2
+1μM FR139317	9/6	8.4±0.2	8.0±0.1	7.6±0.1
+1μM BQ788	7/6	9.9±0.4	8.8±0.2	8.1±0.2
+1μM FR139317 plus 1μM BQ788	9/6	8.4±0.2	8.0±0.1	7.6±0.1
+0.1μM SB209670	4/4	8.6±0.1	8.3±0.1	7.8±0.1
+1μM SB209670	6/6	nc		

Table 5.2 Effect of selective antagonists on the sensitivity to ET-1 in PRAs from sham-operated and coronary ligated rabbit PRAs. Values are mean ± SEM. ET-1, endothelin-1; n/n, number of ring preparations / number of animals; nc, not calculated.

The maximal contraction in PRAs from sham-operated rabbits was not affected but the response to 10nM ET-1 was increased from 49.3±11.6% (% reference contraction to 50mM KCl) to 75.7±3.5% ($P<0.01$; figure 5.1B). FR139317 did not effect the magnitude of the ET-1-evoked vasoconstrictions in vessels from the LVD group (figure 5.2B).

Figure 5.1

Effect of selective antagonists on ET-1-induced responses in PRAs from sham-operated rabbits.

CCRC's to ET-1 (●, n=8/6) and in the presence of 1μM FR139317 (○, n=7/6), 1μM BQ788 (□, n=6/5), and 1μM FR139317 plus 1μM BQ788 (▲, n=7/6).
A Data are expressed as a percentage own maximum contraction.
B Data are expressed as a percentage reference contraction to 50mMKCl.
Each point represents mean ± s.e. mean.

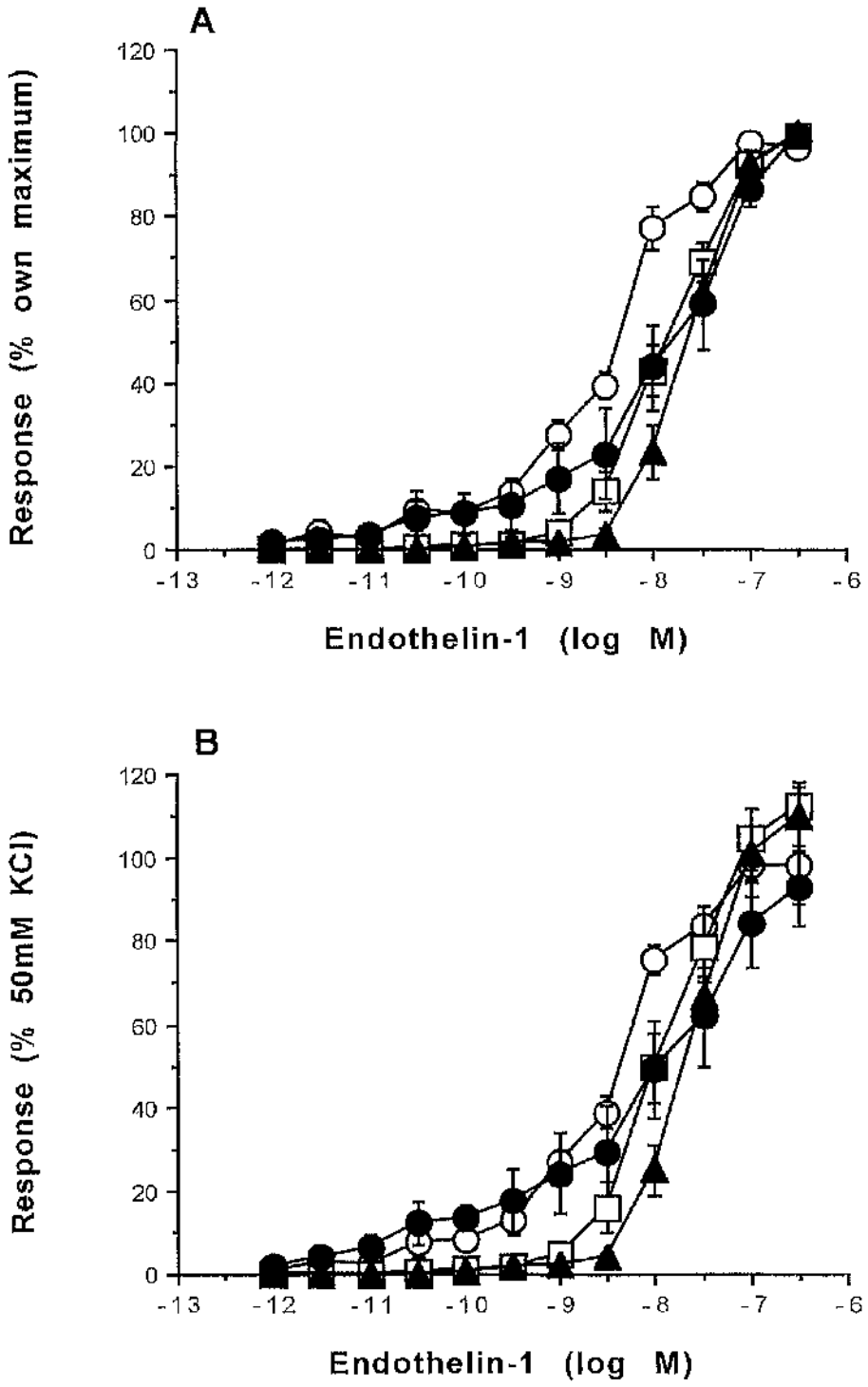


Figure 5.1

Effect of selective antagonists on ET-1-induced responses in pulmonary resistance arteries from 8 week sham-operated rabbits.

Figure 5.2

Effect of selective antagonists on ET-1-induced responses in PRAs from coronary artery ligated rabbits.

CCRC's to ET-1 (●, n=12/7) and in the presence of 1μM FR139317 (○, n=7/6), 1μM BQ788 (□, n=7/6), and 1μM FR139317 plus 1μM BQ788 (▲, n=9/6).
A Data are expressed as a percentage own maximum contraction.
B Data are expressed as a percentage reference contraction to 50mMKCl.
Each point represents mean ± s.e. mean.

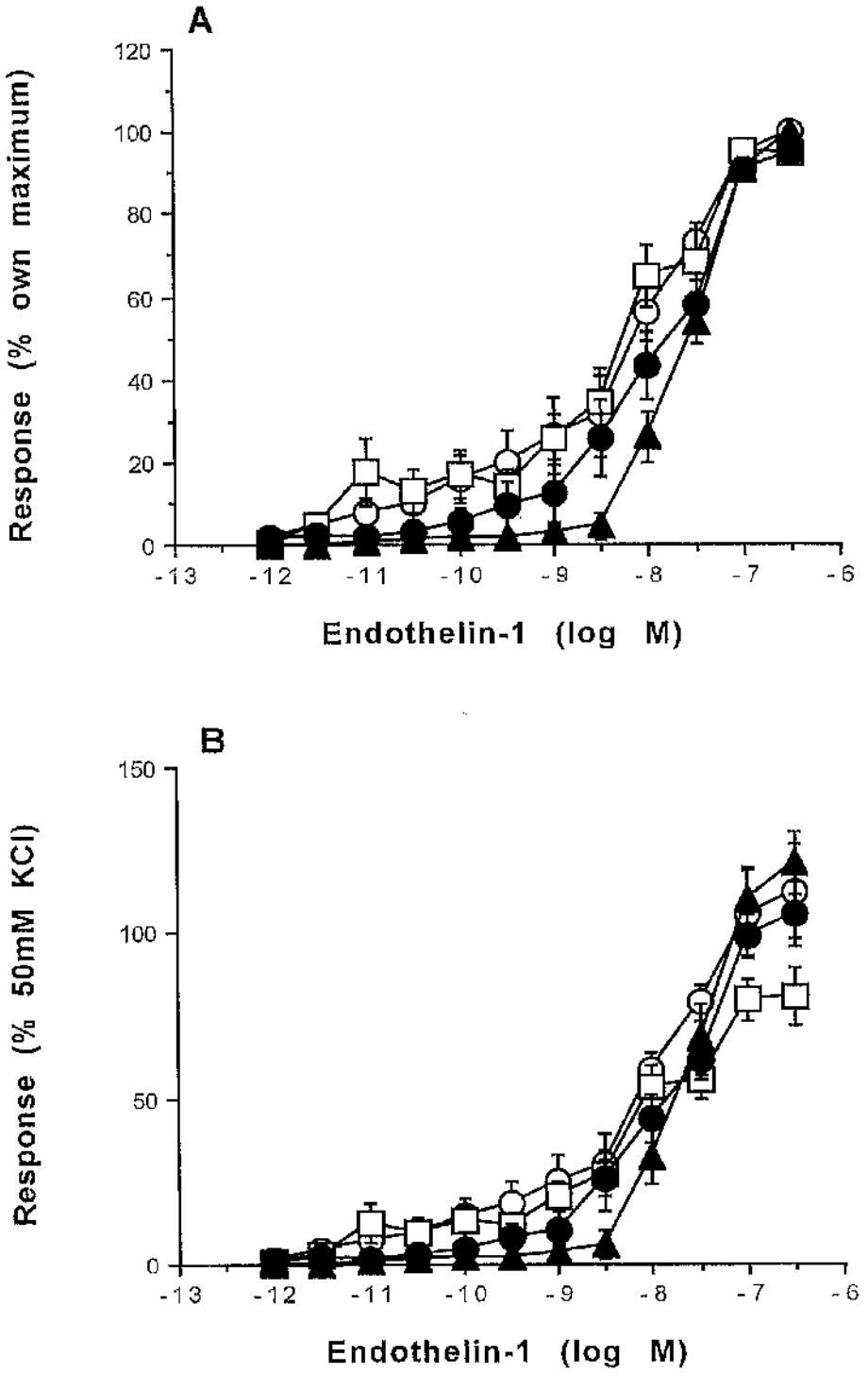


Figure 5.2

Effect of selective antagonists on ET-1-induced responses in pulmonary resistance arteries from 8 week coronary artery ligated rabbits.

BQ788 (vs ET-1)

BQ788 failed to significantly inhibit ET-1 responses in PRAs from both experimental groups (table 5.2). However, in PRAs from sham-operated group, the threshold concentration of ET-1 was increased by BQ788 from 30pmol to 3nmol. Responses to higher concentrations were resistant to BQ788 (Figure 5.1A). BQ788 failed to inhibit responses to any concentration of ET-1 in arteries from the LVD group in fact a tendency for potentiation of the responses was noted at the lower concentration range. The threshold concentration of ET-1 was decreased from 300pmol to 10pmol. Maximum vasoconstriction to ET-1 was not effected by 1μM BQ788 in PRAs sham-operated (93.3±9.5% cf. 112.2±4.7%, figure 5.1B) or coronary-ligated (105.5±9.5% cf. 80.4±8.6%, figure 5.2B) rabbits.

BQ788 + FR139317 (vs ET-1)

Figures 5.1 also shows the effect of BQ788 in combination with FR139317 on responses to ET-1 in PRAs from sham-operated rabbits. In these vessels, BQ788 and FR139317 in combination increased the threshold concentrations of ET-1 which caused contraction from 30pM to 10nM (figure 5.1A). Thus FR139317 did not markedly increase the effect of BQ788. Also the potentiation of the vasoconstrictor response at ET-1 concentrations >1nM which was noted in the presence of FR139317, was not evident when BQ788 was present also. Due to the biphasic nature of the curve, this inhibitory effect was not reflected as a significant change in pEC_{10}, pEC_{25} or pEC_{50} values (table 5.2). The maximum vasoconstriction was not effected. However, as was seen with BQ788 alone, the magnitude of vasoconstrictions to lower ET-1 concentrations were attenuated; for example, response to 1nM ET-1 were reduced from 24.2±9.6% to 2.1±1.0% by FR139317 plus BQ788 (P=0.05, figure 5.1B).

In PRAs from coronary-ligated rabbits, BQ788 plus FR139317 increased the threshold concentrations of ET-1 which caused contraction in vessels from 300pM to

of BQ788 to inhibit responses to lower concentrations of ET-1, as seen in the sham-operated rabbit PRAs. This inhibitory effect was not reflected by a significant change in $p\mathrm{EC}_{10}$, $p\mathrm{EC}_{25}$ or $p\mathrm{EC}_{50}$ values (table 5.2). The maximal responses were not affected by the combination of BQ788 and FR139317 (figure 5.2B).

BQ788 (vs SXS6c)

The effect of 1μM BQ788 on SXS6c-evoked vasoconstrictions in PRAs from sham-operated and coronary-ligated rabbits is shown in figure 5.3. $p\mathrm{EC}_{10}$, $p\mathrm{EC}_{25}$ and $p\mathrm{EC}_{50}$ values are summarised in table 5.3. BQ788 had a marked inhibitory effect on SXS6c-induced vasoconstrictions in PRAs from both groups. The estimated $p\mathrm{K}_B$ values were 7.1±0.2 and 7.0±0.1 for PRAs from sham-operated and coronary-ligated rabbits, respectively. BQ788 had its most profound effect on responses to the lower concentrations of SXS6c and removed the first component of a biphasic response to SXS6c. The magnitude of the maximum vasoconstriction to BQ788 was not significantly altered by BQ788 (figure 5.3B).

BQ788 (vs ET-3)

ET-3-induced responses were significantly inhibited by BQ788 in PRAs from both groups. This is shown in figure 5.4 and $p\mathrm{EC}_{10}$, $p\mathrm{EC}_{25}$ and $p\mathrm{EC}_{50}$ values are summarised in table 5.4. BQ788 had its most profound effect on responses to the lower concentrations of ET-3 and removed the first component of a biphasic response to ET-3 The inhibitory effect on responses to SXS6c concentrations >3nM were more pronounced in coronary-ligated vessels. The estimated $p\mathrm{K}_B$ value was 6.6±0.1 for sham-operated vessels. For coronary-ligated preparations, a $p\mathrm{K}_B$ value of 7.0±0.2 was calculated and this was significantly greater than the value obtained in the PRAs from

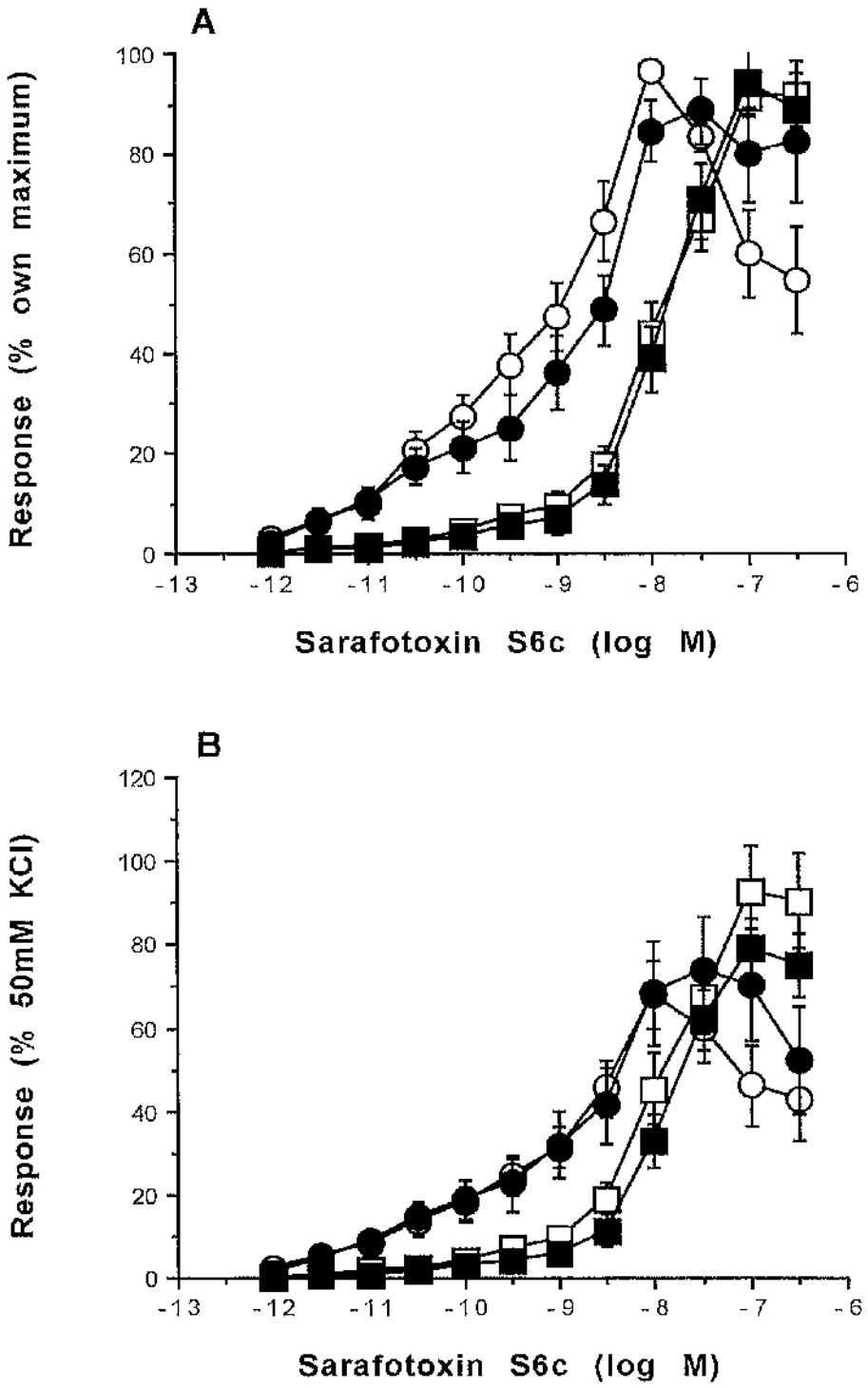


Figure 5.3 Responses to SXS6c in PRAs from sham-operated (○, n=10/9) and coronary ligated (●, n=11/7) rabbits: Effect of 1μM BQ788 in sham-operated (❑, n=7/7) and coronary ligated (■, n=12/8) vessels. **A** Data are expressed as a percentage own maximum contraction. **B** Data are expressed as a percentage reference contraction to 50mMKCl. Each point represents mean ± s.e. mean.

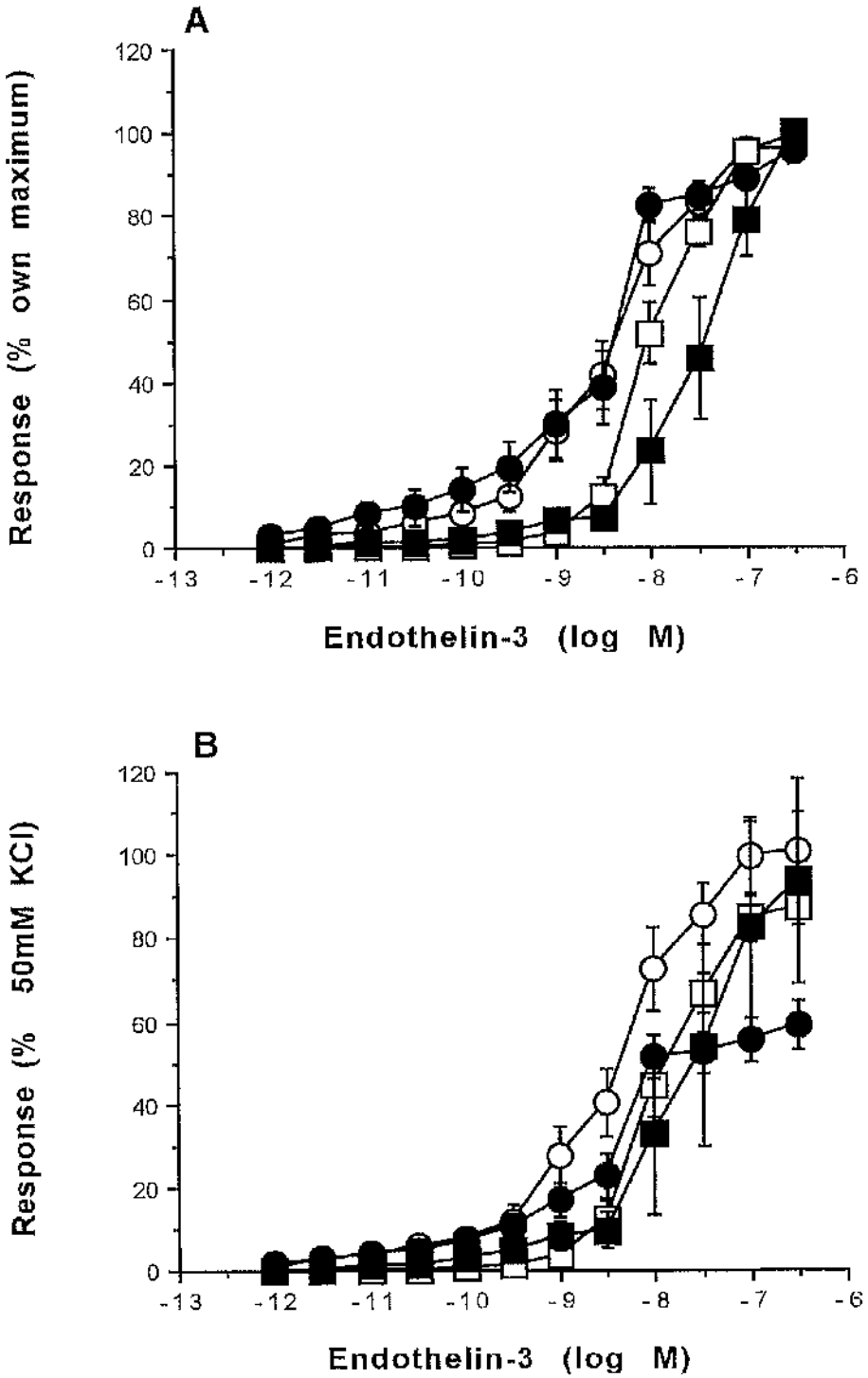


Figure 5.4 Responses to ET-3 in PRAs from sham-operated (○, n=10/9) and coronary ligated (●, n=9/6) rabbits: Effect of 1μM BQ788 in sham-operated (□, n=10/9) and coronary ligated (■, n=5/5) vessels. **A** Data are expressed as a percentage own maximum contraction. **B** Data are expressed as a percentage reference contraction to 50mMKCl. Each point represents mean ± s.e. mean.

sham-operated rabbits ($P<0.05$). Maximal responses were not significantly affected by 1μM BQ788 (figure 5.4B).

	n/n	pEC_{10}	pEC_{25}	pEC_{50}
Sham-operated rabbit PRAs				
SXS6c control	10/9	10.6±0.3	9.9±0.3	9.0±0.2
+1μM BQ788	7/7	9.1±0.2***	8.4±0.1**	7.8±0.1***
+0.1μM SB209670	7/6	9.6±0.4*	8.8±0.2*	8.2±0.1**
+1μM SB209670	7/6	7.9±0.3***	7.4±0.2***	7.1±0.1***
Coronary-ligated rabbit PRAs				
SXS6c control	11/7	10.4±0.3	9.4±0.3	8.6±0.2
+1μM BQ788	12/8	8.7±0.3***	8.3±0.1**	7.8±0.1**
+0.1μM SB209670	6/6	9.2±0.6	8.8±0.4	8.1±0.3
+1μM SB209670	6/6	8.9±0.5*	8.3±0.4*	7.8±0.2*

Table 5.3 Effect of selective antagonists on the sensitivity to SXS6c in PRAs from sham-operated and coronary ligated rabbit PRAs. Statistical comparisons were made by Students' unpaired t-test: antagonist vs. control *$P<0.05$, **$P<0.01$, ***$P<0.001$. Values are mean ± SEM. SXS6c, sarafotoxin S6c; n/n, number of ring preparations / number of animals.

SB209670 (vs ET-1)

The effect of SB209670 on ET-1 responses in rabbit PRAs from sham-operated and coronary-ligated rabbits is shown in figures 5.5 and 5.6, respectively. pEC_{10}, pEC_{25} and pEC_{50} values are summarised in table 5.2. 0.1μM SB209670 did not effect sensitivity to ET-1 in PRAs from either control or LVD groups. However when present at 1μM, it inhibited ET-1 responses in sham-operated rabbit PRAs. A maximum response to ET-1 was not reached within the concentration range studied, therefore pEC values and a pK_B value for ET-1 in the presence of 1μM SB209670 could not be

calculated. However using the maximal response which was attained, this would give an estimated pK_B of 6.8±0.2.

As was noted in sham-operated rabbit PRAs, the ET-1 CCRC did not reach a maximum response within the concentration range studied when coronary-ligated PRAs had been preincubated with 1μM SB209670, thus *p*EC values could not be calculated. However, it is apparent from figure 5.6A that 1μM SB209670 failed to reduce ET-1 sensitivity in this preparation. In fact, a slight potentiation of responses to lower concentrations of ET-1 was noted, in that small responses were observed to 0.03nM and 0.1 nM ET-1 (figure 5.6A). However, when the results are expressed to demonstrate the magnitude of the contractile responses, 1μM SB209670 inhibited the responses to ET-1 in that it caused a rightward shift in the upper component of the CCRC and markedly attenuated the responses to the maximal concentration of ET-1 it was financially viable to use (67.2±9.6% vs. 105.5±9.5%, $P<0.05$; figure 5.6B).

	n/n	pEC_{10}	pEC_{25}	pEC_{50}
Sham-operated rabbit PRAs				
ET-3 control	10/9	9.6±0.3	8.9±0.2	8.4±0.2
+1μM BQ788	10/9	8.5±0.1**	8.3±0.1*	8.0±0.1*
Coronary-ligated rabbit PRAs				
ET-3 control	9/6	9.7±0.5	9.1±0.3	8.5±0.2
+1μM BQ788	5/5	8.3±0.3	7.8±0.2*	7.5±0.2*

Table 5.4 Effect of BQ788 on the sensitivity to ET-3 in PRAs from sham-operated and coronary ligated rabbit PRAs. Statistical comparisons were made by Students' unpaired t-test: antagonist vs. control *$P<0.05$, **$P<0.01$. Values are mean ± SEM. ET-3, endothelin-3; n/n, number of ring preparations / number of animals.

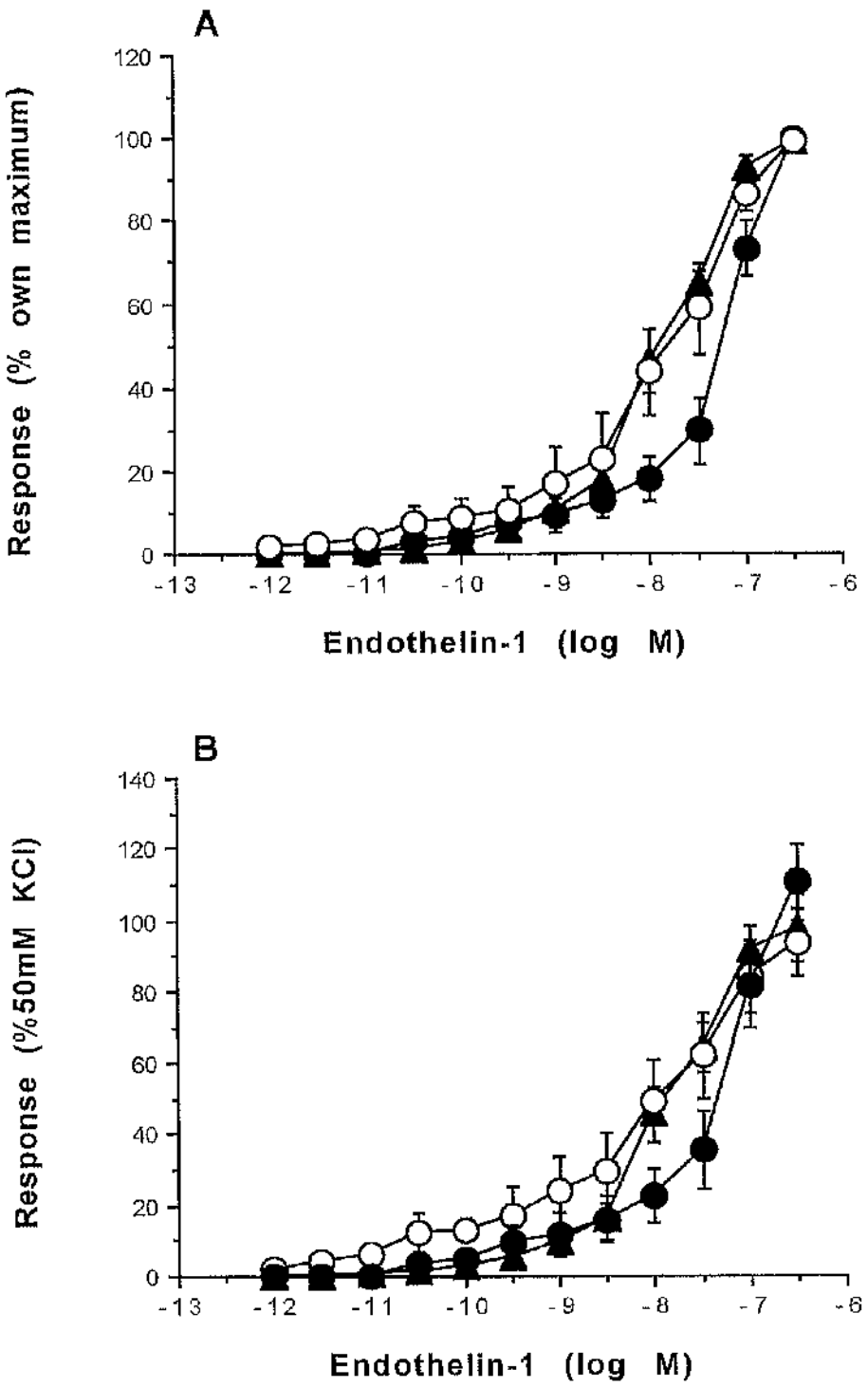


Figure 5.5 Effect of SB209670 on ET-1-induced responses in PRAs from sham-operated rabbits. CCRC's to ET-1 (○, n=8/6), and in the presence of 0.1μM (▲, n=6/6) and 1μM (●, n=8/7) SB209670. A Data are expressed as a percentage own maximum contraction. B Data are expressed as a percentage reference contraction to 50mMKCl. Each point represents mean ± s.e. mean.

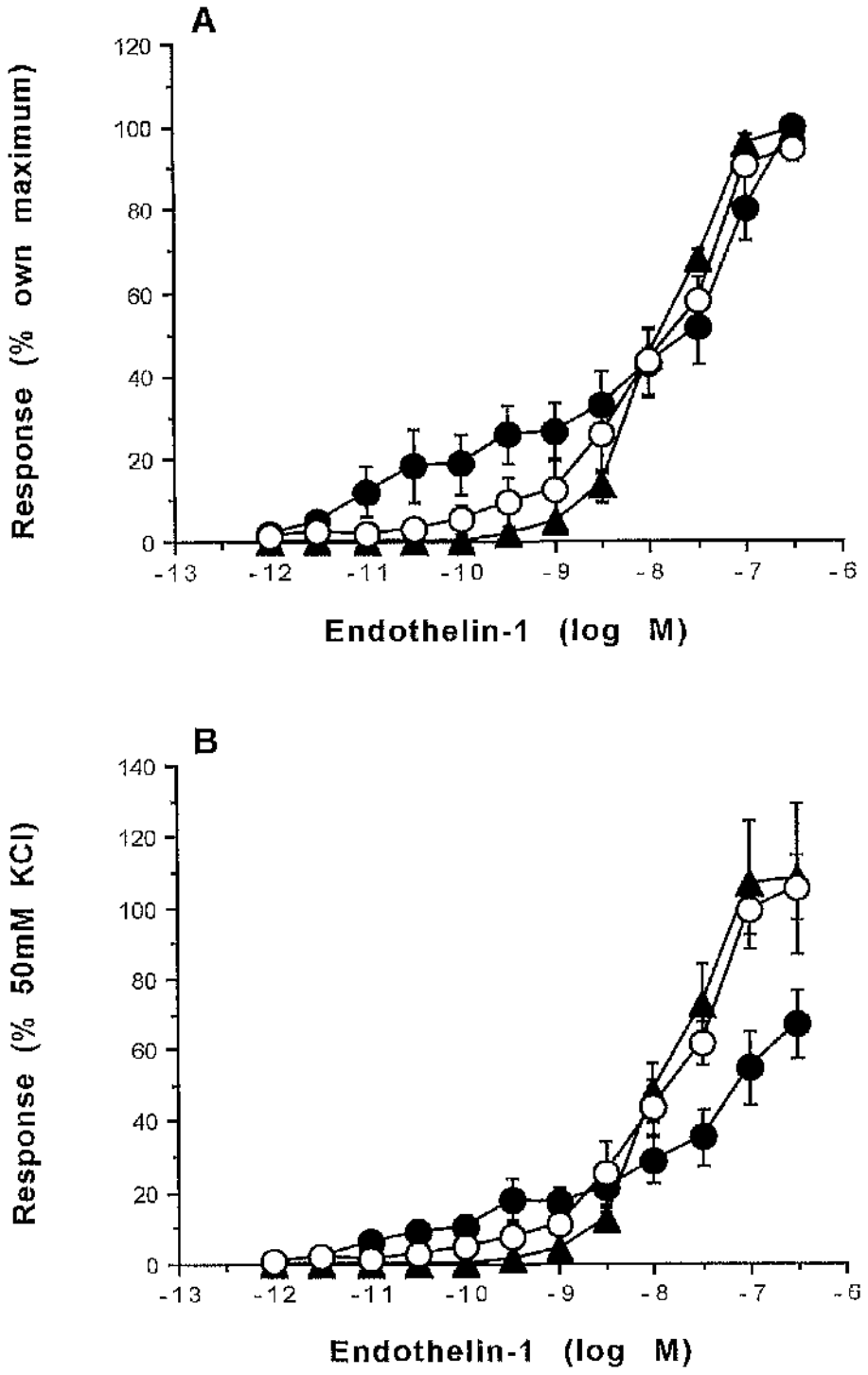


Figure 5.6 Effect of SB209670 on ET-1-induced responses in PRAs from coronary ligated rabbits. CCRC's to ET-1 (○, n=12/7), and in the presence of 0.1μM (▲, n=4/4) and 1μM (●, n=6/6) SB209670. **A** Data are expressed as a percentage own maximum contraction. **B** Data are expressed as a percentage reference contraction to 50mMKCl. Each point represents mean ± s.e. mean.

SB209670 (vs SXS6c)

The effect of SB209670 on SXS6c responses in rabbit PRAs from sham-operated rabbits is shown in figures 5.7. pEC_{10}, pEC_{25} and pEC_{50} values are summarised in table 5.3. In these vessels, SB209670 produced a concentration-dependent inhibition of responses to SXS6c and a profound effect as seen on the early component of the CCRC.. 0.1μM SB209670 produced a significant inhibition of the response. This inhibitory effect was even more pronounced in the presence of 1μM SB209670 and the threshold concentration was increased from 0.3pM to 10nM SXS6c (figure 5.7A, table 5.3). The estimated pK_B values was 7.8±0.2 (1μM SB209670). 0.1μM SB209670 did not effect the magnitude of the contractile response to SXS6c concentrations below 10nM however, the maximum response was significantly increased from 67.9±8.2% to 105.0±11.7% ($P<0.05$) in it's presence. The maximum SXS6c-induced vasoconstriction was not effected by 1μM SB209670 (figure 5.7B).

Figure 5.8A shows that SB209670 also produced a concentration-dependent inhibition of responses to SXS6c in vessels from the LVD group. However the inhibitory effect of this non-selective $ET_{A/B}$ receptor antagonist was markedly less than that observed in PRAs from control vessels. This is demonstrated further by the estimated pK_B value of 6.7±0.1 (1μM SB209670) in PRAs from coronary-ligated rabbits which was significantly less than that observed in the sham-operated rabbit vessels ($P>0.001$). This may be related to the lesser effect on the early component of the CCRC. SB209670 did not effect the magnitude of the maximum contractile response to SXS6c in PRAs from coronary-ligated rabbits (figure 5.8B).

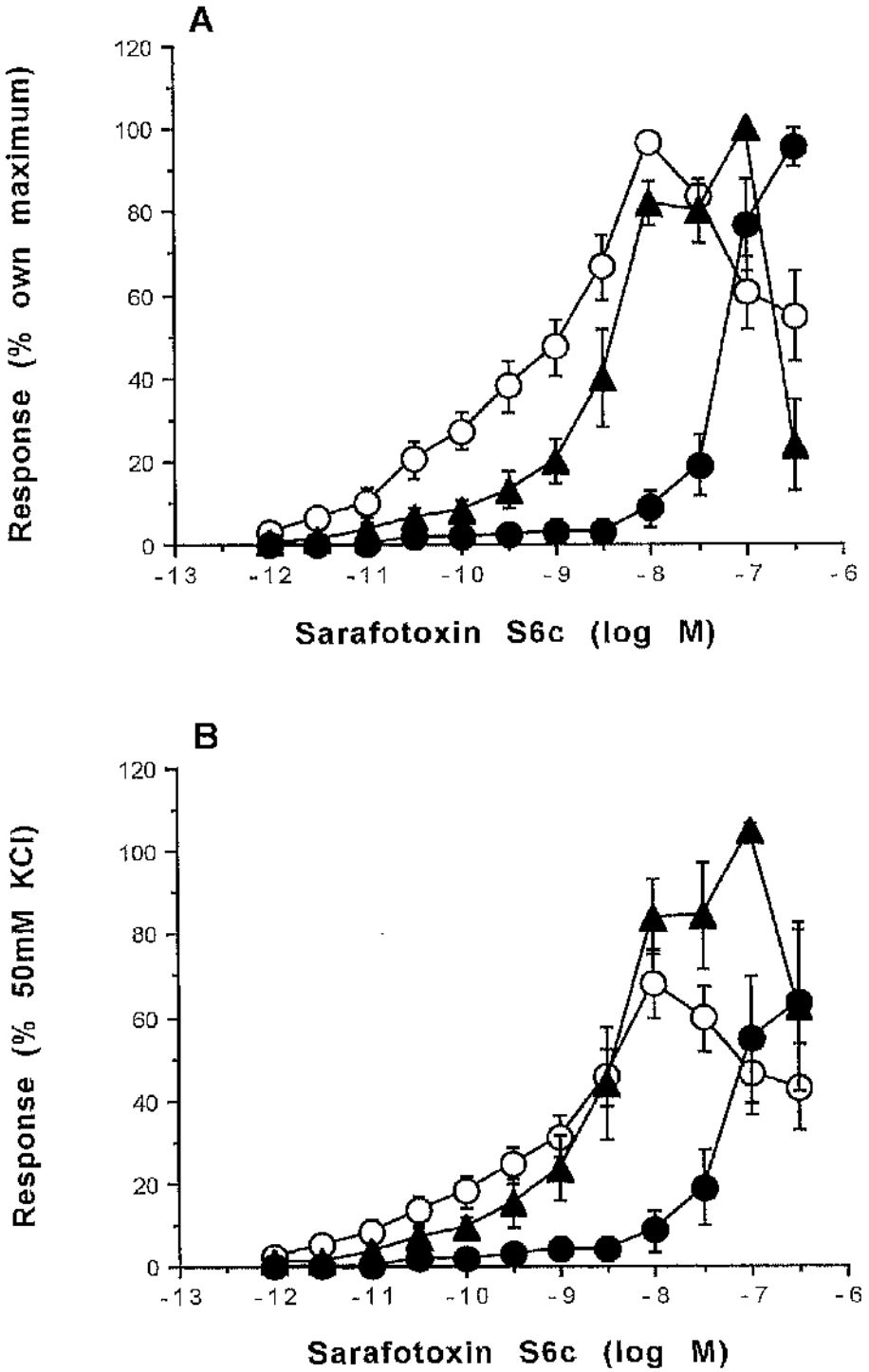


Figure 5.7 Effect of SB209670 on SXS6c-induced responses in PRAs from sham-operated rabbits. CCRC's to SXS6c (○, n=10/9), and in the presence of 0.1μM (▲, n=7/6 and 1μM (●, n=7/6) SB209670. **A** Data are expressed as a percentage own maximum contraction. **B** Data are expressed as a percentage reference contraction to 50mMKCl. Each point represents mean ± s.e. mean.

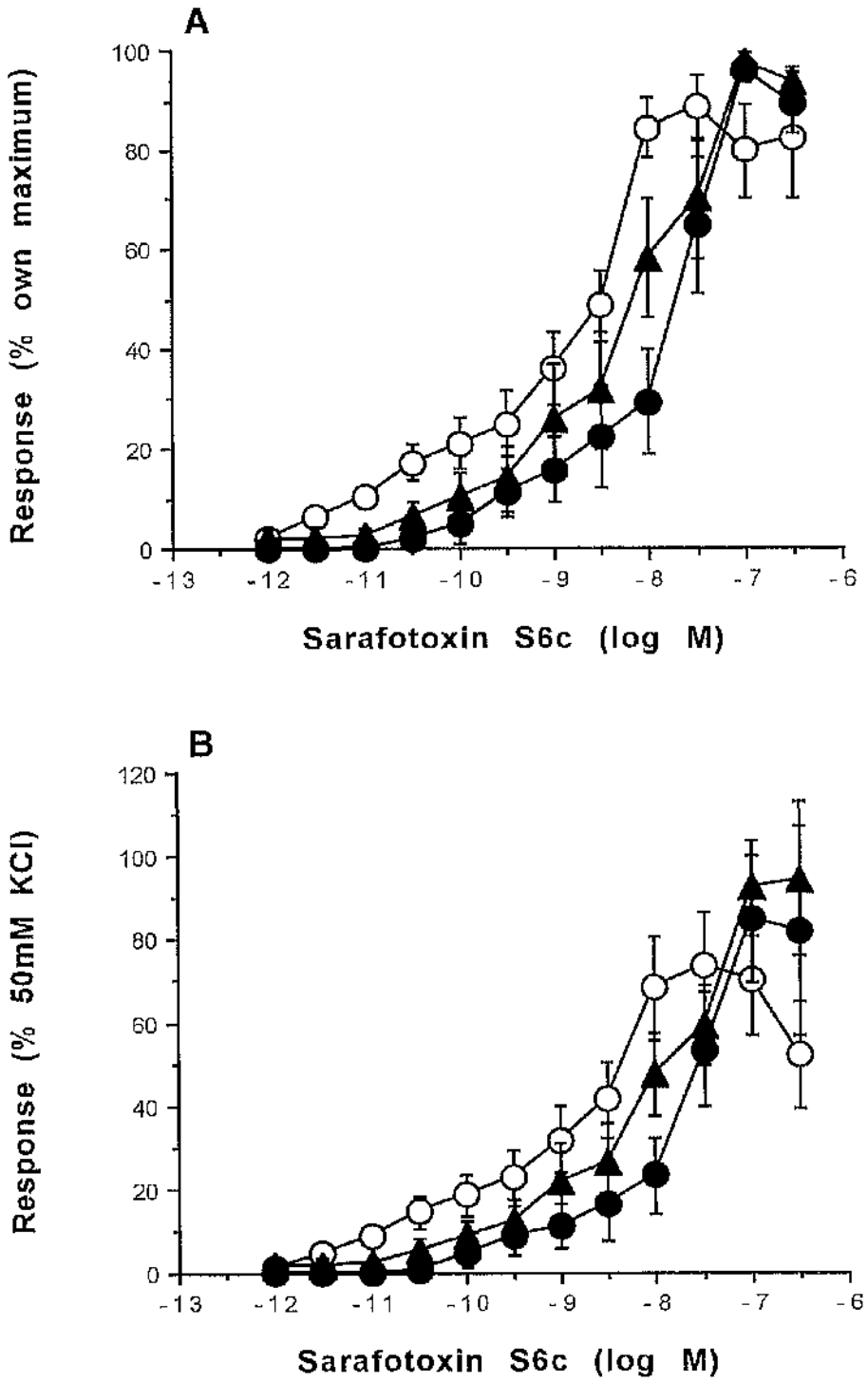


Figure 5.8 Effect of SB209670 on SXS6c-induced responses in PRAs from coronary ligated rabbits. CCRC's to SXS6c (○, n=11/7), and in the presence of 0.1μM (▲, n=6/6 and 1μM (●, n=6/6) SB209670. **A** Data are expressed as a percentage own maximum contraction. **B** Data are expressed as a percentage reference contraction to 50mMKCl. Each point represents mean ± s.e. mean.

Effect of antagonists on ET-receptor mediated contraction of human pulmonary resistance arteries

Both ET-1 and ET-3 induced a concentration-dependent contraction of human PRAs. The potency of ET-3 was significantly greater than that of ET-1 in these vessels and this is shown marked increases in *p*EC values displayed in table 5.5. However, the maximal contraction evoked by ET-3 (103.54±5.7% reference contraction to 50mM KCl, n=5/5) was lower than that induced by ET-1 (183±24.4%, n=8/8; $P<0.05$).

	n/n	pEC_{10}	pEC_{25}	pEC_{50}
ET-1	8/8	9.5±0.2	8.8±0.2	8.1±0.2
ET-3	5/5	11.2±0.1***	10.6±0.3***	9.5±0.3**

Table 5.5 Sensitivity to ET-1 and ET-3 in human PRAs. Statistical comparisons were made by Students' unpaired t-test; ET-3 vs. ET-1 **$P<0.01$, ***$P<0.001$. Values are mean ± SEM. ET-1, endothelin-1; ET-3, endothelin-3; n/n, number of ring preparations / number of animals.

BQ788 (vs. ET-1/ET-3)

Figure 5.9A shows that BQ788 inhibited responses to ET-1 up to 1nM but was without effect at higher ET-1 concentrations in human PRAs. This is also shown by the marked inhibition at the level of the pEC_{10} (table 5.6). The maximum contraction to ET-1 was not altered in the presence of BQ788 (151.7±18.2% (control) and 211.0±38% (in the presence of 1μM BQ788); figure 5.9B).

BQ788 caused a marked rightward shift in the vasoconstrictor response to ET-3. This is shown in figure 5.10A and is further demonstrated by the marked decrease in pEC_{10}, pEC_{25} and pEC_{50} values for ET-3 in the presence of 1μM BQ788 (table 5.6).. Assuming that a maximum response had been achieved, the estimated pK_B value for

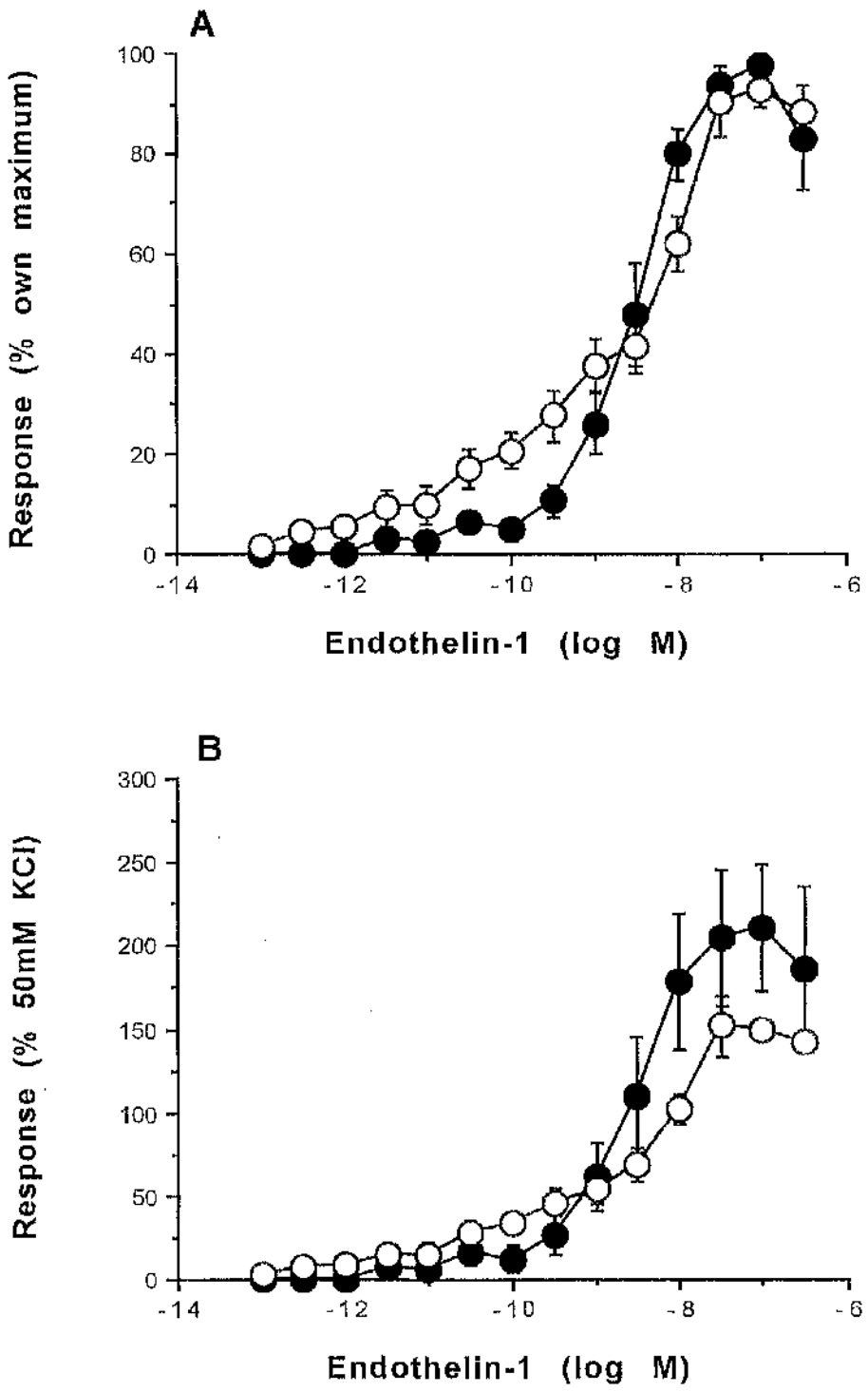


Figure 5.9 Effect of BQ788 on ET-1-induced responses in human PRAs. CCRC's to ET-1 (○, n=4/4) and in the presence of 1μM BQ788 (●, n=4/4). **A** Data are expressed as a percentage own maximum contraction. **B** Data are expressed as a percentage reference contraction to 50mMKCl. Each point represents mean ± s.e. mean.

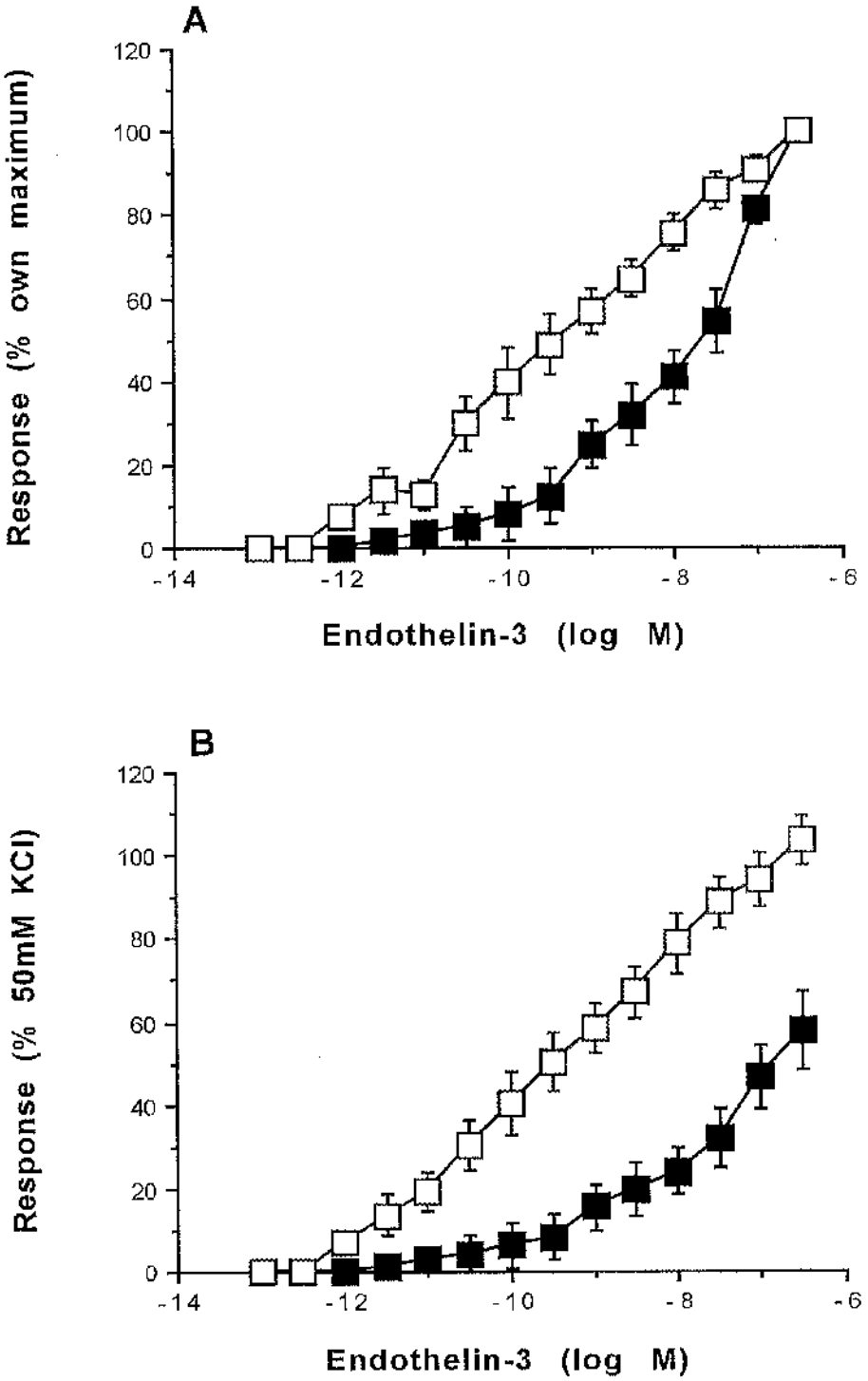


Figure 5.10 Effect of BQ788 on ET-3-induced responses in human PRAs. CCRC's to ET-3 (❑, n=5/5) and in the presence of 1μM BQ788 (■, n=5/5). **A** Data are expressed as a percentage own maximum contraction. **B** Data are expressed as a percentage reference contraction to 50mMKCl. Each point represents mean ± s.e. mean.

	n/n	pEC_{10}	pEC_{25}	pEC_{50}
ET-1	4/4	11.1±0.3	9.6±0.4	8.3±0.2
ET-1+1μM BQ788	4/4	9.6±0.2**	8.9±0.2	8.5±0.1
ET-3	5/5	11.2±0.1	10.6±0.3	9.5±0.3
ET-3+1μM BQ788	5/5	9.6±0.5*	9.0±0.4*	7.7±0.2**

Table 5.6 Effect of BQ788 on sensitivity to ET-1 and ET-3 in human PRAs. Statistical comparisons were made by Students' unpaired t-test; BQ788 vs. control *P<0.05, **P<0.01. Values are mean ± SEM. ET-1, endothelin-1; ET-3, endothelin-3; n/n, number of ring preparations / number of animals.

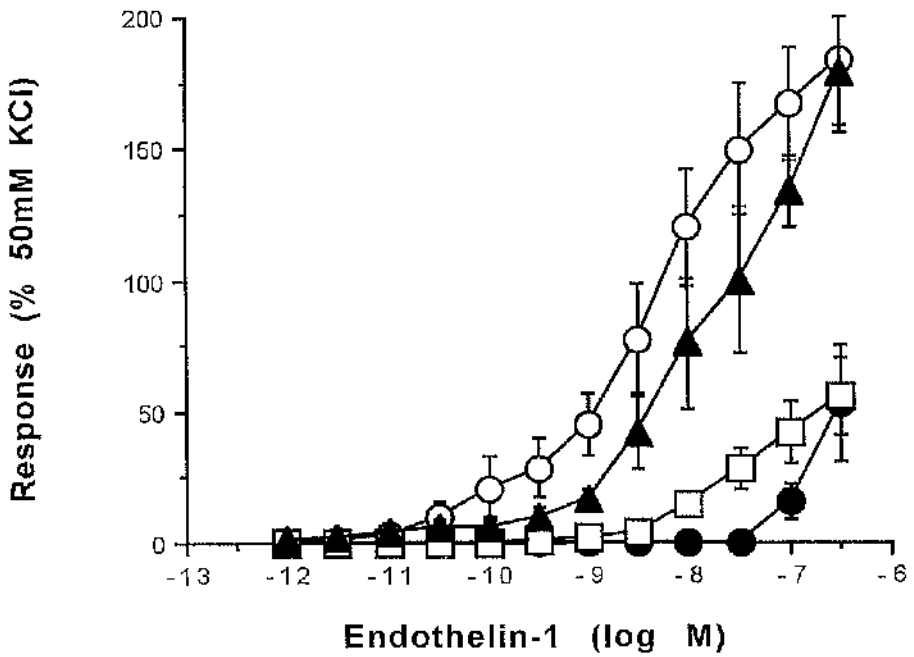


Figure 5.11 Effect of SB209670 on ET-1-induced responses in human PRAs. CCRC's to ET-1 (○, n=8/8) and in the presence of 10nM (▲, n=3/3), 0.1μM (❑, n=7/6), and 1μM (●, n=7/6) SB209670. Data are expressed as a percentage reference contraction to 50mMKCl. Each point represents mean ± s.e. mean.

BQ788 against ET-3 was 7.9±0.1. This selective ET_B receptor antagonist significantly reduced the magnitude of the contractile response to all ET-3 concentrations; the maximum vasoconstriction was decreased from 103.5±5.6% to 57.8±9.2% ($P<0.01$; figure 5.10B).

SB209670 (vs. ET-1)

In small human pulmonary arteries, the presence of SB209670 resulted in a marked inhibition of the entire ET-1-induced CCRC. The antagonistic effect was concentration dependent. This non-selective $ET_{A/B}$ receptor antagonist SB209670 at a concentration of 10nM did not significantly effect the sensitivity of human PRAs to ET-1. The calculated *p*EC values in the absence / presence of 10nM SB209670 were as follows: pEC_{10}, 9.5±0.2 / 9.1±0.2; pEC_{25}, 8.8±0.2 / 8.3±0.2; pEC_{50}, 8.1±0.2 / 7.7±0.2. 10nM SB209670 did not effect the magnitude of ET-1-induced vasoconstrictions (figure 5.11). However a marked inhibition of the ET-1-induced vasoconstriction was evident in the presence of 0.1μM and 1μM SB209670. 0.1μM SB209670 shifted the threshold concentration from 3pM to 1nM ET-1, and 1μM SB209670 abolished ET-1-induced vasoconstrictions to all ET-1 concentrations up to 0.1μM. The control contractile response to 0.1μM ET-1 of 166.8±21.4% was attenuated to 41.8±11.9% and 15.8±7.1% (both $P<0.001$) in the presence of 0.1μM and 1μM SB209670, respectively (figure 5.11). Hence the degree of inhibition was such that a maximum response was not attainable within the ET-1 concentration range which was financially feasible. Due to this, I was unable to calculate *p*EC or pK_B values for these concentrations of antagonist. However, assuming that the maximum vasoconstriction would not be significantly altered by SB209670, one could estimate a pK_B value of ~8 for 1μM SB209670.

5.4 Discussion

Endothelin-receptor subtypes in rabbit PRAs

The results of chapter 4 of this thesis showed that the potency order for the ET agonists in 8 week procedure animals, using pEC_{50} values as an index, was SXS6c>ET-3=ET-1. This is indicative of vasoconstriction being mediated by ET_B receptors, as has been previously shown in the larger pulmonary artery of the rabbit (Fukuroda *et al.*, 1994a; Hay *et al.*, 1996; Maggi, *et al.*, 1989; Panek *et al.*, 1992). In the studies of this chapter, I investigated the ET-receptor subtypes in this preparation further with the use of several selective antagonists.

SB209670 had an estimated pK_B value of 7.8 against SXS6c in the sham rabbit muscular pulmonary arteries. An inhibitory effect of this antagonist was also evident against ET-1 responses in this preparation, but a pK_B value could not realistically be calculated since the response did not reach a maximum. However if it was assumed that a maximum was attained , this provided an estimated pK_B value of 6.8. Hay *et al* (1996) and Beck *et al.* (1995) have shown that SB209670 has similar pK_B values (6.7 and 7.7, 6.6 and 7.8, respectively) against responses to ET-1 and SXS6c in large rabbit pulmonary arteries. Ohlstein *et al* (1994a;b) also showed a pK_B value of 6.7 for SB209670 against ET-1-mediated vasoconstrictions in large rabbit pulmonary arteries. In addition, these investigators reported that SB209670 has a pK_B value of 9.4 against ET_A- mediated responses in the rat aorta. This confirms that, in this study, SB209670 is acting as an antagonist against an ET_B receptor in the rabbit pulmonary resistance artery and this SB209670-sensitive receptor has a similar pharmacological profile to that in the larger pulmonary arteries. As described in the results, the shallow component of the CCRC to ET-1 was resistant to SB209670. Since this antagonism occurred only at the higher concentration range of ET-1, this would suggest that this component of the ET-1 response curve was mediated by SB209670-sensitive ET_B receptors. This may indicate that responses to ET-1 are not mediated by a homogeneous population of ET_B receptors,

consistent with the observations of Hay *et al* (1996) and Beck *et al* (1995) in the large rabbit pulmonary artery and Hay and Luttman (1997) in the guinea-pig bronchus.

The selective ET_B receptor antagonist BQ788 (1μM) removed the initial "shallow component" of the ET-1 CCRC but failed to effect the rest of the CCRC. It did, however, competitively inhibit responses to both SXS6c and ET-3 with estimated pK_B values of 7.1 and 6.6 respectively. Again, this phenomenon was observed by Hay *et al* (1996) in the larger rabbit pulmonary arteries where the pK_B values were 6.2 and 5.1 for SXS6c and ET-3, respectively, whilst there was no effect on ET-1. The authors concluded that the ET_B receptors stimulated by ET-1 in rabbit large pulmonary arteries are not sensitive to BQ788. Similarly, Beck *et al* (1995) demonstrated in the larger rabbit pulmonary artery that BQ788 (10μM) was ineffective against ET-1-induced vasoconstriction, but inhibited SXS6c-elicited contractile responses, with a pK_B value of 5.8. It would appear, therefore, that on the basis of both agonist and antagonist interactions, that the ET_B receptors stimulated by ET-1 in the large and small pulmonary arteries are pharmacologically similar.

Differential effects of BQ788 and SB209670 on the two components of the ET-1 CCRC were evident. The results show that ET-1 responses include a "shallow" component at lower at lower concentrations of ET-1 and this did appear to be resistant to the effects of SB209670 but sensitive to BQ788. Thus there is evidence for heterogeneous populations of ET_B receptors in the pulmonary resistance artery. One is sensitive to BQ788, but not SB209670 and mediates responses to lower concentrations of ET-1. The other is insensitive to BQ788, but sensitive to SB209670 and mediates responses to higher concentrations of ET-1. This resembles the profile of the receptor mediating responses in the large pulmonary artery of the rabbit (Hay, *et al.*, 1996). Thus the simplest explanation for the "shallow" component of the ET-1 CCRC in the rabbit PRAs is that there is a heterogeneous population of ET_B receptors. This is suggested by the shape of the CCRC and the differential effects of BQ788 and SB209670 on the "shallow" component of the ET-1 CCRC. However, this interpretation of the data remains speculative. Curve fitting for a biphasic response is, unfortunately, not possible

where the first component of the curve is less than 30% of the maximum response. This theory cannot, therefore, be assessed mathematically. Nevertheless, competition radioligand binding studies with ET-1 described in chapter 7 of this thesis supports this hypothesis. Previous studies, on several different tissue preparations, have proposed differential ET_B receptor subtypes based on the sensitivity of conventional ET_B receptor antagonists (Sudjarwo, *et al.*, 1993; 1994; Hori, *et al.*, 1994). Hay and Luttmann (1997) demonstrated in isolated guinea-pig bronchus, that the contractions induced by several ET-receptor agonists were mediated predominantly via stimulation of ET_B receptors however, these receptors were also shown to be insensitive to selective ET_B receptor antagonists. The authors concluded that ET-receptor mediated responses in the guinea-pig bronchus are not mediated by the classical ET_B receptor, but rather an atypical receptor population. The existence of additional ET_B receptors or a subtype of the ET_B receptor that is insensitive to BQ788 has also been hypothesised in the systemic circulation *in vivo*. This was proposed by Cirino *et al* (1997) following the observation that combined treatment with BQ123 and BQ788 revealed an action of ET-1 in the pig cardiovascular and renal system which could not be attributed to either ET_A or ET_B receptors blocked by these antagonists.

The results of this chapter also show that, in rabbit PRAs, BQ788 markedly inhibited vasoconstrictions elicited by both ET-3 and SXS6c, confirming that these peptides evoke their vasoconstrictor response via the activation of ET_B receptors. Lower concentrations of both SXS6c and ET-3 were more sensitive to the effects of BQ788 which removed the first component of a biphasic response to these agonists. The estimated pK_B for BQ788 against SXS6c was 7.1, whilst the lower value of 6.6 was estimated against ET-3, indeed, SXS6c has a higher affinity for this receptor. This suggests that SXS6c and ET-3 differentially activate the ET_B receptors which may contribute to the possible heterogeneous population present in this preparation. That ET-1 is also acting at different receptors from SXS6c in rabbit PRAs is suggested by the selective effect of BQ788 on responses to SXS6c. In addition, in these results and those of chapter 4, I showed desensitisation to higher concentration of SXS6c, as indicated by

the "drop off" in it's CCRC, which is not observed in the cumulative responses to ET-1. In addition the vasoconstrictor responses to individual applications of SXS6c were transient, whereas the increase in tone elicited by each ET-1 concentration was sustained. Similar findings have also been reported in other preparations in which the involvement of vasoconstrictor ET_B receptors have also been described, namely rat PRAs (McCulloch & MacLean, 1995) and in rabbit saphenous vein (Sudjarwo, *et al.*, 1994).

SXS6c-mediated responses were even more sensitive to the to the actions of the mixed receptor antagonist SB209670 compared to the selective ET_B receptor antagonist BQ788. SB209670 also produced a greater inhibition of SXS6c-responses in this preparation compared with ET-1-induced vasoconstrictions, as was evident from the 10 fold difference in pK_B values. Ohlstein *et al* (1994a) also reported in the large rabbit pulmonary artery that SB209670 was approximately 4 fold more potent as an ET_B receptor antagonist when SXS6c was the agonist rather than ET-1. Similarly, Beck *et al.* (1995) demonstrated, also in the large rabbit pulmonary artery, that SB209670 produced 10 fold greater shifts in the CCRCs produced by SXS6c than ET-1. These results and also the differential inhibitory effect of BQ788 on responses to SXS6c and ET-3 demonstrated in this study, provide additional evidence that the potencies of ET receptor antagonists depend upon the specific ET agonist. This phenomenon has been reported previously by several investigators (Warner, *et al.*, 1993b; Kizawa, *et al,* 1994; Ohlstein, *et al,* 1994a; Yoneyama, *et al.*, 1995; Hay & Luttmann, 1997) These results may be explained by the existence of heterogeneous populations of ET receptor subtypes, as I have previously suggested, for which different ET receptor antagonists have different affinities. This has also been proposed by Warner *et al* (1993a), who demonstrated in first branch rabbit pulmonary artery that PD142893, a nonselective ET-receptor antagonist, did not inhibit ET-1 mediated vasoconstrictions, but gave a 3 fold antagonism of constriction induced by SXS6c. These apparent differences in ET_B receptors may correspond with the previous reports that different populations of high- and low- affinity ET_B receptors are present in the brain; a novel subtype of the ET_B

receptor that binds ET in the picomolar range was identified and characterised in rat brain and atrium (Solovsky, *et al.*, 1992). Alternatively, the explanation for this difference may be that the different agonists ligands bind to different or multiple accessory binding domains on the ET_B receptor, and receptor antagonists may have differential affinities for these domains (Hiley, *et al.*, 1992; Sakamoto, *et al.*, 1993). Another possibility is that a single population of ET_B receptors exists but in two different conformational states, an active state coupled to contraction and an inactive state that is not coupled to contraction; this has been postulated by Robertson *et al.* (1994) for the angiotensin II receptors.

SB209670 has also been shown to effectively antagonise ET_B-mediated vasodilation (Ohlstein, *et al*, 1994b). The inhibition of these receptors in rabbit PRAs may account for the augmentation in the maximum SXS6c-induced vasoconstriction which was observed in the presence of (0.1μM) SB209670. Ohlstein *et al* (1994a) and Beck *et al* (1995) also demonstrated that the maximal contraction induced by SXS6c in the rabbit isolated large pulmonary artery was potentiated by several different non-selective ET-receptor antagonists including SB209670. However in the studies of chapter 4 of this thesis, I was unable to show ET_B-receptor mediated vasodilations in rabbit PRAs; this is discussed later.

Effect of pulmonary hypertension secondary to left ventricular dysfunction (LVD)

What happens in the rabbits exhibiting PHT secondary to LVD? In isolated PRAs from coronary-ligated rabbits, a potentiation of responses to lower concentrations of ET-1 was observed in the presence of BQ788 and SB209670. This suggests that, after eight weeks of coronary ligation and with the development of PHT, the BQ788-sensitive receptor-mediated response has changed in that it is now potentiated by both BQ788 and by SB209670. This may indicate an alteration in ET_B receptor subtypes with the development of PHT. Both BQ788 and SB209670 inhibit the endothelial ET_B receptor which mediates endothelium-dependent relaxation (Douglas *et al.*, 1995; Ohlstein, *et al.*,

1994b). Endothelial ET_B receptors have been shown to mediate pulmonary vasodilatation in rats and lambs (Eddahibi, *et al.*, 1991; Wong, *et al.*, 1995). Fukuroda *et al* (1992) reported that ET isopeptides caused vasodilation in preconstricted isolated porcine pulmonary arteries. These results suggest, therefore, that there has been an upregulation of this receptor concurrent with the development of PHT. In chapter 4 however, I failed to demonstrate ET_B receptor-mediated vasodilation in this preparation. I also examined the possibility of a relaxatory response to SXS6c in preconstricted rabbit PRAs in the presence of FR139317. However, despite the presence of this selective ET_A receptor antagonist, no vasodilatory response was evident and again, SXS6c evoked a concentration-dependent contractile response. Nevertheless the possibility remains that the existence of this receptor in rabbit PRAs may be masked by the predominance of vasoconstrictor ET_B receptor subtypes, thus preventing it from being identified functionally. Furthermore, the results of chapter 6 suggest an upregulation of basal nitric oxide synthase in eight week ligated rabbit pulmonary resistance arteries. In addition, on a few occasions throughout this study, I observed an increase in basal tone in response to BQ788 alone, this would suggest that endothelial ET_B receptors are also involved in this preparation. The observation that responses to ET-3 were reduced in the PRAs from the ligated rabbits (shown in chapter 4) may be explained by an increase in endothelial ET_B-mediated vasodilatation as ET-3 is extremely potent at this receptor (Douglas *et al.*, 1995).

The question remains as to whether ET_B receptors on the endothelium are functionally distinct from those present on the vascular smooth muscle. Conflicting views have been reported by several investigators. From the findings that the non-selective antagonist PD142893 displayed a markedly greater inhibitory effect against SXS6c-induced vasodilatory compared with vasoconstrictor responses, Warner *et al.* (1993) concluded that the ET_B receptor mediating vasoconstriction was most probably distinct from that on the endothelium mediating the release of nitric oxide. In contrast, from studies in rat tracheal rings, Clozel & Gray (1995) reported that the inability of ET_B receptor blockade to inhibit ET-1-mediated vasoconstrictions could be accounted

for by cross-talk between receptors that allows ET_A receptors to compensate for inhibition of ET_B receptors. Thus these authors concluded that these ET_B receptors may not represent two different subtype. More recently Mizuguchi *et al.* (1997) performed a study using the ET_B receptor gene knockout mouse, to clarify whether the ET receptor subtypes mediating the vasoconstriction and vasodilation response to ET_B receptor agonists were the products of a single ET_B receptor gene. The results of this study demonstrated that the PD142896 (non-selective ET_A/ET_B receptor antagonist)- sensitive vasodilator response of the aorta and the PD142896-resistant contractile response of the gastric fundus to SXS6c were completely absent in the ET_B receptor gene knockout mouse. Thus the authors concluded that the two pharmacological heterogeneous responses to SXS6c are mediated by receptors derived from the same ET_B receptor gene.

The pK_B value for BQ788 against ET-3 was increased from 6.6 to 7.0 in the vessels from the LVD rabbits whilst the pK_B value for SB209670 against SXS6c was reduced from 7.8 to 6.7. In addition, SB209670 no longer evoked a significant inhibition of ET-1-induced vasoconstrictions. The reason for these changes are unclear but it is known that the actual phenotype of the pulmonary vascular smooth muscle is different throughout the pulmonary arterial tree and changes in phenotype are evident with the development of PHT (e.g. Meyrick & Reid, 1978; Frid *et al.*, 1994). Therefore, ligand-receptor interactions may well be influenced by changes in smooth muscle phenotype. In addition, there may also be an alteration in the ET-receptor subtypes present in the pulmonary vasculature in pathophysiological conditions. Indeed, previous investigators have reported an upregulation of smooth muscle ET_B receptors in pathological states which are also associated with increased plasma ET-1 concentrations and an increase in peripheral vascular resistance. In particular, this was reported in heart failure in man (Love, *et al.*, 1996) and dogs (Cannan, *et al.*, 1996) and in systemic hypertension (Kanno, *et al.*, 1993; Batra, *et al.*, 1993). In studies on isolated renal artery from ageing spontaneously hypertensive rats, Seo & Luscher (1995) demonstrated that endothelial ET_B receptor-mediated vasodilation was more efficient in control rat vessels, whilst

ET_B receptors in the vascular smooth muscle were unmasked in spontaneously hypertensive rats. Similarly, Clozel and Breu (1996) showed in rat models of systemic hypertension in vivo, the predominant influence of endothelial ET_B receptors mediating vasorelaxation, whilst ET_B receptors appeared to be mediating vasoconstrictor tone in normotensive rats. Such alteration in ET_B-receptors may account for the results of chapter 4 of this thesis, showing alteration in ET-1- and SXS6c-elicited responses of PRAs from rabbits following 16 and 32 weeks of coronary artery ligation. The results of this chapter may indicate an upregulation of inhibitory ET_B and ET_A receptors in PRAs from LVD rabbits induced by eight weeks of coronary artery ligation. Altered expression of ET_B-receptor mRNA has been reported in the lungs of rats with monocrotaline-induced PHT (Yorikane, *et al.*, 1993). Although this study demonstrated a reduction in ET_B-receptor mRNA, it was unclear which function, vasodilation or vasoconstriction, would be altered by this attenuation. Furthermore, the morphological results, shown in chapter 3 of this thesis, demonstrate structural changes in small pulmonary vessels from pulmonary hypertensive rabbits which are common to many form of PHT. The growth and migration of endothelial cells are the prerequisites for vascular remodelling. Morbidelli *et al* (1995) reported that proliferation and migration of endothelial cells is promoted by ETs via activation of ET_B receptors; thus ETs can contribute to neovascularisation through an autocrine mechanism that requires ET_B receptor activation. Furthermore, Azuma *et al.* (1995) demonstrated, in a preparation of angioplasty-induced lesion formation in the rabbit carotid artery, that an increase in ET-1 receptors, especially ET_B and/or a putative non-ET_A/non-ET_B receptors, were evident in the hyperplastic vascular wall.

The studies with FR139317 suggest that the pharmacology of the ET receptor in rabbit pulmonary resistance arteries is even more complex. FR139317 did not inhibit responses in either sham-operated or coronary ligated rabbit vessels. Another selective ET_A receptor antagonist, BQ123, was also shown to be ineffective in inhibiting ET-1-induced vasoconstrictions in the larger rabbit pulmonary artery (Warner, *et al.*, 1993). This is consistent with the suggestion that ET_A receptors do not form the major

population of ET receptors. This has also been demonstrated in binding assays of rabbit pulmonary artery membranes which showed that this preparation contained ET_A and ET_B receptors in the ratio 23:77 (Fukuroda, *et al.*, 1994a). In contrast, Cardell *et al.* (1993) showed that ET-1 vasoconstrictor responses in guinea-pig main pulmonary artery were competitively antagonised by FR139317. This observation along with the decreased potency of ET-3 compared with ET-1, indicates the predominance of ET_A receptors in this preparation. Thus, there appears to be both species and vessel-related differences in the ET-receptor subtypes of pulmonary arterial system. In this study, in both sham-operated and ligated rabbit vessels, FR139317 caused a tendency to potentiate the responses to ET-1 at higher ET-1 concentrations and this tendency was also observed for lower concentrations of ET-1 in the ligated rabbit vessels. This indicates that ET-1 is not acting through a typical interaction with ET_A receptors and suggests the presence of a putative inhibitory ET_A receptor, as first described by Gray and Clozel (1993) in the rat fundic strip. In this previous study, responses to ET-1 were potentiated in the presence of the selective ET_A receptor BQ123. The presence of such a receptor has also previously been reported in the rat pulmonary resistance artery, where the selective ET_A receptor antagonist, BMS 182874, potentiated ET-1 responses (McCulloch & MacLean, 1996).

The concept of this receptor "crosstalk" has previously been proposed to explain the observations that dual inhibition of ET_A and ET_B receptors is required to inhibit ET-1 induced vasoconstrictions in rabbit pulmonary artery (Fukuroda, *et al.*, 1994c) and in human bronchi and rat trachea (Fukuroda, *et al.*, 1996; Clozel & Gray, 1995). More recently, this postulate has been suggested by Mickley *et al.* (1997) to explain the requirement of dual inhibition of ET_A and ET_B receptors in order to expose ET_B receptor mediated vasoconstriction of rat isolated small mesenteric arteries. In this hypothesis, the ET_A receptor can compensate when the ET_B receptor is inhibited and mediate responses to ET-1 and vice versa; thus explaining the decreased potency of antagonists from that which would be anticipated from results of previous binding and functional studies. For example, this hypothesis may be used to explain why a selective

antagonist such as BQ788, which has been shown by numerous binding and pharmacological studies to be a selective antagonist for ET_B receptors, is unable to inhibit ET_B receptor mediated vasoconstrictor responses, in that interaction or "crosstalk" of the receptor subtypes occurs such that the ET_A receptor subtype can compensate for the ET_B receptor blockade and therefore the vasoconstrictor response can still be manifested. The inability of FR139317 and BQ788 to antagonise ET-1-mediated responses, which was shown in the results of this chapter, may also be explained by a possible "crosstalk" between ET_A and ET_B receptors. However, due to the apparent predominance of vasoconstrictor ET_B receptors in rabbit pulmonary arteries, it seems unlikely that on inhibition of these receptors, that ET_A receptors could evoke a similar CCRC; but, the possibility also remains that the vasoconstrictor ET_B receptor predominantly responsible for this vasoconstriction may be insensitive to BQ788. In addition, Clozel & Gray, (1995) reported that this "crosstalk" does not take place when an ET_B receptor agonist is used or when only ET_B receptors mediate the response. This may explain the marked inhibition of SXS6c responses by BQ788 in this preparation but the comparative ineffectiveness against the non-selective agonist ET-1. The mechanism for the plausible receptor "crosstalk" is not fully understood. Fukuroda *et al.* (1996) suggested interactions at in the signal transduction systems between ET_A and ET_B receptors. This proposal was based on the observations by Ozaki *et al.* (1997) in human Giradi heart cells expressing ET_A and ET_B receptors. This study showed that stimulation of ET_A receptors with ET-1 results in a lowering in the affinity of BQ3020 and BQ788 for ET_B receptors, thus suggesting conversion of the ET_B receptor to a BQ788-insensitive form through ET_A-mediated intracellular signalling. Allosteric interactions between ET receptors has been suggested to account for the results of radioligand binding in rat heart (Sokolovsky, 1993).

The ineffectiveness of FR139317 and BQ788 in antagonising ET-1-mediated responses in adult rabbit PRAs was also demonstrated in PRAs from 7 day old rabbits (chapter 9). However, these findings are in contrast to other results of chapter 9, which showed a significant inhibitory effect of FR139317 in the same preparation from

newborn and 4 day old rabbits. Similarly, BQ788 produced a marked inhibition of responses to ET-1 in vessels from fetal to 4 day old rabbits. This suggests an alteration in the interaction of ET receptor subtypes with developmental age, and this is discussed in chapter 9 of this thesis.

In PRAs from sham-operated rabbits, FR139317 had no significant effect on the ability of BQ788 to inhibit responses to ET-1. However, the potentiation of the response to higher ET-1 concentrations which was noted in the presence of FR139317, was no longer evident when BQ788 was present also. This suggests either that ET-1 is acting on vasoconstrictor ET_B receptors although this response is masked by the actions of possible inhibitory ET_A receptors, or that ET-1 is not acting at typical ET_A and ET_B receptors. The combined effects of FR139317 and BQ788 might be expected to be the same as the effect of SB209670. The differential results might be explained by SB209670 being a much more potent antagonist at an ET_B receptor than BQ788 (Ohlstein, *et al.*, 1994b; Sogabe, *et al.*, 1993). Or it could be that the ET_B vasoconstrictor receptor population is uniquely sensitive to SB209670. In coronary-ligated PRAs, the potentiation of the responses to lower ET-1 concentration which was noted in the presence of either FR139317 or BQ788 alone, was no longer evident when both these antagonists were present together. However, the decrease in sensitivity to lower ET-1 concentration still did not reach significance. As discussed previously, synergy between ET_A and ET_B receptors has been reported in larger rabbit pulmonary arteries where administration of both an ET_A and ET_B receptor antagonist is required to inhibit responses to ET-1 (Fukuroda *et al.*, 1994c). Necessity of dual receptor blockade has also been demonstrated in human bronchi (Fukuroda *et al.*, 1996). These findings suggest the presence of normally quiescent ET_A receptors and receptor cross-talk between ET_A and ET_B receptors through intracellular signalling cascades (Fukuroda *et al.*, 1994c; Fukuroda *et al.*, 1996).

In conclusion, these results show that the pharmacology of the ET-1 receptor in rabbit pulmonary resistance arteries is extremely complex. There would appear to be a quiescent ET_A receptor which synergises with a vasoconstrictor ET_B receptor which is

BQ788 sensitive (SB209670 insensitive) and mediates responses to lower concentrations of ET-1. There is also a BQ788-insensitive receptor which is sensitive to SB209670 and mediates responses to higher ET-1 concentrations. Furthermore, evidence is provided for inhibitory ET_B and ET_A receptors which may be upregulated in the vessels from the rabbits with LVD. This may be a physiological compensatory mechanism in response to the early elevation in pulmonary pressure to maintain responses to ET-1 constant, particularly at the lower physiologically relevant concentration range. Antagonism of ET-receptor mediated vasoconstriction may provide a possible therapy in established pulmonary hypertensive states, or prophylactic against pulmonary hypertension secondary to other pathologies such as heart failure. However, the effectiveness of ET-receptor antagonism therapeutically will depend on the level of endothelial ET_B receptor stimulation and on the relative selectivity for endothelial and smooth muscle ET_B receptor subtypes.

Endothelin-receptor subtypes in human PRAs

ET-1 had similar sensitivity in human PRAs to that observed in rabbit vessels of a similar internal diameter (pEC_{50} 8.1 and 7.9, respectively). The results show that the CCRCs to ET-1 are biphasic in human PRAs. This has also been shown by previous studies in human and rat PRAs (McCulloch & MacLean, 1995; McCulloch *et al*, 1996). I also demonstrated this in rabbit PRAs in chapter 4 of this thesis. In the previous report in rat PRAs, the authors suggest that the biphasic response may be due to a heterogeneous population of ET_B receptors or due to the presence of inhibitory ET_A receptors (McCulloch & MacLean, 1995). From the results in this chapter in rabbit PRAs, the initial component appears to be due to a heterogeneous population of ET_B receptors. Furthermore, in human PRAs, the first component of the response to ET-1 appears to be due to a population of ET_B receptors mediating vasoconstriction, as was indicated by the inhibitory effect of the selective ET_B receptor antagonist BQ788 at lower ET-1 concentrations (<1nM). The previous study by McCulloch & MacLean

(1995) in human PRAs showed that the selective ET_A receptor antagonist BMS 182874 inhibited ET-1 concentrations greater than 1nM. This suggests that vasoconstriction evoked by higher ET-1 concentrations is mediated through stimulation of ET_A receptors. A previous report in human pulmonary arteries (~1mm outer diameter), demonstrated that ET-1-induced contractions were competitively antagonised by BQ123 (an ET_A receptor antagonist) whereas the ET_B receptor agonist BQ3020 was without effect (Fukuroda, *et al.*, 1994a). Inhibition of ET-1-responses of human larger pulmonary arteries by BQ123 has also been reported by Hay *et al* (1993). These findings indicate differential ET-receptor subtypes in human pulmonary arteries of different sizes. Davenport *et al.* (1995) reported from radioligand binding studies, that ET_B receptors only represented less than 15% of the of the ET receptors in the larger human pulmonary artery. Fukuroda *et al* (1994a) showed from binding assays that human pulmonary arteries of ~1mm outer diameter contained ET_A and ET_B receptors in the ratio 93:7; this compares with the ratio of 23:77 reported in rabbit pulmonary arteries of similar diameter. The influence of vessel size on ET-receptor mediated responses, in terms of antagonist effectiveness, has also been demonstrated in rat pulmonary arteries (MacLean, *et al.*, 1994a; Bonvallet, *et al.*, 1993).

Concentration-dependent contractile responses were also observed to ET-3. ET-1 and ET-3 would be expected to be equipotent at a classical ET_B receptor; the results of chapter 4 of this thesis demonstrated equipotency of these isopeptides in rabbit PRAs. However, from these results, it can be seen that ET-3 was ~5 fold more potent than ET-1; this has also been reported in previous studies in isolated human PRAs (McCulloch, *et al.*, 1996). A receptor selective for ET-3 over ET-1 (denoted ET_C) has been cloned in *Xenopus laevis* dermal melanphores (Karne, *et al.*, 1993). The relative potency of ET-3 over in human PRAs may suggest the presence of such an ET_C receptor. Indeed, there is pharmacological evidence for this receptor in other vascular tissue including rabbit saphenous vein (Douglas, *et al.*, 1995). However, a mammalian vascular counterpart of this putative receptor has yet to be identified. The magnitude of the vasoconstriction evoked by ET-3 was markedly smaller than that of ET-1 in human PRAs; I also showed

this in rabbit PRAs from coronary-ligated rabbits (chapter 4). As was noted in rabbit PRAs, the ability of BQ788 to antagonise ET-3-induced responses in human PRAs confirms that ET-3 is mediating its responses via ET_B receptors. This provides further evidence for the involvement of ET_B receptors in the contractile responses of human PRAs. In addition, the differential effect of BQ788 on ET-1 and ET-3 responses in this preparation provides additional evidence that the potency of BQ788 depends upon the ET-receptor antagonist (this is discussed earlier).

The involvement of both ET_A and ET_B receptor subtypes was shown by the marked inhibitory effect of the non-selective antagonist SB209670. The degree of inhibition of ET-1-responses by SB209670 in human PRAs was markedly greater than that observed in rabbit PRAs. Of the previous studies using selective ET-receptor antagonist and the effect of BQ788 shown in these results, the most profound inhibitory effects on ET-1-induced vasoconstrictions of human pulmonary arteries were noted to this non-selective antagonist. As was suggested in rabbit PRAs, this difference may be explained by a heterogeneous population of ET_B receptors. The limited potency of BQ788, demonstrated in this study, suggests that ET_B receptors in PRAs are not as sensitive to the prototypical ET_B receptor antagonist BQ788 as they are to the non-selective antagonist SB209670. Support for this hypothesis requires molecular biological and further pharmacological evidence of the nature and location of ET-receptor subtypes on the human pulmonary vasculature.

Chapter 6

Influence of nitric oxide on ET-receptor-mediated responses in rabbit pulmonary resistance arteries: Effect of left ventricular dysfunction

6.1 Introduction

I have previously introduced in chapter 1 of this thesis, the physiological role of endothelium-derived nitric oxide (NO) and endothelin-1 (ET-1) in the pulmonary circulation and also the mechanisms of action and effects of this oxidant radical and peptide. The importance of the functional role of the endothelium in the control of pulmonary vascular tone, via the release of vasoactive peptides such as NO and ET-1, has become increasingly evident. Both these endothelial derived agents have marked functional and structural effects on the developing (Kinsella, *et al.*, 1994; Roberts, *et al.*, 1995) and adult pulmonary circulation (Barnard, *et al*, 1991; Janakidevi, *et al.*, 1992). The discovery that NO is endothelium derived relaxing factor (EDRF) (Palmer *et al.*, 1987) was followed in rapid succession by the use of inhaled NO in humans as therapy for pulmonary hypertension (PHT). Many previous studies have demonstrated that NO is the first substance capable of selectively reducing PHT, of various etiologies in both children and adults, without reducing systemic arterial blood pressure (Pepke-Zaba, *et al.*, 1991; Roberts, *et al.*, 1992; Rich, *et al.*, 1993). The role of NO in ET-receptor mediated responses in transitional pulmonary circulation of the fetal and neonatal rabbit is examined in chapter 8 of this thesis. In the studies described in this chapter, I examined the role of NO in ET-receptor mediated responses in the adult rabbit pulmonary resistance arteries (PRAs). Furthermore, the effect of PHT secondary to left ventricular dysfunction (LVD) was investigated also using a rabbit coronary artery ligation model (Deuchar, *et al*, 1997; Pye, *et al*, 1996).

A dysfunction of the endothelium has been implicated in the pathophysiology of a number of cardiovascular diseases, including PHT (Rabinovitch, *et al.*, 1986). Impairment of NO synthesis, and/or increased ET-1 synthesis, or increased smooth muscle sensitivity to ET-1, could account the many features associated with PHT, including the increased pulmonary vascular tone and vascular hypertrophy. As I have previously stated, ET-1 has been implicated in several pathological conditions including heart failure and PHT of various etiologies (Stewart, *et al.*, 1991). Thus, in chapters 4

and 5 of this thesis I examined ET-receptor subtypes in small muscular pulmonary arteries from the rabbit coronary ligation model of left ventricular dysfunction. The involvement of endothelium-derived NO in several pathophysiological states of the pulmonary circulation involving PHT has also been reported; including cystic fibrosis, Eisenmenger's syndrome and chronic obstructive lung disease (Dinh-Xuan, *et al.*, 1989; 1990; 1991). There is evidence that nitric oxide synthase (NOS) may be upregulated in patients with heart failure and inhibition of NOS in these patients increased pulmonary vascular resistance (Habib, *et al,* 1994). Whereas an impairment of NO production was reported in isolated pulmonary artery from patients with chronic obstructive lung disease (Dinh-Xuan. *et al.*, 1993).

The ability of ET to induce the release of EDRF (NO) from the isolated perfused mesentery and prostacyclin from the isolated lung has been demonstrated by De Nucci *et al.* (1988). Since previous studies suggest an important role of these endothelial derived relaxing factors in the maintenance of low tone in the pulmonary circulation, it may be postulated that the potent vasoconstrictor effect of ET-1 on the pulmonary vascular smooth muscle is modulated by their concomitant release, with such interaction providing a feedback mechanism in vascular control. Indeed, previous studies suggests that EDRFs, such as NO, may attenuate ET-1-induced arterial vasoconstriction. Raffestin *et al.* (1991) showed that the rise in pulmonary artery pressure in response to ET-1 in isolated rat lungs was potentiated by several inhibitors of EDRFs. This was also demonstrated in rat aorta by Lang and Lewis (1991) and in pulmonary artery branches of the rat by MacLean, *et al.* (1994b). Hence, an imbalance between NO and ET-1 may contribute to the alteration in vascular tone characteristic of cardiovascular disease.

Therefore in this chapter, I examined the presence of basal and/or agonist-induced NO release in sham-operated and coronary ligated PRAs by examining the effect of the NOS synthase inhibitor on resting tension and on responses to ET-1 and the selective ET_B receptor agonist SXS6c. A vascular contraction mediated by NOS inhibition could be due to inhibition of basal NO, the presence of endogenous tone, or a combination of both effects. To clarify this, I also sought inherent tone in rabbit PRAs

by determining the response of these vessels, under basal tension, to sodium nitroprusside (SNP).

6.2 Methods

Rabbit pulmonary resistance arteries

Lungs were obtained from the rabbit coronary ligation model of left ventricular dysfunction which has been extensively characterised (Pye *et al*, 1996) and is described in detail in section 2.1.1 of this thesis. In the 8 week and 16 week procedure period groups, age matched animals underwent the same methodology as the experimental animals (referred to in results and discussion as "ligated") except the ligatures placed around the coronary artery were not secured. These are subsequently referred to as "sham-operated". Also, age matched animals in which no operational procedures were performed were used as controls in the 16 week and 32 week procedure period groups These are subsequently referred to as "stock". Measurements of ejection fraction were made in all animals and animals were killed by sodium pentabarbitone 8, 16 or 32 weeks following the procedure. The lungs were promptly removed and small intralobar muscular pulmonary arteries (referred to as pulmonary resistance arteries (PRAs) ~160 μm ID; see table 6.1) were dissected out according to the methods stated in section 2.2.3.1. These were mounted as ring preparations (~2mm long) on a wire myograph, bathed in Krebs solution at 37°C. Using the normalisation process explained in section 2.2.5, vessels were tensioned to an equivalent transmural pressure of ~ 16 mmHg. This pressure was chosen as it is similar to *in vivo* pressures of rabbit pulmonary arterioles. Preliminary studies in our laboratory showed that bubbling with 95% O_2 inhibits responses to vasoconstrictors in rabbit PRAs so we bubbled with 16% O_2/ 5% CO_2 balance N_2. These gas mixtures were chosen as they are similar to those which the vessels would be exposed to *in vivo*.

Experimental protocol

Vessels were allowed to equilibrate for 1 hour prior to the addition of any drugs. The response of the PRAs to 50mM KCl was determined twice; once the contractile response had reached a plateau, the vessels were washed out at least 6 times with fresh Krebs solution. Following adequate time to reach baseline tension, Cumulative concentration-response curves (CCRCs) were constructed to ET-1 or SXS6c (1pM-0.3μM) following either a 30 minute "rest period" or a 30 minute incubation period with 100μM L-NAME. In a proportion of SXS6c experiments in vessels from 8 week procedure groups, 0.1μM ET-1 was added to the bath at the end of the SXS6c CCRC, before washing, so as to estimate the SXS6c response as a percentage of response to a maximal ET-1 concentration. In addition, in a separate group of 8 week rabbit PRAs, following equilibration and before the addition of any agents, 1μM SNP was added to the bath to examine possible endogenous tone in the vessels.

Data analysis

Results are expressed graphically as percentage of their own maximum contraction, or percentage of reference contraction to second application of 50mM KCl., or percentage of contraction to 0.1μM ET-1, or absolute response as mg weight tension.. $p\mathrm{EC}_{10}$,$p\mathrm{EC}_{25}$ and $p\mathrm{EC}_{50}$ values (where appropriate) were calculated according to the methods stated in section 2.5.2, and expressed as -log M concentration. Statistical comparison of the means of groups of data were made by Student's unpaired t test when two groups were compared, or ANOVA when three or more groups were compared; $P<0.05$ was considered statistically significant. Throughout, data are expressed as mean±SEM and n /n = number of ring preparations / number of animals.

4.3 Results

The ejection fraction of animals from the various experimental groups, from which lungs were obtained and PRAs dissected for the studies described in this chapter, are shown in table 6.1. The effective pressure to which these vessels were tensioned and the resulting estimate of internal diameter are also shown in table 6.1. The ejection fraction was markedly reduced in ligated animals compared with sham-operated/stock rabbits in 8, 16 and 32 week procedure groups. Also, the reduced ejection fraction was significantly lower in 16 week ligated compared to 8 and 32 week ligated rabbits. Vessels from all experimental groups were tensioned to a similar effective pressure and internal diameter.

	(n)	Internal diameter (μM)	Effective pressure (mmHg)	Ejection fraction (%)
8 week				
sham-operated	(36)	170.1±7.5	18.8±0.8	69.3±1.2
ligated	(40)	174.7±7.1	21.2±0.6	45.7±0.9***
16 week				
sham-operated	(24)	146.7±7.5	20.8±1.3	74.4±0.8
ligated	(36)	161.3±6.6	17.8±0.6	40.2±1.2***+
32 week				
stock	(16)	159.9±7.4	15.8±1.1	74.1±0.9
ligated	(20)	156.2±12.5	19.4±1.1	47.5±1.0***

Table 6.1 (1) Ejection fraction of animals from which PRAs were dissected and studied in this chapter and (2) internal diameter and effective pressure of PRAs from this cohort of rabbits. Statistical comparisons were made by Student's unpaired t-test: ligated vs. sham-operated/stock, ***$P<0.001$; ANOVA: 16 week ligated vs. 8 and 32 week ligated, +$P<0.001$. Values are mean±SEM. n, number of ring preparations.

SNP (1μM) had no effect on the baseline tension in PRAs from either 8 week sham-operated or coronary-ligated rabbits (n=4/4 in each case). The effect of 100μM L-NAME alone on baseline tension of small pulmonary arteries from the various experimental groups was assessed also. This nitric oxide synthase inhibitor evoked variable responses. Of the vessels examined in the 8 week procedure group, 23.5% and 66.7% of the vessel from sham-operated and coronary-ligated rabbits showed a vasoconstrictor response, respectively. In the 16 week procedure group, an increase in baseline tone was evident in 66.6% and 50% of the vessel from sham-operated and coronary-ligated rabbits, respectively. Whilst no response to L-NAME was noted in any of the vessels studied from the 32 week procedure group. The magnitude of the increase in baseline tension, which was noted in the vessels which responded, is shown in figure 6.1. When this increase in tone was evaluated as a percentage of the reference contraction to the second application of 50mM KCl, the response of 8 week sham-operated and coronary ligated rabbit vessels was similar, whilst that of the small pulmonary arteries from 16 week ligated rabbits was significantly reduced compared with corresponding sham-operated tissue (17.4±4.1% vs. 33.0±4.1%, $P<0.05$; figure 6.1A). However, on assessing this vasoconstriction in absolute terms as mg weight tension, no significant difference was noted between sham-operated and coronary-ligated rabbit PRAs from either group (figure 6.1B). The increase in tension noted in the 8 week ligated rabbit PRAs tended to be greater than that of control vessels in the same group (91.9±21.9 mgwt. vs. 50.0±16.9 mgwt.), however, this did not reach the level of statistical significance.

8 week experimental group

Figures 6.2 and 6.3 show the effect of L-NAME on ET-1 responses in PRAs from the 8 week procedure group. pEC_{10}, pEC_{25} and pEC_{50} values are summarised in table 6.2. 100μM L-NAME had no effect on the sensitivity of 8 week sham-operated PRAs to ET-1 (figure 6.2A, table 6.2.) However, in vessels from coronary-ligated

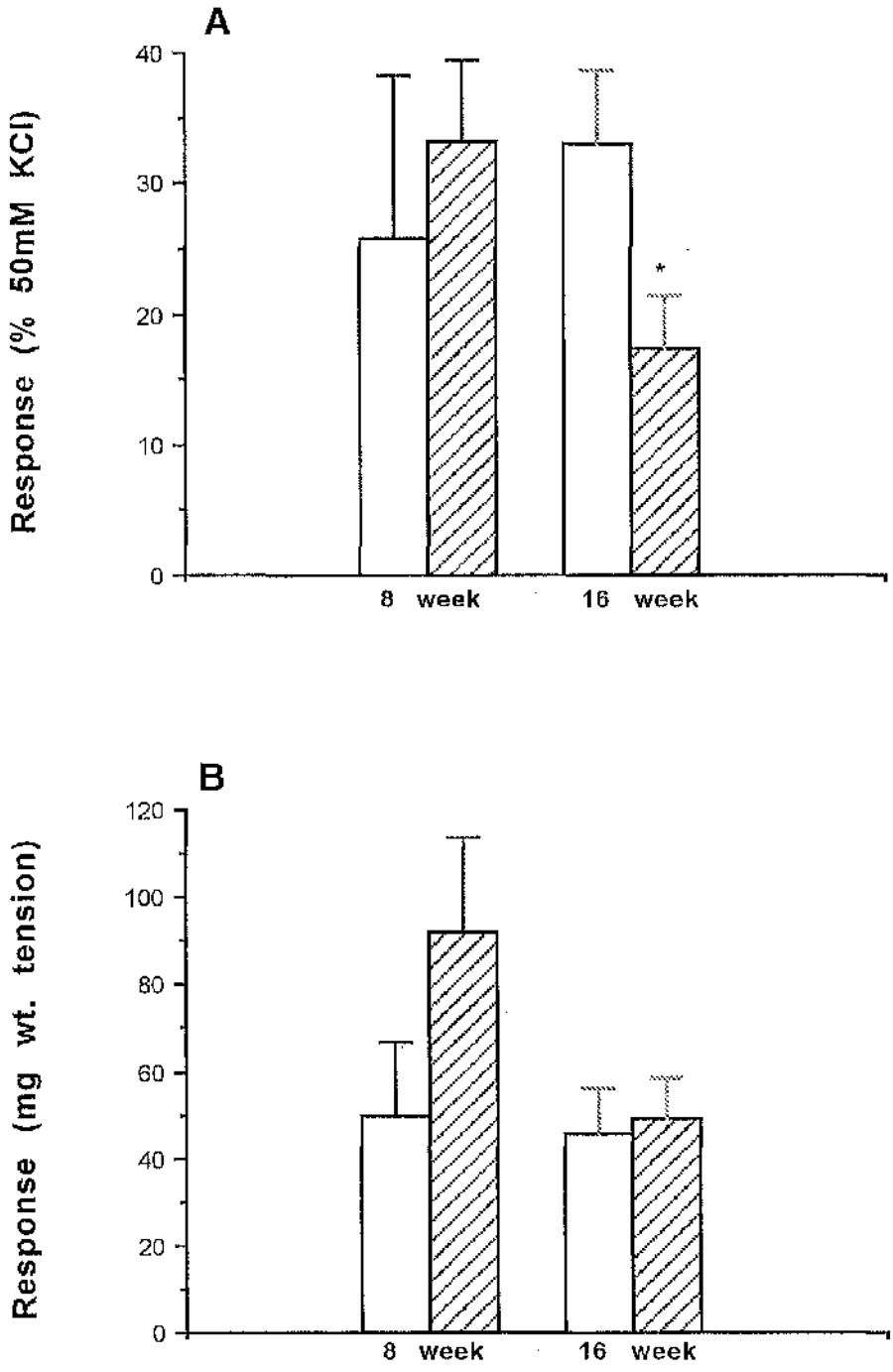


Figure 6.1 Effect of 100μM L-NAME on baseline tension in PRAs from sham-operated (open columns) / coronary-ligated (hatched columns) rabbits from 8 week (n=4/4 / n=10/10) and 16 week (n=8/8 / n=7/7) procedure groups. **A** Data are expressed as a percentage reference contraction to 50mM KCl. **B** Data are expressed as mg weight tension. Each point represents mean±s.e. mean.

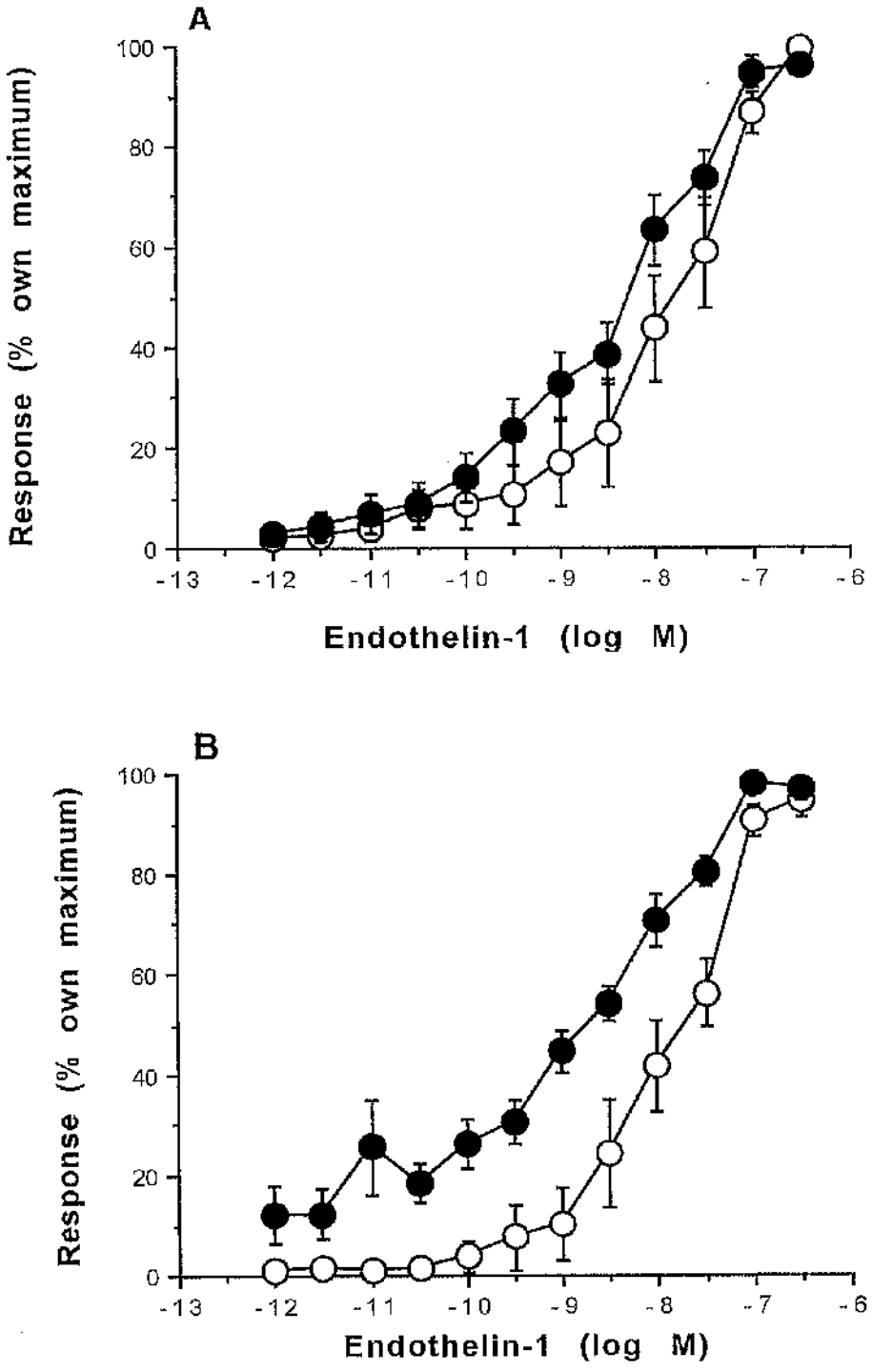


Figure 6.2 Effect of 100μM L-NAME on ET-1 responses in PRAs from 8 week experimental group animals. Data are expressed as a percentage own maximum contraction. **A** sham-operated rabbit PRAs: control (○, n=8/6); +L-NAME (●, n=9/7). **B** coronary-ligated rabbit PRAs: control (○, n=12/7); +L-NAME (●, n=7/6). Each point represents mean ± s.e. mean.

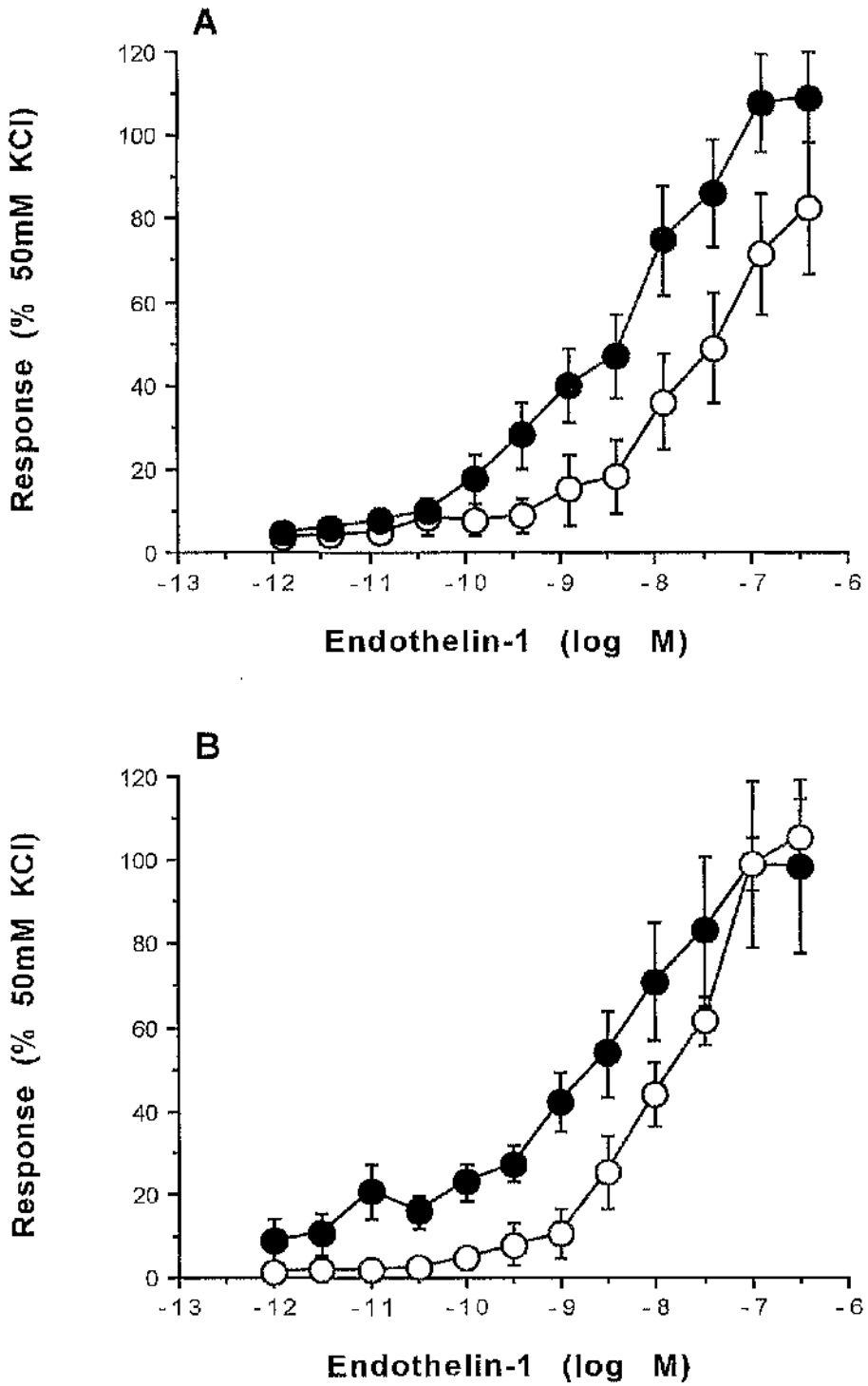


Figure 6.3 Effect of 100μM L-NAME on ET-1 responses in PRAs from 8 week experimental group animals. Data are expressed as a percentage reference contraction to 50mM KCl. **A** sham-operated rabbit PRAs: control (○, n=8/6); +L-NAME (●, n=9/7). **B** coronary-ligated rabbit PRAs: control (○, n=12/7); +L-NAME (●, n=7/6). Each point represents mean ± s.e. mean.

rabbits in this group, a marked potentiation was noted in the ET-1-induced vasoconstriction. In this preparation, L-NAME caused a leftward shift in the entire CCRC, although this effect was more profound over the lower concentrations of ET-1 (figure 6.2B). An ~13 fold and ~8 fold increase was noted in the pEC_{25} and pEC_{50} values, respectively. (table 6.2). The magnitude of the contractile responses were augmented in vessels from both groups. In sham-operated preparations, this was noted particularly to ET-1 concentrations greater than 1nM (figure 6.3A), whilst in PRAs from coronary-ligated rabbits, the enhancement of vasocontractile responses was more pronounced to lower concentration of this peptide (figures 6.3B). However the maximal vasoconstriction was not effected in vessels from either group.

Figures 6.4 and 6.5 show the effect of L-NAME on SXS6c responses in PRAs from the 8 week procedure group. pEC_{10}, pEC_{25} and pEC_{50} values are also summarised in table 6.2. As was noted in the ET-1 response, inhibition of NOS with 100μM L-NAME had no effect on sensitivity of sham-operated rabbit PRAs to SXS6c (figure 6.4A; table 6.2). However the magnitude of the contractile responses to higher SXS6c concentrations were greater in the presence of L-NAME and the maximum response was significantly increased in this preparation (control 67.9±8.2% vs. presence of L-NAME 101.8±13.7%, $P<0.05$; figure 6.5A). In coronary-ligated rabbit PRAs, L-NAME caused a significant potentiation of the responses to SXS6c, causing a leftward shift of the entire CCRC (figure 6.4B). This effect was more pronounced over the lower SXS6c concentrations, such that a pEC_{10} value could not be calculated. An ~20 fold and ~15 fold increase was noted in the pEC_{25} and pEC_{50} values, respectively (table 6.2). The increase in the amplitude of the vasoconstrictions was also more evident at lower SXS6c concentrations. The threshold for contraction increased from 3pM to less than 1pM and the response to 10pM increased 5 fold, however, the maximal contraction to SXS6c was not effected in coronary-ligated rabbit vessels (figure 6.5B). The influence of L-NAME on the magnitude of the SXS6c vasoconstriction was also assessed in terms of the response to 0.1μM ET-1 in the same preparation. Again, a marked increase was noted in the vasoconstriction of PRAs coronary-ligated rabbit to all SXS6c concentration up to

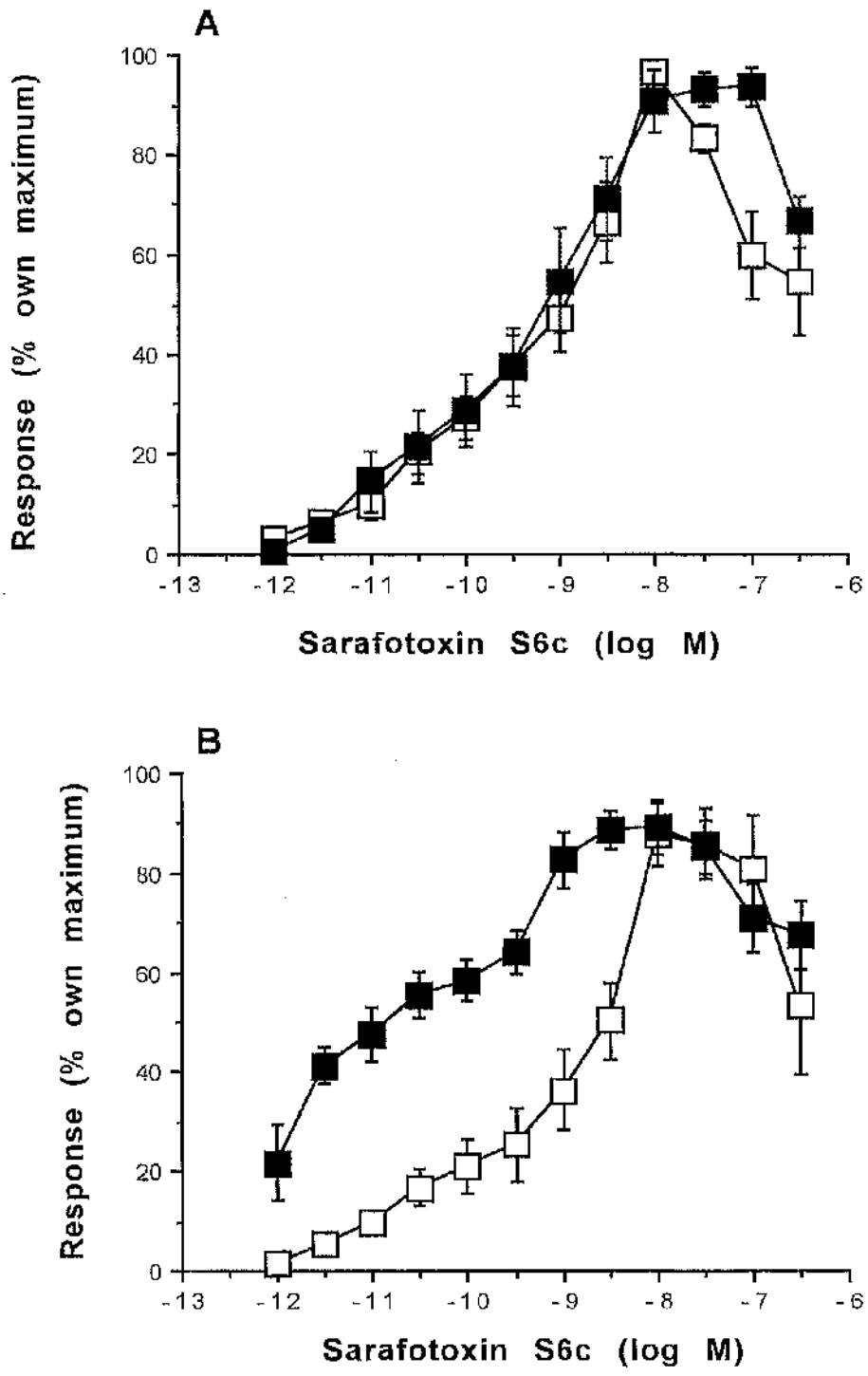


Figure 6.4 Effect of 100μM L-NAME on SXS6c responses in PRAs from 8 week experimental group animals. Data are expressed as a percentage own maximum contraction. **A** sham-operated rabbit PRAs: control (❑, n=10/9); +L-NAME (■, n=5/5). **B** coronary-ligated rabbit PRAs: control (❑, n=11/7); +L-NAME (■, n=8/6). Each point represents mean ± s.e. mean.

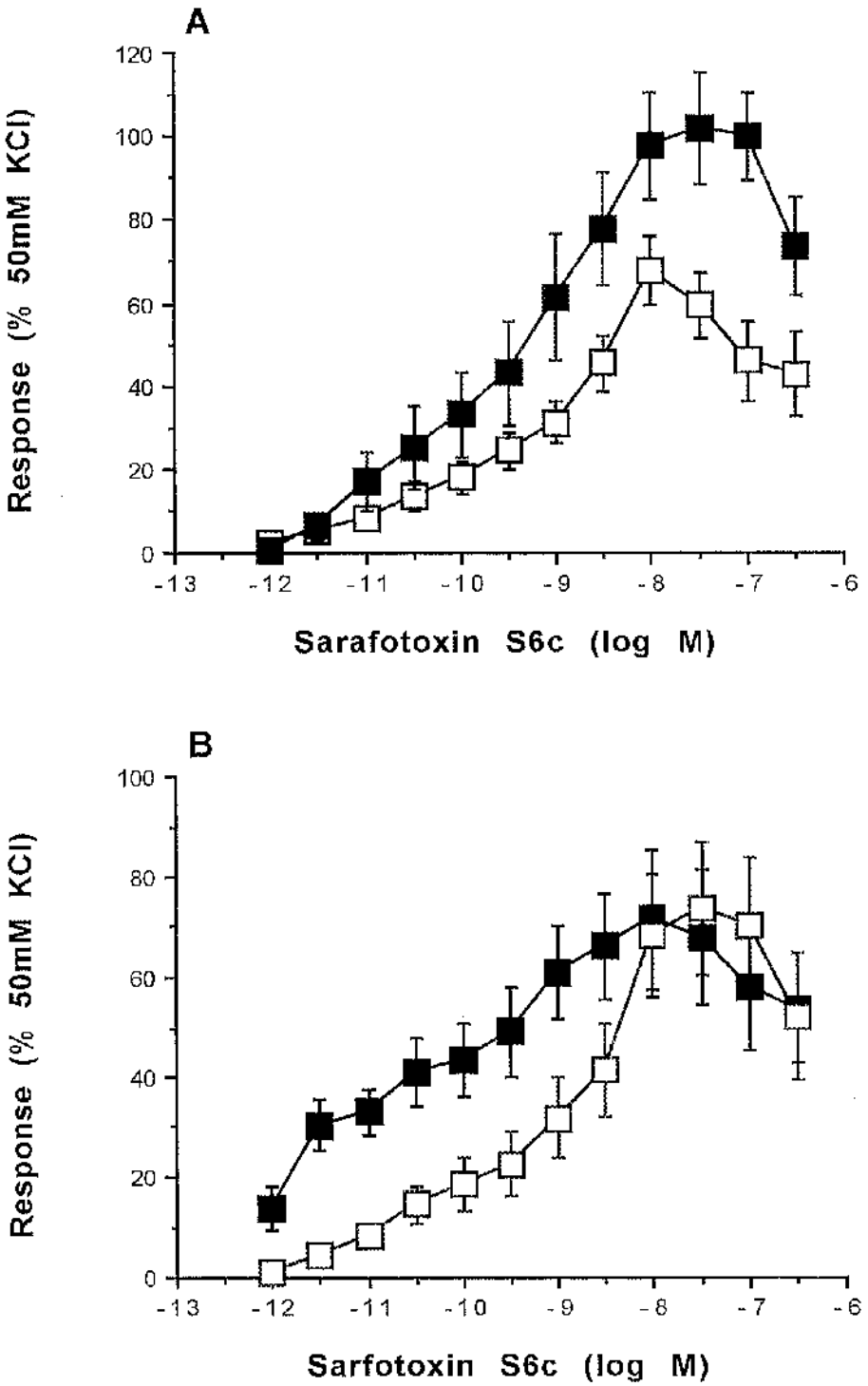


Figure 6.5 Effect of 100μM L-NAME on SXS6c responses in PRAs from 8 week experimental group animals. Data are expressed as a percentage reference contraction to 50mM KCl. **A** sham-operated rabbit PRAs: control (❑, n=10/9); +L-NAME (■, n=5/5). **B** coronary-ligated rabbit PRAs: control (❑, n=11/7); +L-NAME (■, n=8/6). Each point represents mean ± s.e. mean.

30nM (results not shown). The maximum contractile response, in terms of percentage of response to 0.1μM ET-1, tended to be greater in the presence of L-NAME, however this was not significant (sham-operated: 87.2±15.8 % vs. 65.4±5.4%; coronary-ligated: 87.7±9.3% vs. 65.3±8.9%).

	n/n	pEC_{10}	pEC_{25}	pEC_{50}
Sham-operated rabbit PRAs				
ET-1 control	8/6	9.4±0.6	8.6±0.4	7.9±0.3
+100μM L-NAME	9/7	9.5±0.3	9.3±0.3	8.3±0.2
SXS6c control	10/9	10.6±0.3	9.9±0.3	9.0±0.2
+100μM L-NAME	5/5	10.9±0.3	10.1±0.3	9.2±0.3
Coronary-ligated rabbit PRAs				
ET-1 control	12/7	8.9±0.4	8.3±0.2	7.9±0.2
+100μM L-NAME	7/6	10.4±0.3*	9.7±0.3***	8.7±0.2
SXS6c control	11/7	10.4±0.3	9.4±0.3	8.6±0.2
+100μM L-NAME	8/6	nc	11.1±0.1***	10.4±0.3***

Table 6.2 Effect of L-NAME on the sensitivity to ET-1 and SXS6c in PRAs from 8 week sham-operated and coronary ligated rabbit PRAs. Statistical comparisons were made by Students' unpaired t-test: +L-NAME vs. control $*P<0.05$, $**P<0.01$, $***P<0.001$. Values are mean ± SEM. ET-1, endothelin-1; SXS6c, sarafotoxin S6c; L-NAME, N^{ω}-nitro-L-arginine methylester; n/n, number of ring preparations / number of animals; nc, not calculated.

16 week experimental group

Figures 6.6 and 6.7 show the effect of L-NAME on ET-1 responses in PRAs from the 16 week procedure group. pEC_{10}, pEC_{25} and pEC_{50} values are summarised in table 6.3. The sensitivity to ET-1 in either 16 week stock or sham-operated PRAs was not effected by 100μM L-NAME (figure 6.6A and B, table 6.3). However, in vessels from coronary-ligated rabbits in this group, a marked potentiation was noted in the ET-1-induced vasoconstriction. This effect was evident to concentrations of ET-1 greater

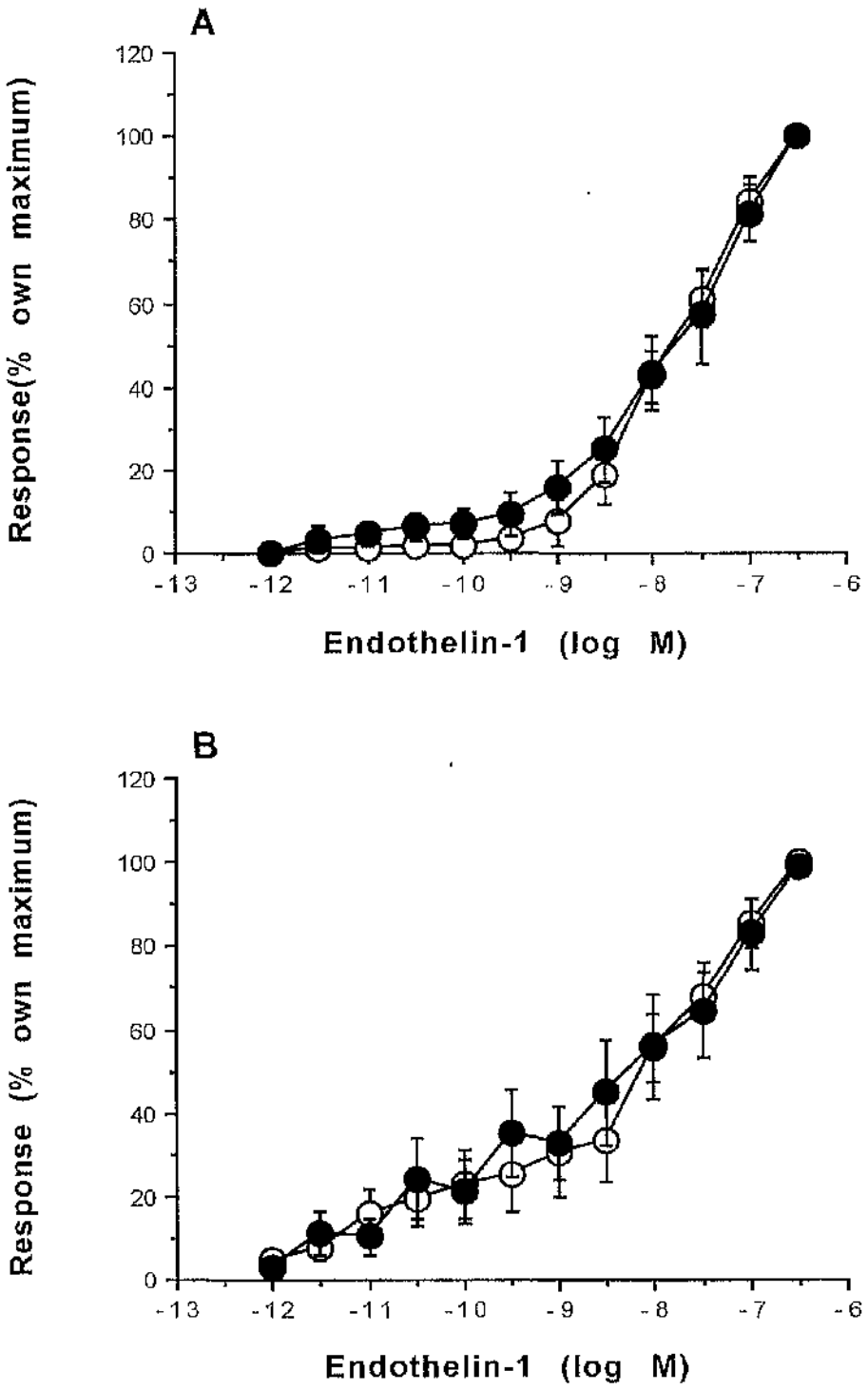


Figure 6.6 Effect of 100μM L-NAME on ET-1 responses in PRAs from 16 week stock and sham-operated rabbits. Data are expressed as a percentage own maximum contraction. **A** stock rabbit PRAs: control (○, n=6/5); +L-NAME (●, n=3/3). **B** sham-operated rabbit PRAs: control (○, n=5/5); +L-NAME (●, n=7/6). Each point represents mean ± s.e. mean.

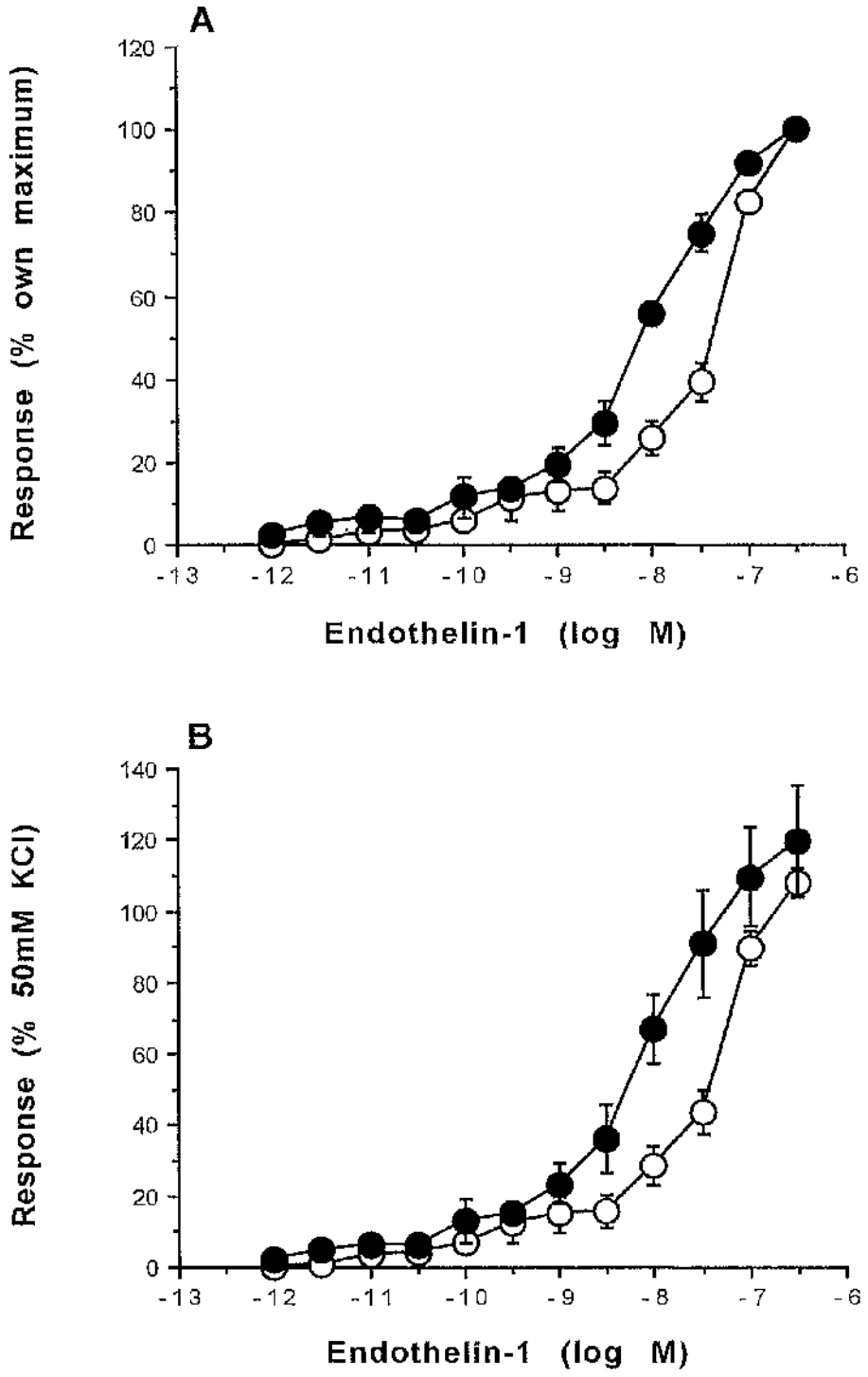


Figure 6.7 Effect of 100μM L-NAME on ET-1 responses in PRAs from 16 week coronary-ligated rabbits. Control (○, n=13/7); +L-NAME (●, n=6/6). **A** Data are expressed as a percentage of their own maximum. **B** Data are expressed as a percentage reference contraction to 50mM KCl. Each point represents mean ± s.e. mean.

than 1nM (figure 6.7A). An ~6 fold increase was noted in the pEC_{25} and pEC_{50} values (table 6.3). This compares with the results in PRAs from 8 week coronary-ligated rabbits where the potentiation effect of L-NAME was most pronounced at lower ET-1 concentrations. Neither the magnitude of the vasoconstrictions nor the maximum response to ET-1 were effected in PRAs from 16 week stock/sham-operated rabbits (not shown). In coronary-ligated rabbit PRAs from this group, the magnitude of the contractile responses in the second component of the CCRC were augmented, however, no significant change occurred in the maximum (Figures 6.7B).

Figures 6.8 and 6.9 show the effect of L-NAME on SXS6c responses in PRAs from the 16 week procedure group. pEC_{10}, pEC_{25} and pEC_{50} values are also summarised in table 6.3. (The effect of L-NAME on SXS6c responses was not examined in vessels from 16 week stock animals). Inhibition of NOS with 100μM L-NAME had no effect on the sensitivity of sham-operated rabbit PRAs to SXS6c (figure 6.8A; table 6.3). In coronary-ligated rabbit PRAs, a leftward shift in the rising component of the SXS6c CCRC was evident (figure 6.8B). This augmentation resulted in a significant 6 fold increase in the pEC_{25} (table 6.3). The potentiating effect was more pronounced at higher SXS6c concentrations, in terms of the amplitude of the contractile response. The threshold for concentration increased from 3pM to less than 1pM and a significant increase was evident in the contractile response to all SXS6c concentration up to 30nM (figure 6.9B). This is in contrast to results in 8 week ligated rabbit arteries, which showed the comparatively greater increase to be most pronounced at lower concentrations of SXS6c. The magnitude of SXS6c-induced vasoconstrictions were not effected in arteries from sham-operated animals and the maximum response was similar (figure 6.9A). Whereas in vessels from LVD rabbits, a significant increase was noted in the maximum response (control 57.5±11.5% vs. 105.6±13.6%, $P<0.05$; figure 6.9B).

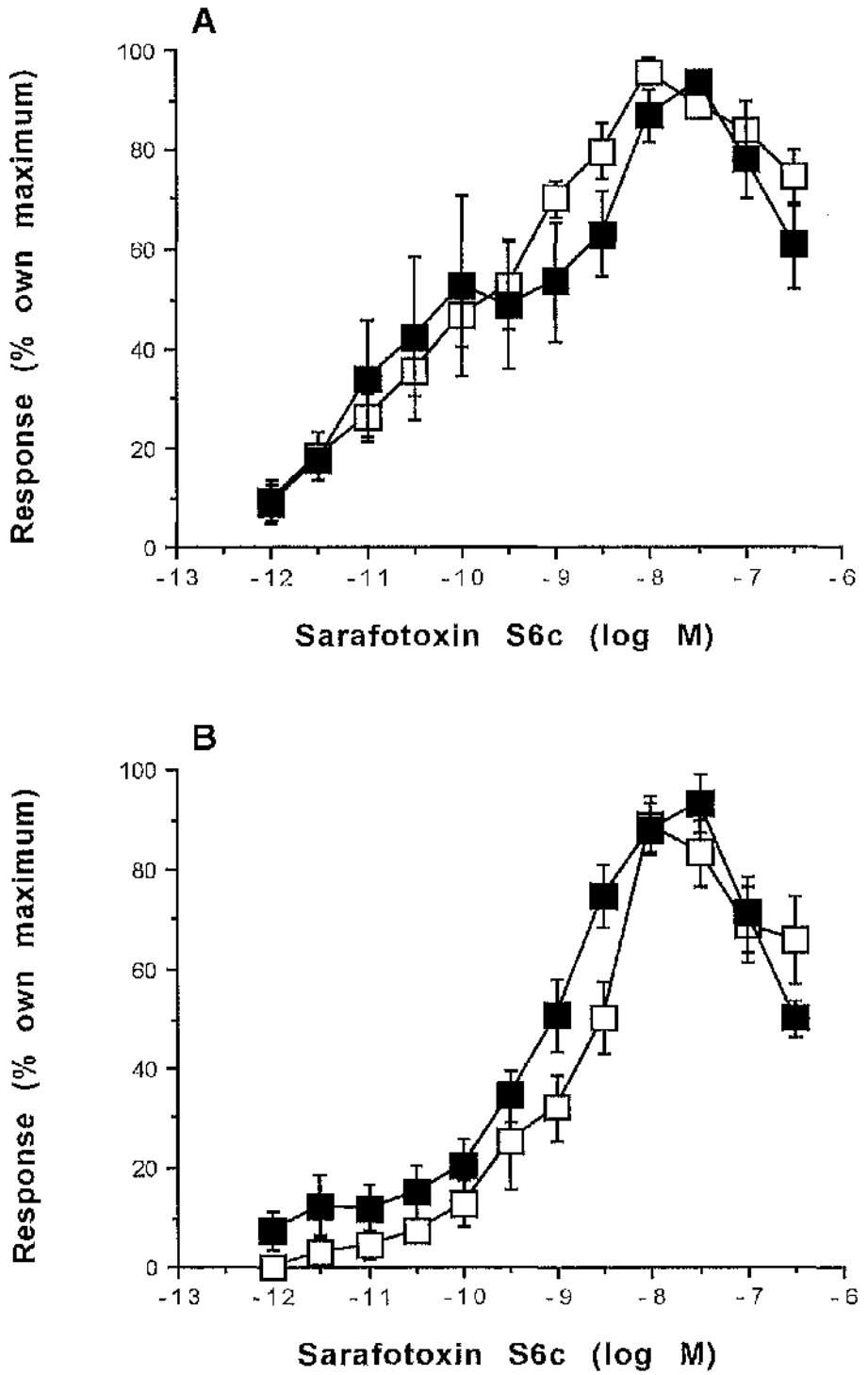


Figure 6.8 Effect of 100μM L-NAME on SXS6c responses in PRAs from 16 week experimental group animals. Data are expressed as a percentage of their own maximum contraction. **A** sham-operated rabbit PRAs: control (❑, n=6/6); +L-NAME (■, n=4/4). **B** coronary-ligated rabbit PRAs: control (❑, n=9/6); +L-NAME (■, n=7/6). Each point represents mean ± s.e. mean.

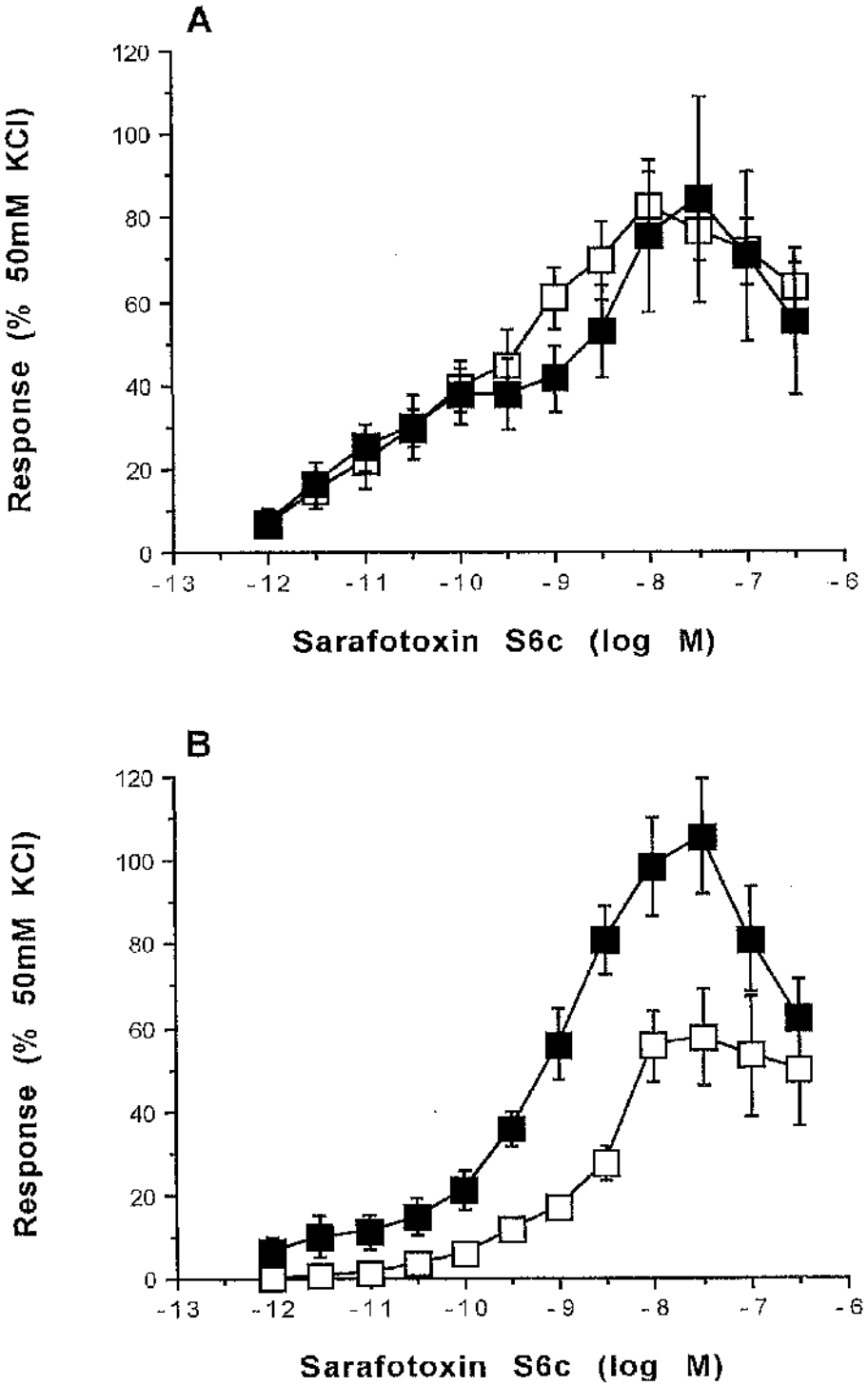


Figure 6.9 Effect of 100μM L-NAME on SXS6c responses in PRAs from 16 week experimental group animals. Data are expressed as a percentage reference contraction to 50mM KCl. **A** sham-operated rabbit PRAs: control (❑, n=6/6); +L-NAME (■, n=4/4). **B** coronary-ligated rabbit PRAs: control (❑, n=9/6); +L-NAME (■, n=7/6). Each point represents mean ± s.e. mean.

	n/n	pEC_{10}	pEC_{25}	pEC_{50}
Stock rabbit PRAs				
ET-1 control	6/5	8.9±0.3	8.4±0.2	7.8±0.2
+100μM L-NAME	3/3	9.5±0.4	8.5±0.3	7.7±0.3
Sham-operated rabbit PRAs				
ET-1 control	5/5	9.7±0.6	10.1±1.0	8.2±0.2
+100μM L-NAME	7/6	9.9±0.6	9.5±0.6	8.5±0.5
SXS6c control	6/6	11.5±0.3	10.8±0.3	9.8±0.2
+100μM L-NAME	4/4	11.5±0.4	10.5±0.5	9.7±0.7
Coronary-ligated rabbit PRAs				
ET-1 control	13/7	9.0±0.3	8.2±0.2	7.5±0.1
+100μM L-NAME	6/6	10.0±0.4*	8.9±0.2*	8.0±0.03**
SXS6c control	9/6	9.9±0.2	9.1±0.2	8.6±0.2
+100μM L-NAME	7/6	9.3±0.3	9.7±0.1*	9.1±0.2

Table 6.3 Effect of L-NAME on the sensitivity to ET-1 and SXS6c in PRAs from 16 week stock/sham-operated and coronary ligated rabbit PRAs. Statistical comparisons were made by Students' unpaired t-test: +L-NAME vs. control *P<0.05, **P<0.01. Values are mean ± SEM. ET-1, endothelin-1; SXS6c, sarafotoxin S6c; L-NAME, N^{ω}-nitro-L-arginine methylester; *n/n*, number of ring preparations / number of animals.

32 week experimental group

Figures 6.10 and 6.11A show the effect of L-NAME on ET-1 responses in PRAs from the 32 week procedure group. pEC_{10}, pEC_{25} and pEC_{50} values are summarised in table 6.4. L-NAME (100μM) had no significant effect on the sensitivity to ET-1 in PRAs from either 32 week stock or coronary-ligated rabbits (figure 6.10A and B, table 6.4). The magnitude of the vasoconstrictions were not effected in vessels from age-matched stock animals (not shown); maximum control response 106.7±9.0% vs. 137.6±41.2%. However, Figure 6.11A shows, in coronary-ligated rabbit PRAs, that the

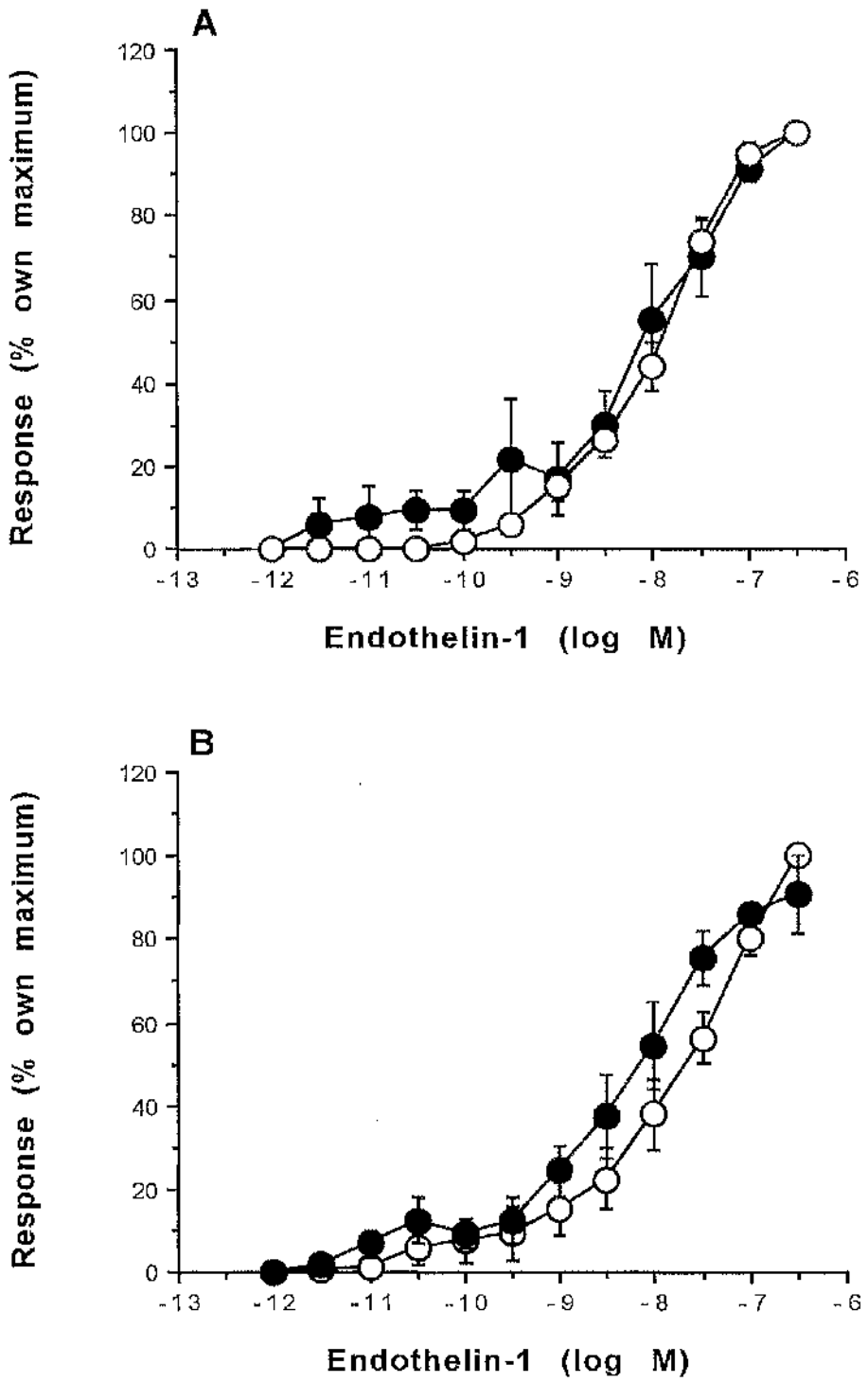


Figure 6.10 Effect of 100μM L-NAME on ET-1 responses in PRAs from 32 week experimental group animals. Data are expressed as a percentage own maximum contraction. **A** stock rabbit PRAs: control (○, n=5/5); +L-NAME (●, n=3/3). **B** coronary-ligated rabbit PRAs: control (○, n=5/5); +L-NAME (●, n=5/5). Each point represents mean ± s.e. mean.

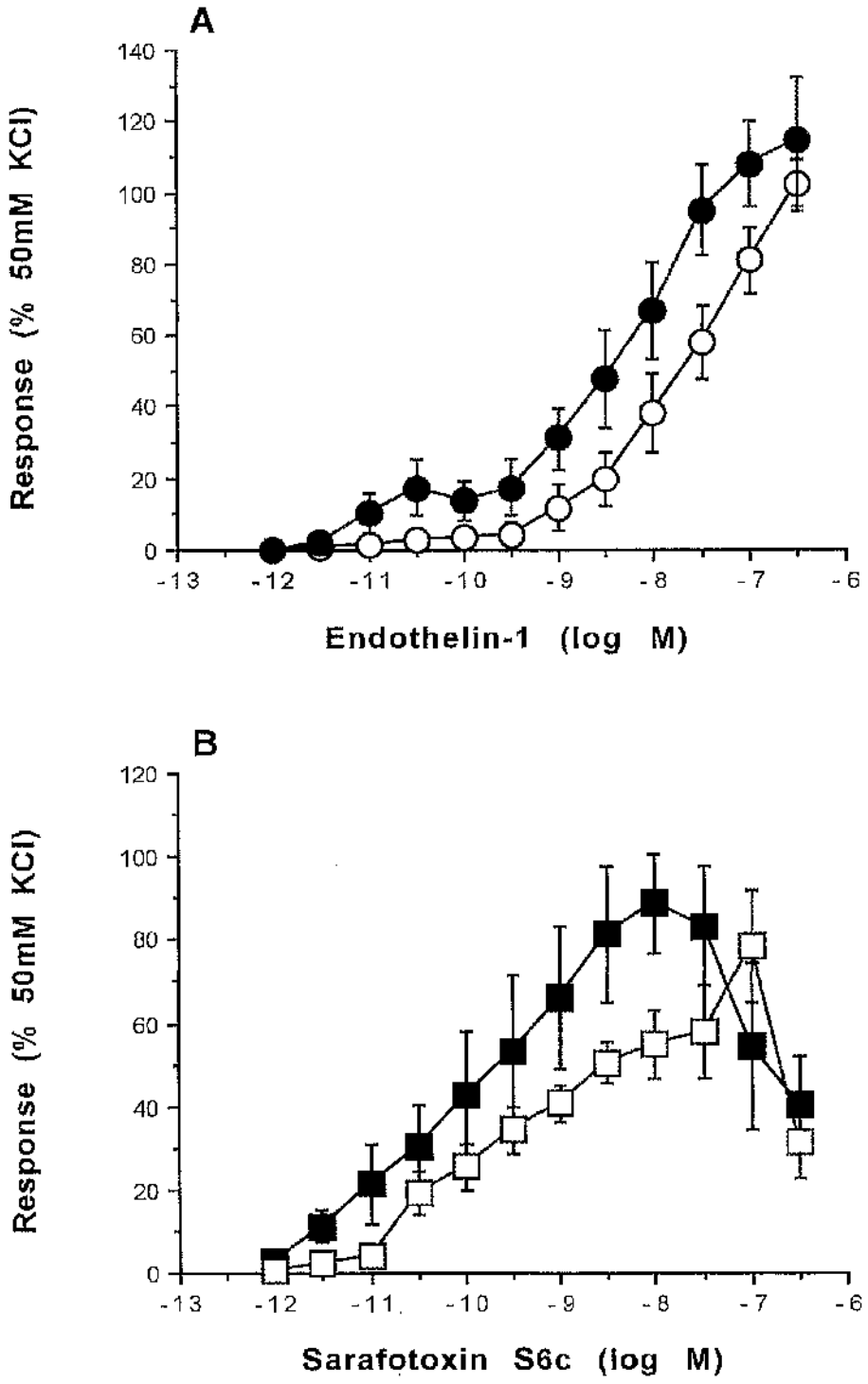


Figure 6.11 Effect of 100μM L-NAME on ET-1 and SXS6c responses in PRAs from 32 week coronary ligated rabbits. Data are expressed as a percentage reference contraction to 50mM KCl. **A** ET-1 control (○, n=5/5); +L-NAME (●, n=5/5). **B** SXS6c control (□, n=6/6); +L-NAME (■, n=5/5). Each point represents mean ± s.e. mean.

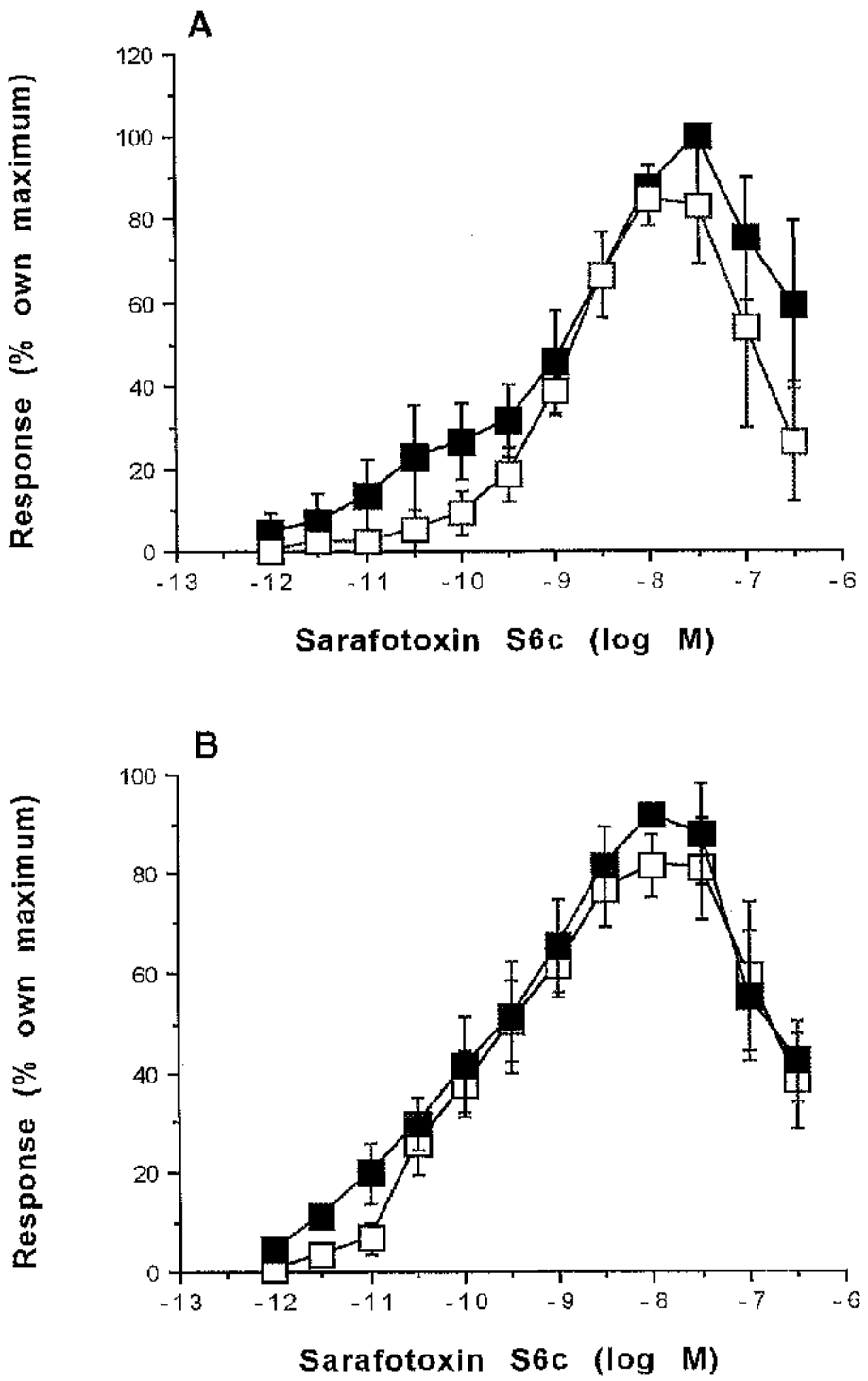


Figure 6.12 Effect of 100μM L-NAME on SXS6c responses in PRAs from 32 week experimental group animals. Data are expressed as a percentage own maximum contraction. **A** stock rabbit PRAs: control (❑, n=5/5); +L-NAME (■, n=3/3). **B** coronary-ligated rabbit PRAs: control (❑, n=6/6); +L-NAME (■, n=5/5). Each point represents mean ± s.e. mean.

amplitude of the ET-1 responses tended to be greater in the presence of L-NAME but the maximum response was not effected.

Figures 6.12A and B and table 6.4 show the effect of L-NAME on the sensitivity of PRAs from the 32 week procedure group. As was noted in the ET-1 response, inhibition of NOS with L-NAME had no significant effect on the sensitivity of stock or coronary-ligated rabbit PRAs to SXS6c (figure 6.12A and B, respectively ; table 6.4). In coronary-ligated rabbit PRAs, an ~ 4 fold increase was noted in the pEC_{10}, however this was not significant (table 6.4). The magnitude of SXS6c-induced vasoconstrictions were not effected in arteries from stock animals (not shown). In vessels from LVD rabbits, the amplitude of the vasoconstrictions tended to be greater in the presence of L-NAME, particularly to higher peptide concentrations however, the maximum response was not significantly increased (control 57.9±10.9% vs. 88.7±12.1%; figure 6.11B). These results compare with those in PRAs from 8 and 16 week coronary-ligated rabbits, where L-NAME was seen to cause a marked potentiation of both ET-1 and SXS6c responses.

	n/n	pEC_{10}	pEC_{25}	pEC_{50}
Stock rabbit PRAs				
ET-1 control	5/5	9.2±0.2	8.5±0.1	7.9±0.1
+100μM L-NAME	3/3	9.5±0.4	8.8±0.3	8.0±0.2
SXS6c control	5/5	10.0±0.3	9.4±0.2	8.7±0.1
+100μM L-NAME	3/3	10.5±0.5	10.0±0.6	8.9±0.3
Coronary-ligated rabbit PRAs				
ET-1 control	5/5	9.3±0.5	8.7±0.4	7.7±0.1
+100μM L-NAME	5/5	9.8±0.4	8.8±0.2	8.1±0.2
SXS6c control	6/6	10.8±0.2	10.3±0.3	9.4±0.2
+100μM L-NAME	5/5	11.2±0.2	10.4±0.3	9.5±0.3

Table 6.4 Effect of L-NAME on the sensitivity to ET-1 and SXS6c in PRAs from 32 week stock and coronary ligated rabbit PRAs. Values are mean ± SEM. ET-1, endothelin-1; SXS6c, sarafotoxin S6c; L-NAME, N^{ω}-nitro-L-arginine methylester; n/n, number of ring preparations / number of animals.

6.4 Discussion

The results of this chapter demonstrated that the NOS inhibitor, L-NAME, potentiates responses to ET-1 and SXS6c in small muscular pulmonary arteries from rabbits with LVD, induced by coronary-artery-ligation, but not from age-matched stock or sham-operated rabbits. However, the extent of this potentiation varied depending on the length of time of the coronary artery ligations. The increase in sensitivity to these peptides was most pronounced in the 8 week procedure group, was still evident in vessels from 16 week coronary-ligated vessels, whereas, in the 32 week procedure group, L-NAME had no significant effect on either ET-1- or SXS6c-induced responses. These results suggest that there is an increase in NO production associated with the development of PHT in these rabbits. This may be basally released NO or NO released by the ET-1 and SXS6c via the endothelial ET_B receptor. I failed to show vasorelaxatory responses to SXS6c in preconstricted rabbit PRAs in the studies of chapter 4 of this thesis. However, there is the possibility that the apparent predominance of contractile ET-receptor subtypes in this preparation may be preventing the exposure of these receptors functionally. The results in PRAs from 8 week coronary-ligated rabbits demonstrated that the profound potentiation of the responses to SXS6c and ET-1 following inhibition of NOS was greatest over the first component of the CCRC. The results of chapter 5 of this thesis provide several points of evidence for an upregulation of endothelial ET_B receptors mediating vasodilation in 8 week coronary-ligated rabbit PRAs, particularly in first component of the CCRC: (1) a potentiating effect of BQ788 and SB209670 on ET-1-induced responses, particularly to lower concentrations of ET-1, was noted in PRAs from 8 week coronary-ligated rabbits; both these antagonists are known to inhibit this ET_B receptor subtype (Douglas, *et al.*, 1995; Ohlstein, *et al.*, 1994a). Also, (2) SB209670 was comparatively less effective in antagonising SXS6c-induced vasoconstrictions in coronary-ligated PRAs compared with the marked inhibition noted in sham-operated PRAs. Finally, (3) the contractile response to ET-3 was significantly reduced in coronary-ligated rabbit PRAs compared with vessels from

control animals and ET-3 is extremely potent at this receptor (Douglas, *et al.*, 1995). Furthermore, in this chapter, L-NAME caused a comparatively greater increase in the contractile response to the ET_B selective agonist SXS6c compared to the non-selective agonist ET-1. The differential effect of L-NAME on SXS6c and ET-1 suggests a heterogeneous population of receptors, differentially influenced by NO. It suggests that SXS6c has a high potency for a particular ET_B-like receptor. A heterogeneous population of receptors in these vessels is also indicated by the results of chapters 4 and 5 of this thesis.

In addition, results from chapter 3 of this thesis demonstrated that the PRAs removed from rabbits with LVD, and increased NOS, demonstrated a decreased sensitivity to sodium nitroprusside (SNP); this is an endothelium-independent relaxatory agent which activates NO directly. In man, an increase in stimulation of NO by long term nitrate administration is also followed by a tolerance to the effects of nitrates (Needleman & Johnson, 1973). MacLean & MacMillan (1993) reported that in rats chronically treated with L-NAME such that endothelial NOS (eNOS) is depleted, the pulmonary arteries were hypersensitive to SNP. Hence there is evidence that when there is an increased stimulation of NO, either by upregulation of eNOS or administration of nitrates, the vascular smooth muscle compensates by a reduction in sensitivity to NO. Whereas the findings of MacLean & MacMillan (1993) suggests that when eNOS is inhibited, the reverse case holds, with the smooth muscle becoming more sensitive to NO.

The nature of the increase in the ET-1- and SXS6c-induced responses produced by NOS inhibition varied in 8 and 16 week coronary-ligated rabbit PRAs. As stated, in preparations from 8 week LVD animals this augmentation was most pronounced to lower concentrations of these peptides; this suggests that this may actually be important in *in vivo* conditions. However in PRAs from 16 week coronary-ligated rabbits, the potentiation of both ET-1 and SXS6c responses occurred to higher agonist concentrations and this was further demonstrated by the significant increase in the maximum vasoconstriction to SXS6c. This suggests that the possible upregulation of

endothelial ET_B receptors, activated by lower concentrations of ET-1 and SXS6c, in vessels from 8 week LVD animals (which is suggested by the results of this chapter and those of chapter 5), is no longer present in 16 week LVD rabbits. From these findings, one could hypothesis that an increase in endothelial ET_B receptors mediating vasodilation is an early compensatory mechanism which occurs in early stages of increased pulmonary pressure induced by LVD. An ET-1 limiting process on vasoconstriction mediated by ETB receptors has also been suggested in the systemic circulation (Allcock & Warner, 1995). In the cohort of animals used in this chapter, the ejection fraction of the 16 week coronary-ligated rabbits was significantly lower than the 8 week LVD animals but this attenuation did not progress to the 32 week experimental groups. Thus it appears unlikely that the rabbits ligated for a longer duration represent a more severe LVD condition.

Previous investigators have reported an influence of endothelial derived factors, such as NO, on ET receptor-mediated responses in various arterial preparation from several species. For example, in the isolated canine liver arterial circuit pressor responses to ET-1 and SXS6b were shown to be potentiated by infusion of L-NAME (Faro., *et al.*, 1995). Lang and Lewis (1991) reported that NO inhibited the ET-1-induced increase in protein kinase C in rat aorta. Removal of the endothelium was shown to increase the maximum effectiveness of both ET-1 and ET-3 on rat pulmonary artery and thoracic aorta rings (Rodman, *et al.*, 1989). Finally, Zellers and co-workers (1994) reported that ET-1-induced vasoconstrictions in small porcine pulmonary arteries and veins were augmented by L-NNA, an L-arginine antagonist, and this was enhancement was greater in veins. In addition, my own study showed maximal contractile responses to SXS6c to be markedly enhanced in the presence L-NAME in "control" PRAs from 8 week procedure animals, thus indicating the involvement of NO in responses to SXS6c in this preparation. Also, in chapter 8 of this thesis, I showed that L-NAME evokes a marked potentiation of ET-1 and SXS6c responses in neonatal rabbit PRAs.

The "drop off" in tension which occurred at higher concentrations in the SXS6c-induced vasoconstriction, was still evident in the presence of L-NAME, thus suggesting that NO is not involved in its occurrence. The most plausible explanation for this is receptor desensitisation. It has previously been shown that the ET_B receptor is strongly tachyphylactic (Henry, 1993). In another preparation in which responses were concluded to be mediated via ET_B receptors, rabbit saphenous vein, SXS6c evoked a vasoconstrictor response, however a second application of this peptide was ineffective at inducing contraction (Sudjarwo, *et al.*, 1994). In addition, cumulative addition of SXS6c also resulted in a bell-shaped concentration response curve. The authors concluded that high concentrations of SXS6c selectively activated the ET_B receptor and then desensitised this receptor, resulting in a transient contraction or a bell-shaped CCRC. Indeed, in my own studies, I was unable to expose the same preparation to more than one single application of ET-receptor agonists, due to the lack of response on a second application and also the persistence of the contractile response, even after numerous washes, when the vessel had been constricted with higher ET-1 concentrations.

In this chapter, I also examined the possible influence of NO on endogenous tone in rabbit PRAs from the various experimental groups, by examining the effects of L-NAME alone on baseline tension. L-NAME induced a vasoconstriction in only a proportion of the PRAs examined. In the 8 week experimental group, a greater number of PRAs from coronary-ligated rabbits exhibited a contractile response compared with age-matched sham-operated rabbit vessels, whereas approximately half of the total number of 16 week group vessels from both LVD and control rabbits contracted in response to L-NAME alone. However, none of the vessels from the 32 week procedure group exhibited a response. The increase in vascular tone following inhibition of NOS tended to be greater in 8 week coronary-ligated PRAs compared with control vessels in the same group however, due to variability in the amplitude of the response, this increase was not significant. Whereas in vessels from 16 week procedure animals, the amplitude of the increase in vascular tone was reduced in vessels from LVD rabbits compared with age-matched sham-operated preparations. The possible explanations for

these findings are that either NO is basally released from PRAs and this is altered in coronary-ligated PRAs or the inherent tone in these vessels is altered in LVD animals. However, SNP alone was shown to have no effect in baseline tension in PRAs from either 8 week sham-operated or coronary ligated rabbits, suggesting a lack of endogenous tone in these vessels. Yet the vasoconstrictions to L-NAME may be due to an "uncovering" of the inherent tone in these PRAs. I did not examine the influence of SNP in vessels from 16 and 32 week experimental group animals, therefore I am unable to comment on the possible existence of inherent tone in these vessels. Sheer stress has been shown to stimulate NO production (Tesfamariam & Cohen, 1988). However, all the vessels in this study were set up at similar transmural pressures, thus the differences in the responses to L-NAME alone cannot be accounted for by such an effect. The phenomenon of endogenous tone is not often observed in isolated vascular preparations, particularly in pulmonary vessels since these are relatively relaxed *in situ* (see chapter 1). In the isolated rat lung, Raffestin *et al.* (1991) demonstrated that baseline perfusion pressure was not increased in response to several different EDRF inhibitors, including L-NMMA which also inhibits synthesis of NO. Furthermore, these investigators also demonstrated a potentiation of the increase in pulmonary artery pressure induced by ET-1 in the presence of these inhibitors. From these two findings, the authors concluded that the potentiation of the ET-1 response was due to an inhibition of ET-1-induced EDRF release from the lung, rather than a decrease of basal NO activity. However, previous investigators have reported alterations in basal NO and/or inherent tone in the pulmonary vasculature in a pulmonary hypertensive state. In chronic hypoxic rat models of pulmonary hypertension, MacLean *et al.* (1995) demonstrated that there is an increase in endogenous vascular tone in large pulmonary and PRAs from pulmonary hypertensive rats compared with controls, and this was shown to be normally attenuated by NO production. Barer and co-workers (1993) showed that in isolated blood-perfused lungs of rats, L-NAME induced an increase in pulmonary perfusion pressure in chronic hypoxic rats but not in controls. A dissociation between basal and stimulated NO release has also been suggested to play a pathophysiological role in the early stages of heart

failure. In a rat model of heart failure, Teerlink *et al* (1994) showed that ACh-induced vasorelaxations were similar in isolated aorta from control and heart failure rats, thus suggesting that stimulated NO was not altered. However, the effects of L-NAME and measurements of basal cGMP indicated that basal NO was decreased in heart failure rats.

In chapter 3 of this thesis, I showed that endothelium-dependant relaxations to various agents, including acetylcholine (ACh), could not be demonstrated in preconstricted adult PRAs from either stock, sham-operated or coronary-ligated rabbits. Furthermore, the ineffectiveness of ACh appears to be an age-related phenomenon in this preparation as I demonstrated vasorelaxations to this agent in fetal and neonatal rabbit PRAs (chapter 8). However, studies of other investigators show that agonist-induced NO release is diminished in pathophysiological states. The ability of ACh to relax preconstricted pulmonary arteries was shown to be reduced in vessels from chronic hypoxic rats compared with those from control animals (Adnot, *et al.*, 1991; MacLean, *et al.*, 1995). A decrease in ACh-induced NO release has also been reported in human pulmonary arteries from patients with chronic obstructive lung disease (Dinh-Xuan, *et al.*, 1993). The results of these previous studies suggest that agonist-induced release of NO is diminished in PHT, whereas the results of this study indicate an enhancement of basal NO. This difference in findings may be due to the causative factor of the pulmonary hypertensive state. In the aforementioned studies, PHT was induced by an hypoxic insult and hypoxia has been shown to reduce NO release (Johns, *et al.*, 1989; Warren, *et al.*, 1989). However, the initial change in the pulmonary circulation secondary to left-sided cardiac disease is a passive response to a rise in the pulmonary venous (left atrial) pressure. Thus hypoxia does not play a major role in the aetiology of the PHT model examined in this study.

The results of this chapter are compatible with the results of other studies which have demonstrated an increase in NO associated with PHT. For example, there is evidence that NOS may be upregulated in patients with heart failure and inhibition of NOS increased pulmonary vascular resistance in these patients (Habib, *et al.*, 1994).

Pulmonary NO production has also been shown to increase from the lungs of chronically hypoxic rats and there is evidence that inducible and endothelial NOS is upregulated in the pulmonary resistance arteries from these pulmonary hypertensive rats (Isaacson, *et al.*, 1994; Xue, *et al.*, 1994). In a chronic hypoxic rat model of PHT, MacLean *et al* (1995) showed that L-NAME increased the sensitivity to ET-1 in main pulmonary arteries from chronic hypoxic rats but not in control vessels.

Similar findings have also been reported in other cardiovascular disease states, involving the systemic circulation. Intra-arterial infusions of the inhibitor of NO formation, L-NMMA, in human forearm circulation was reported to cause greater vasoconstriction in patients with congestive heart failure than in healthy controls (Drexler, *et al.*, 1992). Noll and co-workers (1994) demonstrated in the isolated aorta from cardiomyopathic syrian hamsters with pulmonary congestion, that contractions to noradrenaline were enhanced in the presence of L-NAME and maximum vasorelaxations to ACh were more pronounced compared to control aorta responses. In addition, low concentrations of ET-1 were shown to cause stronger contractions in aorta from cardiomyopathic hamsters compared with controls, suggesting that this could contribute to vasoconstriction in heart failure. The authors concluded that the increased agonist-induced release of NO may be a compensatory mechanism in this model.

In conclusion, the results of this study suggest that in rabbit PRAs, secondary release of NO may oppose the direct constrictor effect of ET-1 and SXS6c. Inhibition of NOS caused a significant potentiation of ET-receptor mediated responses in PRAs from rabbits with PHT secondary to LVD, which was induced by 8 weeks and 16 weeks of coronary artery ligation. However, this was not evident in PRAs from 32 week LVD animals; these animals are more likely to represent survivors in this experimental group rather than a more severe form of LVD. The increase in basal and/or agonist stimulated NO production may be an early compensatory mechanism in response to the early elevation in pulmonary pressure with LVD in order to maintain responses to ET-1 constant.

Chapter 7

Effects of ET-1 on [^{125}I]ET-1 binding and intracellular cyclic nucleotide levels in rabbit pulmonary arteries

7.1 Introduction

There is unequivocal evidence from pharmacological, biochemical and molecular biological studies that the diverse effects of endothelins (ETs) are mediated by distinct receptor subtypes (Yanagisawa & Masaki, 1989; Sakurai, *et al.*, 1992b). At present the existence of two ET-receptor subtypes has been confirmed (see section 1.4.5). To briefly recap, the order of affinity of the ETs for the first receptor subtype, designated ET_A, is ET-1(= ET-2)>ET-3. The second receptor subtype, designated ET_B, has equal affinity for all three ETs and for sarafotoxins. On the basis of functional studies, it has been proposed that multiple ET_B receptors exist (Warner, *et al.*, 1993a; Douglas, *et al.*, 1994). ET_{B1}-like and ET_{B2}-like are distinguished by their anatomic location and function; the former located on the endothelium, mediating vasodilation, whereas the latter is located on the vascular smooth muscle mediating vasoconstriction. ET receptor subtypes have also been distinguished by their pharmacological profiles, in terms of sensitivity to various antagonists. In addition, there is also evidence for a third type, designated ET_C, which is selective for ET-3 (Martin, *et al.*, 1990; Samson, *et al.*, 1990; Douglas, *et al.*, 1995).

In the studies of chapters 4 and 5 of this thesis, ET-1 was shown to evoke biphasic vasoconstrictions over a broad concentration range in small rabbit muscular pulmonary arteries. This comprised of a "shallow component" at lower concentrations of ET-1 and a steep rising component at ET-1 concentrations above 1nM. Furthermore, these components were differentially influenced by various ET-receptor antagonists. These findings were suggestive of negative cooperativity, i.e. an interaction with multiple sites, in this preparation. The possible existence of a heterogeneous population of ET receptors has been previously reported, including in the rabbit larger pulmonary artery and trachea (Hay, *et al.*, 1996; Yoneyama, *et al.*, 1995). However this interpretation of my data remains speculative. Since the first component of the ET-1 cumulative concentration response curve was less than 30% of the maximum, curve fitting analysis for a biphasic response was not possible and so the theory could not be

mathematically. In this chapter, I describe investigation into the nature of ET-1 induced response in rabbit pulmonary arteries by examination of competition radioligand binding studies with ET-1 in rabbit pulmonary artery membrane preparations.

In this chapter, I also address regulation of cyclic nucleotide levels. Regulation of intracellular cyclic nucleotide levels is one of the important mechanisms in the regulation of pulmonary vascular tone (see section 1.1.4.4). Exogenous cyclic nucleotides, guanosine 3':5'-cyclic monophosphate (cGMP) and adenosine 3':5'- cyclic monophosphate (cAMP) themselves, are potent pulmonary vasodilators (Haynes, *et al.* 1992; McMahon, *et al.*, 1992; 1993). cGMP is the key second messenger of nitric oxide (NO)-induced pulmonary vasodilation. Pussard *et al* (1995) demonstrated that cGMP levels were significantly reduced in human pulmonary vessels treated with the NO synthase inhibitor N^{ω}-nitro-L-arginine methylester (L-NAME) and indomethacin, whereas an elevation was shown in SNP treated vessels. The results of chapter 6 and 8 demonstrated the involvement of NO in ET-receptor mediated responses in PRAs from neonatal and adult rabbits. In particular L-NAME, an agent which acts to decrease intracellular concentrations of cGMP, was shown to markedly potentiate ET-receptor mediated vasoconstrictions in rabbit PRAs from coronary artery ligated animals but not sham-operated rabbits. This finding indicated an increase in basal and/or agonist induced NO in vessels from LVD animals.

It has been reported that the expression of ET receptors is regulated by various agents, including cAMP (Nambi, *et al.*, 1992; Kanno, *et al.*, 1993). Dibutyryl cAMP, a cell-permeable cAMP analogue, was shown to induce an upregulation of ET_B receptor mRNA in primary rat cultured astrocytes (Hamma, *et al.*, 1992), whilst a down regulation was shown in ROS 17/2 rat osteosarcoma cells (Sakurai, *et al.*, 1992a). cAMP plays a central role in the vasoactive responses to various agents (Exton, 1985). Sumatriptan (GR43175) is a selective 5-hydroxytryptamine $1_{B/D}$ ($5\text{-}HT_{1B/D}$) -like receptor agonist which is also believed to evoke vascular smooth muscle contraction through inhibition of adenylate cyclase and therefore a decrease in intracellular concentrations of cAMP ($[cAMP]_i$) (Humphrey, *et al.*, 1988; Sumner & Humphrey,

1990). In the studies of chapter 3 of this thesis, maximal vasoconstrictions to sumatriptan were significantly reduced in PRAs from 8 week LVD rabbits compared with response of corresponding arteries from sham-operated rabbits. Furthermore, the responses to 5HT were shown to be markedly decreased in PRAs from 8 and 32 week coronary-ligated rabbits compared with vessels from age matched stock/sham-operated animals. Both of these cyclic nucleotides, cAMP and cGMP, have been reported to be involved in the second messenger systems of ET-receptor mediated responses. For example, Pussard *et al.* (1995) reported at ET-1 markedly reduced the cGMP increase in SNP-stimulated human pulmonary arteries. This previous finding suggested that ET-1 may play a role in the control of muscle tone in the human pulmonary vascular bed by modifying cGMP levels associated with vasorelaxant agonist stimulation.

In this present study, I also examined the effect of ET-1 on intracellular concentrations of cGMP and cAMP within pulmonary arteries from 8 week sham-operated and coronary-ligated rabbits. Forskolin was used to activate adenylate cyclase directly (Koch, 1982) and the effect of ET-1 on forskolin-induced levels was investigated also. Ignarro *et al.* (1987) demonstrated regional variation in cGMP levels in different sized bovine pulmonary arteries. Similarly, intracellular levels of cAMP and cGMP, and the effect of chronic hypoxia on these, were shown to vary depending on size of pulmonary arteries in the rat (MacLean, *et al.*, 1996b). Therefore in this present study I measured the intracellular level of these cyclic nucleotides in vessels of differing sizes; namely main intrapulmonary artery and pulmonary resistance arteries.

7.2 Methods

Radioligand binding

Membranes from pulmonary arteries, dissected from the main intrapulmonary artery to those of diameter of ~100μm, of the lungs from 8 week sham-operated rabbits were prepared as described in section 2.4.2 of chapter 2. Briefly, membrane suspensions

were prepared from the pulmonary arteries from 3-4 lungs and stored at -80°C for use in competition assays and protein measurement. Protein content was determined by the assay described in section 2.4.3, using bovine serum albumin as a standard.

The protocol for competition assays with unlabelled ET-1 and [^{125}I]-ET-1 is also explained fully in chapter 2, section 2.4.4. Briefly, assay tubes contained final concentration of 2 pM [^{125}I]-ET-1) and appropriate volume and concentration of unlabelled ET-1 (0.01pM-30nM). Final volume of each tube was 250µl, made up with assay buffer. The binding reaction was initiated by the addition of the membrane preparation (~10µg protein / tube). After a 2 hour incubation at 22°C, separation of bound from free ligand was carried out by rapid vacuum filtration through Whatman GF-B filters. Filter were washed 4 times with assay buffer and radioactivity associated with the filter was determined with a Gamma counter. Nonspecific binding was determined simultaneously in the presence of 200 nM unlabelled ET-1 and specific binding was defined as total binding minus non-specific binding.

Assay of intracellular cyclic nucleotide levels

Lungs were obtained from the rabbit coronary ligation model of left ventricular dysfunction which has been extensively characterised (Pye, *et al.*, 1996) and is described in detail in section 2.1.1 of this thesis. 8 weeks following sham-operation or coronary artery-ligation rabbits were sacrificed and main intrapulmonary arteries and small muscular pulmonary arteries (pulmonary resistance arteries (PRAs)) were dissected out (see section 2.2.3.1). Tissue samples were processed and assayed to measure intracellular levels of cAMP using modified binding protein technique of Brown *et al.* (1972). [cAMP]i was measured under several different conditions: (1) basal, and in the presence of (2) 0.1µM ET-1 or (3) 10µM forskolin or (4) both simultaneously. Tissue samples were also processed and assayed to measure intracellular levels of cGMP levels using modified radioimmunoassay technique of Cailla *et al.* (1976). [cGMP]i was measured under (1) basal conditions and (2) in the presence of 0.1µM ET-1. In both

cyclic nucleotide assays, all incubations were performed in the presence of 1mM isobutylmethylxanthine (IBMX), a non-selective phosphodiesterase inhibitor. A detailed description of the tissue preparation and assays is given in the methods sections 2.3.4 and 2.3.5 of chapter 2 of this thesis.

Data analysis

Competition binding data was analysed by non-linear least-square curve fitting, for a one-site or two-site model, using the Graphpad Prism Program. This provided best fit for a two-site model and K_i values for each site were calculated using IC_{50} values provided by the program and the Cheng Prussof equation (Cheng & Prussof, 1973). Results are expressed graphically as percentage of the specific binding of the maximum and each point represents mean ± SEM. n= number of animals.

Results for biochemical assays for the measurement of intracellular cyclic nucleotide levels are expressed graphically as pmol cAMP/ mg tissue or pmol cGMP/ mg tissue. Statistical comparison of the mean groups of data were made by ANOVA followed by Tukeys post test; $P<0.05$ was considered statistically significant. Throughout data are expressed as mean ±SEM and n=number of animals.

7.3 Results

Competition assay

The specific binding of [^{125}I]ET-1 to rabbit pulmonary artery membranes was completely inhibited by unlabelled ET-1 in a biphasic manner; this is shown in figure 7.1. Two components were evident; an ultra-high affinity ET binding site at unlabelled ET-1 concentrations below 1pM and a second high affinity site within the range of 1pM to 30nM unlabelled ET-1. Curve fitting analysis using the Graphpad Prism program revealed a best fit for a two site model. K_i values were 6.43×10^{-14}M for the ultra high

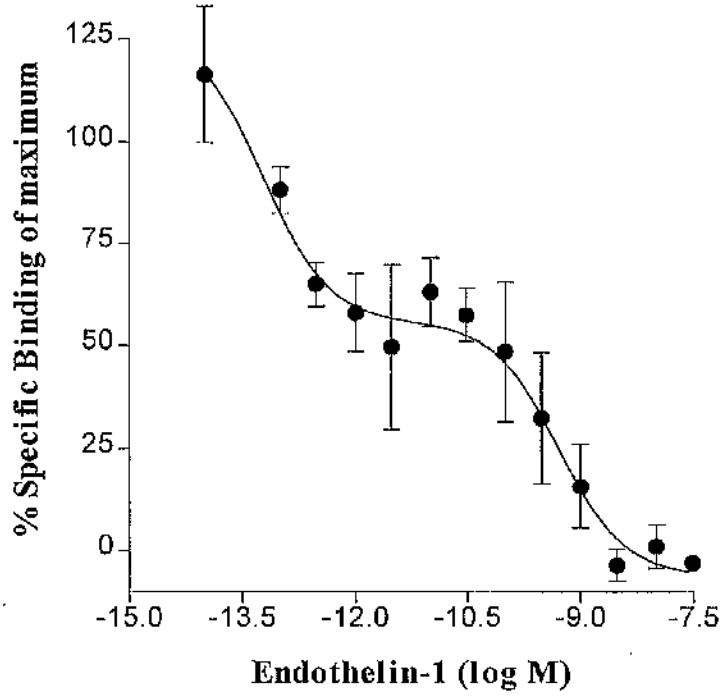


Figure 7.1

Inhibitory effect of unlabelled ET-1on the specific binding of [125ET-1] to rabbit intrapulmonary artery membranes. Results are expressed as a percentage of specific binding of maximum. Each point represents the mean+/- SEM of five experiments.

affinity first component and 4.83 ± 10^{-10}M for the high affinity second component. B_{max} values of 48.84 fmol/mg of protein and 19.34 fmol/mg of protein were estimated for site one and site two, respectively. Thus, approximately 70% of the ET-receptor density was represented by the ultra high affinity site.

Intracellular levels of cyclic AMP

The effect of 0.1μM ET-1 on basal levels of $[cAMP]_i$ in main intrapulmonary arteries (MIPAs) and pulmonary resistance arteries (PRAs) from sham-operated and coronary-ligated rabbits are shown in figures 7.2A and 7.2B, respectively. In MIPAs from both experimental groups, ET-1 did not significantly alter intracellular cAMP levels from basal levels (figure 7.2A). In PRAs from both groups of rabbits, ET-1 consistently decreased $[cAMP]_i$ but again this failed to reach statistical significance (figure 7.2B).

Forskolin (10μM) produced a marked increase in intracellular cAMP levels in all arteries from both sham-operated and coronary-ligated rabbits. In MIPAs from sham-operated rabbits, forskolin increased $[cAMP]_i$ from 0.38±0.1 to 17.47±6.4 pmol/mg tissue ($P<0.05$); in the same vessels from coronary-ligated rabbits, this increase was from 0.97±0.3 to 25.42±6.8 pmol/mg ($P<0.001$). Whilst in PRAs, forskolin increased $[cAMP]_i$ from 0.85±0.4 to 27.72±12.3 pmol/mg ($P<0.05$) and from 2.9±1.2 to 34.8±9.6 pmol/mg ($P<0.01$) in sham-operated and coronary-ligated preparations, respectively. Thus, the stimulatory effect of forskolin was greater in all arteries from coronary-ligated compared to sham-operated animals.

The effects of 0.1μM ET-1 on 10μM forskolin-stimulated intracellular cAMP levels in MIPAs and PRAs from sham-operated and coronary-ligated rabbits are shown in figures 7.3A and 7.3B, respectively. As can be seen, the combined presence of ET-1 and forskolin in the incubation solution, resulted in a similar reduction in forskolin-stimulated $[cAMP]_i$ in MIPAs from sham-operated (52.6±12.2% forskolin plus ET-1 vs. 100% for paired forskolin alone) and coronary-ligated rabbits (44.6±93.9%; figure

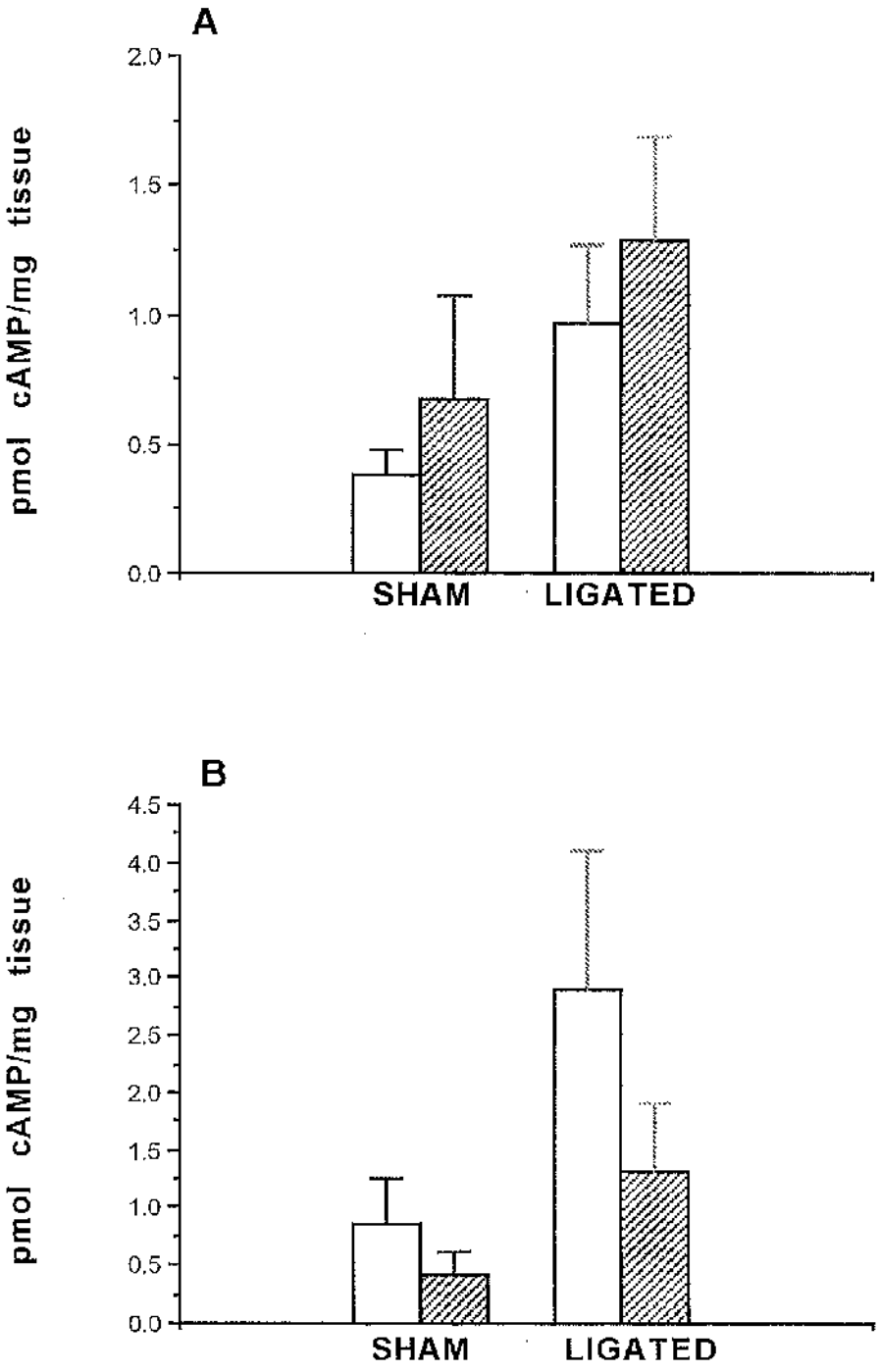


Figure 7.2 Effect of ET-1 (hatched columns) on control (open columns) intracellular cAMP levels in pulmonary arteries from sham-operated (n=6) and coronary artery-ligated rabbits (n=8). Results are expressed as pmol cAMP/mg tissue. Levels in (**A**) main intrapulmonary arteries and (**B**) pulmonary resistance arteries. Each column represents the mean ±SEM.

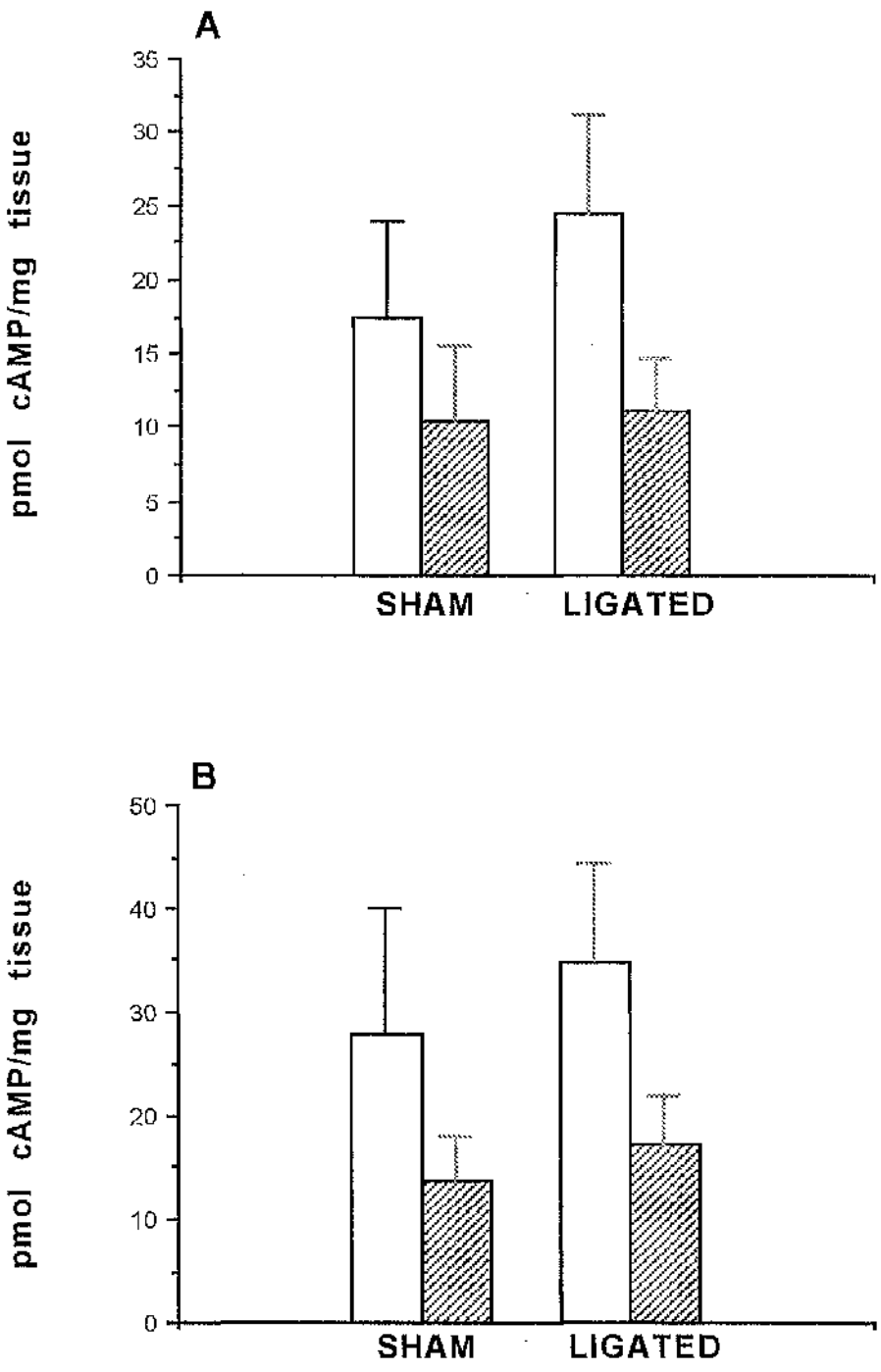


Figure 7.3 Effect of ET-1 (hatched columns) on forskolin-stimulated (open columns) cAMP production in pulmonary arteries from sham-operated (n=6) and coronary artery-ligated (n=8) rabbits. Results are expressed as pmol cAMP/mg tissue. Levels in (**A**) main intrapulmonary arteries and (**B**) pulmonary resistance arteries. Each column represents the mean ±SEM.

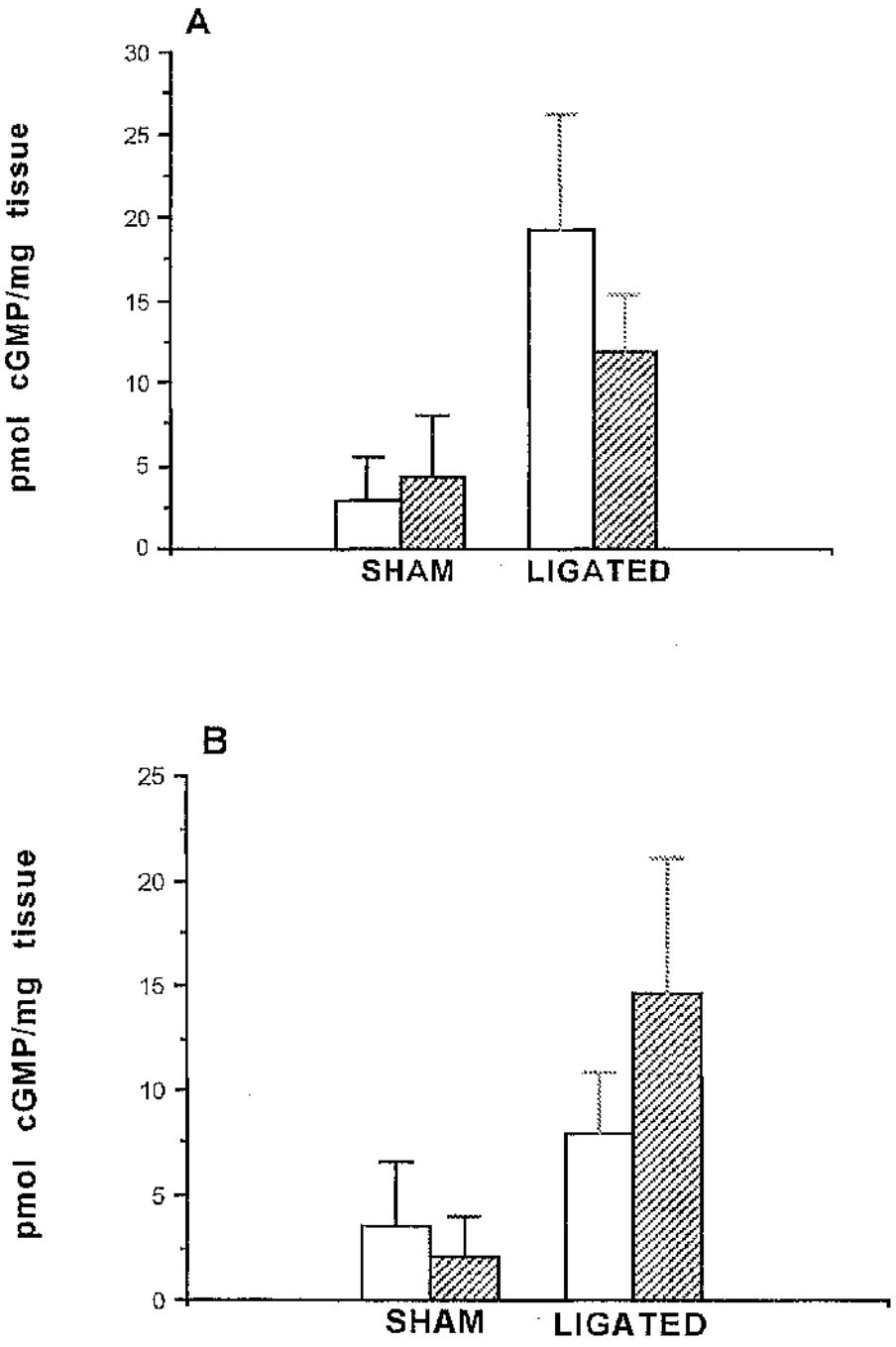


Figure 7.4 Effect of ET-1 (hatched columns) on control (open columns) intracellular cGMP levels in pulmonary arteries from sham-operated (n=4(A)/5(B)) and coronary artery-ligated rabbits (n=4(A)/6(B)). Results are expressed as pmol cGMP/mg tissue. Levels in (**A**) main intrapulmonary arteries and (**B**) pulmonary resistance arteries. Each column represents the mean ±SEM.

7.3A). Similarly, the presence of ET-1 inhibited forskolin stimulated [cAMP]i in PRAs from sham-operated (57.1±11.4% of paired forskolin alone) and coronary-ligated rabbits (53.1±10.5%; figure 7.3B). However, these apparent reductions were not significantly different from forskolin alone levels. ET-1 plus forskolin -stimulated intracellular cAMP levels were significantly greater than basal levels in PRAs from sham-operated rabbits ($P<0.05$) and in MIPAs and PRAs from coronary-ligated rabbits ($P<0.05$).

Intracellular levels of cGMP

Intracellular levels of cyclic GMP in the absence and presence of 0.1μM ET-1 in MIPAs and PRAs from sham-operated and coronary artery-ligated rabbits are shown in Figures 7.A and 7.4B, respectively. In MIPAs from coronary artery-ligated animals, incubation with 0.1μM ET-1 consistently decreased $[cGMP]_i$. However, ET-1 did not significantly alter $[cGMP]_i$ (figure 7.4A).

In small muscular pulmonary arteries from sham-operated rabbits, incubation with ET-1 consistently reduced intracellular cGMP levels (31.47±11.9% compared to 100% for paired control). In comparison, vessels from the same anatomical location from coronary-ligated rabbit lungs, showed a consistent increase in intracellular cGMP following incubation with ET-1 (187.6±6.8% compared to 100% for paired control). However again, there was great variability between tissues and these alterations failed to reach statistical significance.

7.4 Discussion

Effect of ET-1 on [^{125}I]-ET-1 binding

In rabbit intrapulmonary artery membranes, unlabelled ET-1 completely inhibited [^{125}I]ET-1 binding in a concentration-dependent, biphasic manner. Curve fitting analysis provided a best fit for a two-site model, thus indicating the existence of

two distinct ET receptor populations in this preparation. This finding is consistent with functional studies of isolated rabbit pulmonary resistance arteries, discussed in chapters 4 and 5 of this thesis, in which ET-1 was shown to evoke a potent biphasic concentration-dependent vasoconstriction These earlier results showed that cumulative concentration response curves to ET-1 included a "shallow component" at lower ET-1 concentrations (below 1nM) and a "steeper component" at higher concentrations of ET-1. Furthermore, both of these functional components were differentially affected by selective antagonists, thus suggesting a heterogeneous population of ET receptors in this preparation. The biphasic inhibitory effect of ET-1 demonstrated in the competition binding assays of this present chapter provides further evidence for this. This finding may correspond with previous binding assay data which showed different populations of high- and low-affinity ET_B receptors in brain tissue (Sokolovsky, *et al.*, 1992). Also consistent with this is the report of Perreault and Baribeau (1995). In this previous study in separate membrane preparations from pulmonary arteries and veins from the newborn piglet, [^{125}I[-ET-1 binding was completely displaced by either ET-1 or ET-3 in a concentration dependent manner. ET-1 competition was shown to correspond to a one-site model but computer analysis revealed two binding sites for that of ET-3: one in the picomolar range, and the other in the nanomolar range. These authors suggested that site one and site two corresponded to an ET_{B1} and ET_{B2} receptor, respectively. Furthermore, the binding characteristic of this ET_{B1} receptor may account for the fact that vasodilator responses to ET-receptor agonists are normally only seen at very low concentrations, as indeed was also shown in the piglet pulmonary vasculature.

The existence of an ultra-high affinity ET binding site was evident at unlabelled ET-1 concentrations below 1pM and a K_i value of 6.43×10^{-14}M was calculated. The second site was evident within the range 1pM to 30nM unlabelled ET-1 and also displayed high affinity with a K_i value of 4.83×10^{-10}M. This concentration for the K_i for site two is the same as K_i (0.5±0.1nM) demonstrated by Panek *et al.* (1992) for competition binding experiments for ET-1 vs. [125]ET-1 in membranes also prepared from rabbit pulmonary arteries. The K_i for site two is also similar to the nanomolar

affinities of ET-1 for ET_A and ET_B receptors which have been previously defined (Arai, *et al.*, 1990; Sakamoto, *et al.*, 1991, Ohlstein, *et al.*, 1996). However, the K_i calculated for site one is in stark contrast to these values, hence this ultra high affinity receptor population appears to be an untypical ET_A/ET_B receptor. Indeed the findings of other investigators also indicate that ET receptor agonists may exert their effects via different receptors that do not fit the current classification of ET_A and ET_B receptors (e.g. Sumner, *et al.*, 1992; Eglezos, *et al.*, 1993; Harrison, *et al.*, 1992).

Functional studies of ET-receptor mediated responses in rabbit PRAs (chapters 4 and 5) did not reveal such high affinity receptors. Threshold concentration to elicit a vasoconstrictor response was ~12pM and EC_{50} value was ~12nM (chapter 4). Different order of potency between binding and functional studies could be explained by the exposure of ultra high affinity ET-receptors, using binding techniques, which are not functional and therefore cannot be revealed using this latter technique. In addition, the EC_{50} for ET-1 compares more closely with the K_i of 4.8nM calculated for the second site. Therefore, the ET-receptors of this second lower affinity population may represent those which produce vascular responses following activation. In addition, other investigators have proposed that receptors may exist in two conformational states, an active state coupled to contraction and an inactive state not coupled to contraction (Robertson, *et al.*, 1994; Beck, *et al.*, 1995). Thus, one could propose that the apparent existence of ultra high affinity ET-receptors in rabbit pulmonary arteries, which cannot be exposed in functional assays, may be due to these receptors not being coupled to smooth muscle contraction and may be related to a different function within the pulmonary vasculature. Disparity between the findings of functional and binding studies is also seen in previous studies by other investigators. In functional studies in the rabbit saphenous vein, Douglas, *et al.* (1995) reported that vasoconstrictions to ET-1 were resistant to BQ123 (selective ET_A antagonist). However, radioligand binding studies clearly demonstrated the presence of BQ123-sensitive [^{125}I]ET-1 binding sites and ET_A receptor mRNA in this tissue (Webb, *et al.*, 1993; Gray, *et al.* , 1993b). As another example, Vigne *et al* (1993) demonstrated in rat brain capillary endothelial cells, that the

IC_{50} for BQ123 inhibition of ET-1 induced intracellular Ca^{2+} transients was much lower than the IC_{50} determined in binding experiments. The differences in methodology could explain the discrepancies between binding studies and other physiological /pharmacological studies.

The existence of two distinct ET-1-receptor populations, indicated by the two site model of inhibition of [^{125}I]-ET-1 binding, is in disagreement with the finding of previous ET-receptor binding studies which I have previously referred to, where only a single population of receptors was indicated. The K_i values of 0.064pM and 0.48nM calculated for site one and site two, respectively, contrasts with values obtained in other binding studies in rabbit pulmonary artery preparations. ET-1 was previously shown to completely inhibit [^{125}I]-ET-1 binding in a monophasic manner in membranes prepared from endothelium-denuded rabbit pulmonary arteries with IC_{50} values of 0.13nM and 0.38nM (La Douceur, *et al.*, 1993; Fukuroda, *et al.*, 1994a). The reasons for theses differences in results are unknown. The details of the methods for these previous binding assays and my own protocol were as follows. [^{125}I]-ET-1 was present at a concentration of 2 pM (this study), 10pM (Fukuroda, *et al.*, 1994a) and 20 pM (La Douceur, *et al.*, 1993); the IC_{50} is dependent on the concentration of the radioligand used and the calculation of K_i involves this value, hence the different radioligand concentrations used may be involved in the differences between studies. However other parameters were similar. The concentration of unlabelled ET-1 used to define nonspecific binding was 100-200 nM for all three studies. La Douceur and co-workers (1993) do not give details of protein content of membrane preparation, however a similar concentration was employed in the study of this chapter and that of Fukuroda *et al.* (1994a). Incubations of reaction mixtures were for either 2 or 4 hours at 22-37^{o}C, thus adequate conditions for equilibration were allowed in all three studies. Hence, it is unlikely that the discrepancy in findings between these studies are due to differences in these aspects of methodology.

Fukuroda *et al.* (1994b) showed that [^{125}I]-ET-1 binding to rabbit pulmonary arterial preparations (prepared from arteries of 1-5mm outer diameter) was inhibited by

BQ3020, with mean IC_{50} ~0.45nM. Similarly, La Douceur *et al.* (1993) reported that SXS6c inhibited [^{125}I]ET-1 in this preparation (prepared from secondary branches) with an IC_{50} of 0.32nM Thus the K_i value of 0.48nM calculated for site two in this binding study in rabbit arterial membranes demonstrated in this chapter, was similar to that previously reported for selective ET_B receptor agonists. In addition, from also examining the inhibitory effect of selective ET_A receptor antagonist BQ123 on inhibition of [^{125}I]-ET-1 binding, both these groups demonstrated the predominance of ET_B receptors in this preparation, as was indicated by the finding of the functional assay studies conducted in chapter 5 of this thesis.

Previous binding studies of ET-1 in other species and vascular preparations have also reported a monophasic response. In saturation experiments using slide mounted sections, [^{125}I]-ET-1 bound with high affinity to the media of human pulmonary artery, coronary artery, aorta and internal mammary artery and a one site model was preferred; this suggested the presence of either a single population of receptors or a heterogeneous population with the same affinity for the peptide (Davenport, *et al.*, 1995). D' Orleans-Juste *et al.* (1992) showed in rat lung membranes that ET-1 inhibited [^{125}I]-ET-1 binding with an IC_{50} value of ~0.2nM. Marsden *et al.* (1989) demonstrated that unlabelled ET-1 inhibited [^{125}I]-ET binding to cultured rabbit aortic smooth muscle cells with K_i of ~0.4nM.

The reason for this discrepancy in findings is unclear however, several possibilities may account for this. In the findings of my study the unusual first site was apparent at ET-1 concentrations below 1pM whereas, previous binding studies used this as the lowest concentration of ET-1 (e.g. Perreault & Baribeau, 1995; Nogughi, *et al.*, 1997). Therefore, there is the possibility that the ultra high affinity binding site was not revealed in these previous studies because an insufficient range of unlabelled ET-1 was used. However this is unlikely as the anomaly remains that in the findings of other investigators, the percentage of the specific [^{125}I]-ET-1 binding was ~100% (maximum) at ~1pM unlabelled ET-1; whereas this percentage was only around 60% at ~1pM and ~100% was observed at 100pM unlabelled ET-1 in this present study. On the other

hand, the divergence of findings may be related to the preparation of the arterial membranes. In the binding studies of Fukuroda *et al* (1994a), La Douceur *et al.* (1993) and Davenport *et al* (1995) in rabbit and human pulmonary arteries, membranes were prepared from endothelium-denuded arteries, whereas the membranes used in my own study were prepared from arteries in which the endothelium was not removed. (Davenport *et al.* also removed the adventitia). Thus, the previous studies would represent binding to receptors which existed on the vascular smooth muscle, whilst those present on the endothelium would also be bound in the study of this chapter. Furthermore, there is the possibility that the ET receptors of the ultra high affinity site were present on the vascular endothelium. This postulate is compatible with the report of Perreault and Baribeau (1995) which suggested that the first, high affinity, ET-3 binding site corresponded to an ET_{B1} receptor subtype.

Additional previous competition binding studies with ET-receptor agonists, other than ET-1, have demonstrated the possible existence of multiple, different affinity sites. Nakimichi *et al.* (1992) reported that in porcine bronchus and lung parenchyma membranes, ET-3 inhibited [^{125}I]ET-1 binding with hill coefficients considerably lower than unity. This same study also demonstrated in these preparations, that 4AlaET-1 and BQ123 displaced specific [^{125}I]ET-1 binding in a biphasic manner, indicating high and low affinity sites. Similar findings for ET-3 have also been reported in lung and tracheal preparations (Tschirhart, *et al.*, 1991; Takayanagi, *et al.*, 1991). Furthermore, previous functional studies have demonstrated the existence of distinct ET receptor populations. Douglas *et al.* (1995) demonstrated that contractile responses to ET-1 (SXS6c and ET-3 also) were best fitted to a two site model in the rabbit isolated saphenous vein. A high affinity (pM) site, showing characteristics of an ET_B receptor, and a low affinity (nM) site, showing characteristics of an ET_C-like receptor, were reported. Furthermore, this study also showed that the two components of the cumulative concentration response curves were differentially influenced by selective antagonists, as indeed I also found in the functional studies in rabbit pulmonary resistance arteries in chapter 5 of this thesis.

The heterogeneity in ET-receptors indicated by the biphasic inhibitory effect of ET-1 on [^{125}I]-ET-1 binding may be related to the different sized pulmonary arteries from which the membrane preparation was produced. Previous functional studies have demonstrated differential responses to ET-receptor agonists and antagonists in rat pulmonary arteries from different anatomical locations, thus demonstrating a diversity in the ET-receptor subtype at different levels of the pulmonary circulation (MacLean, *et al.*, 1994a; Higashi, *et al.*, 1997). In addition, Frid *et al.* (1994) reported that the bovine pulmonary arterial media is composed of multiple phenotypically distinct smooth muscle cells; differential ET-receptor subtypes may exist on different smooth muscle cells. For the competition binding studies shown in this chapter, I prepared the membrane preparation from isolated rabbit pulmonary resistance arteries of various diameters which were all dissected out from within the lung. In this thesis, I only studied functional responses in isolated pulmonary resistance arteries from the rabbit, however, the effects of ET-receptor agonists and antagonists were similar to findings in larger rabbit pulmonary arteries by other investigators (e.g. Hay, *et al.*, 1996; see chapter 5). Moreover, membrane preparations for the ET-1 vs. [^{125}I]ET-1 competition binding studies of Perreault and Baribeau (1995), were prepared from pulmonary arteries dissected from the hilum down to 100μm diameter, and a one site fit was shown. Furthermore, Noguchi *et al.* (1997) carried out [^{125}I]-ET-1 binding studies (autoradiographic technique) separately in elastic and muscular neonatal porcine pulmonary arteries and demonstrated similar IC_{50} and K_D values for these two preparations which also differ in size. Therefore, the fact that binding was performed on membranes from rabbit pulmonary arteries of different sizes seems unlikely to account for the apparent existence of two distinct ET-receptor populations.

In summary, competition binding data from this study suggests the presence of two distinct ET receptor populations in rabbit pulmonary arteries; one displaying ultra-high affinity and second, also demonstrating high affinity in the nanomolar range. This is consistent with the results of functional studies of ET-receptor mediated responses in isolated rabbit pulmonary resistance arteries (shown in chapters 4 and 5) in which ET-1

evoked vasoconstriction in a biphasic manner and the existence of heterogeneous ET receptors was suggested by the differential effects of various ET-receptor agonist and antagonists. However, confirmation by conventional protein purification/ molecular cloning is necessary before their existence can be firmly established.

Effect of ET-1 on intracellular levels of cyclic nucleotides

Although ET-1 has been shown to be a potent vasoconstrictor in pulmonary arteries from various species, including rabbit and human (chapters 4 and 5 this thesis), the second messenger system behind this ET-receptor mediated response is still unclear. The findings of this chapter may suggest the ability of ET receptors to interact with cyclic nucleotide second messenger systems in rabbit pulmonary arteries.

Although intracellular cAMP levels were not significantly altered by ET-1, unidirectional changes were consistent; an increase in main intrapulmonary arteries, whilst a decrease was evident in small muscular pulmonary arteries from both groups of animals. ET-1 did not significantly change intracellular cGMP levels in either group. However in coronary-ligated rabbit vessels, a consistent decrease with ET-1 was evident in MIPAs, whilst a consistent increase was evident in PRAs. In sham-operated rabbit preparations, [cGMP]i levels were unaltered by ET-1 in MIPAs but, this peptide consistently decreased levels in PRAs. Pussard *et al.* (1995) also reported that ET-1 had no effect on basal cGMP levels in intact human pulmonary arteries or veins.

Forskolin stimulates adenylate cyclase directly (Koch, 1982). As would be expected, the presence of forskolin resulted in a marked increase in all pulmonary arteries from both groups of animals. This finding is in agreement with previous reports. Sweeney *et al.* (1995) demonstrated that forskolin induced a concentration dependent increase in $[cAMP]_i$ in bovine pulmonary arteries. In addition, the results of this chapter demonstrated that ET-1 tended to inhibit forskolin-stimulated cAMP production in rabbit pulmonary arteries. Such negative coupling to adenylate cyclase would cause vasoconstriction via a reduction in intracellular Ca^{2+} levels. Phosphodiesterase (PDE)

enzymes are responsible for the breakdown of cyclic nucleotides (Wright, *et al.*, 1994; Eckly & Lugnier, 1994). Since cAMP accumulation was measured in the presence of the general PDE inhibitor IBMX, this data should reflect a direct inhibition of adenylate cyclase activity rather than degradation of cAMP by increased phosphodiesterase activity. This inhibitory effect of ET-1 on forskolin-stimulated cAMP was not statistically significant, however other investigators have shown evidence for this. Eguchi *et al.* (1993) demonstrated that ET-1 (and ET-3) dose-dependently inhibited cAMP formation stimulated by forskolin and isoproterenol (another adenylate cyclase activator) in cultured bovine endothelial cells. In transfected chinese hamster ovary cells, inhibition of forskolin-stimulated cAMP was shown to be mediated by ET_B receptors, whereas ET_A receptors were responsible for the accumulation of cAMP. Therefore the findings of the study of this chapter may provide yet more evidence for the predominance of ET_B receptors in rabbit pulmonary arteries, which was indicated from the results of the functional studies of chapters 4 and 5. In human Girardi heart cells expressing ET_A and ET_B receptors (ratio 4:6), ET-1 was shown to suppress forskolin-stimulated cAMP through activation of ET_A and ET_B receptors (Ozaki, *et al.*, 1997). In addition, this reduction in forskolin-stimulated cAMP was only partially blocked (20%) by ET_A or ET_B receptor alone, however was completely inhibited by a mixture of these selective antagonists. Thus suggesting intracellular cross-talk between ET_A and ET_B receptor subtypes in these cells. I discussed the possible existence of such a cross-talk mechanism in rabbit pulmonary arteries and other preparations in chapter 5 of this thesis.

Furthermore, the results in this chapter showed that the effect of ET-1 on intracellular cAMP and cGMP levels was altered, though not significantly, in pulmonary arteries from LVD rabbits compared with those from age-matched sham-operated animals. Basal, ET-1-induced and forskolin-stimulated $[cAMP]_i$ tended to be greater in coronary-ligated rabbit arteries compared to those from sham-operated animals. Comparatively greater levels in coronary-ligated compared to sham-operated rabbit arteries was also apparent for basal and ET-1 induced intracellular cGMP levels. This

latter tendency may relate to the increased NO levels in coronary-ligated rabbit PRAs which was suggested by the results in chapter 6 of this thesis. In this earlier chapter, inhibition of NO synthase by L-NAME resulted in a marked potentiation of ET-1 and SXS6c-induced vasoconstrictions of PRAs from coronary-ligated but not sham-operated rabbits. This contrasts with a previous study of isolated aorta from a rat model of heart failure, where NO synthase inhibition with L-NAME, as well as measurements of basal cGMP, demonstrated that basal NO release was decreased in chronic heart failure rats, however, stimulated NO release was not affected (Teerlink, *et al.*, 1994b). Similarly, in chronic hypoxic rat models, Shaul *et al.* (1993b) reported a decrease in $[cGMP]_i$ in the main pulmonary arteries removed from chronic hypoxic rats, and MacLean *et al* (1996b) demonstrated changes in basal cyclic nucleotide levels following hypoxic insult and this effect varied depending on vessel size. In this latter study, neither $[cAMP]_i$ nor $[cGMP]_i$ was changed by chronic hypoxia in pulmonary resistance arteries, however a decrease in the level of both these cyclic nucleotides was demonstrated in both first branch and main pulmonary arteries. The result of this previous study reflects further the evidence that the phenotype of the vascular smooth muscle cells in the pulmonary resistance arteries is very different from those in the larger arteries and that further changes occur with the onset of pulmonary hypertension (Meyrick & Reid, 1978; Sakai, *et al.*, 1996).

The studies in this chapter do not provide any insight into mechanism by which ET-1 may influence cyclic nucleotide levels. However other studies indicate the involvement of guanine nucleotide-binding proteins (G proteins). G proteins serve as the transducing communicator between agonist occupation of extracellular receptors and their effector systems (Gilman, 1987). In cultured porcine kidney epithelial (LLC-PK1) cells, ET-1 enhanced cGMP production but reduced vasopressin- and forskolin-stimulated cAMP production (Ozaki, *et al.*, 1994). Furthermore, both of these effects of ET-1 were antagonised by BQ788 but not by BQ123. Pertussis toxin (which results in uncoupling of membrane receptors and inhibits certain G protein-dependent receptor-mediated responses) inhibited the ET-1-induced cAMP decrease but not the increase in cGMP, thus indicating that the cAMP response is mediated by pertussis toxin- sensitive

Gi protein, whilst the cGMP response is mediated by a pertussis toxin-insensitive G protein. These authors concluded therefore that ET_B receptors in LLC-PK1 cell couple to two types of signal transduction cascades to reduce cAMP and stimulate cGMP production via distinct G proteins. From studies in cultured bovine endothelial cells, Eguchi *et al* (1993) suggested that endothelial ET_B receptors are functionally coupled to adenylate cyclase, possibly via G_i protein. It has also been demonstrated that ETs stimulate cAMP formation through G_s-protein coupled to ET receptors or through PGE_2 production secondary to ET-induced intracellular Ca^{2+} mobilisation (Domae, *et al.*, 1994; Takigawa, *et al.*, 1995). However in comparison, in a previous *in vivo* study in canine pulmonary circulation, pretreatment with pertussis toxin was shown to have no effect on subsequent ET-1-induced vascular response on resistance or compliance This indicated that pertussis toxin sensitive G protein signalling does not mediate ET-1 induced pulmonary vasoconstriction (Barman & Pauly, 1995).

Many previous studies indicate the involvement of the phosphotidylinositol (4, 5) -bisphosphate (PIP_2) pathway in the mechanism of action of ETs. Both ET_A and ET_B receptors have been described to be coupled to PIP_2 hydrolysis via G protein-coupled phospholipase C (PLC), and to the generation of inositol phosphates (IP) and diacylglycerol (DAG). The actions of IP3 and DAG, via the activation of PKC, then result in an increased concentration of intracellular Ca^{2+} (Emori, *et al.*, 1990; Eguchi, *et al.*, 1992; see chapter 1). Yoshida *et al.* (1995) reported that in small branches of rabbit pulmonary artery, the amplitude of ET-1-induced vasoconstrictions were suppressed by pretreatment with Calphostin C, a selective PKC inhibitor, and additional application of BQ788 completely abolished it. These authors concluded that stimulation of the ET_A receptor induces contraction mainly through activation of PKC in this preparation. PKC was also reported to involved in ET-receptor mediated contractions in rabbit saphenous vein (Sudjarwo & Karaki, 1995). In cultured rabbit vascular smooth muscle cells, ET-1 induced a rapid (15 sec.) transient formation of IP_3 (Marsden, *et al.*, 1989). The rapidness of this alterations suggests that the PIP_2 pathway is an initial event following ET-receptor activation.

In this study I investigated the involvement of cyclic nucleotides in rabbit pulmonary arteries but not that of the PIP_2 pathway. However recent evidence has established the phenomenon of cross-talk between distinct signal transduction mechanisms to be of importance (Spence, *et al.*, 1995). Asada *et al.* (1995) reported that ET-1-stimulated cAMP accumulation in ROS 17/2 cells occurred as a secondary effect of PIP_2 hydrolysis/ Ca^{2+} transduction cascade. Although the results of this chapter suggest a possible involvement of cyclic AMP and cyclic GMP in rabbit pulmonary arteries of various sizes, and under both basal and ET-1 stimulated conditions, the alterations were not sufficient to be statistically significant due to variability in results within the individual groups. This may be related to the limitations of the assay. Alternatively, as bovine pulmonary arterial media has been shown to be composed of multiple phenotypically distinct smooth muscle cells (Frid, *et al.*, 1994), if this was also the case in the rabbit, then the variability in cyclic nucleotide levels may relate to differences in levels/ involvement in different cell types. This possibility is suggested by previous evidence which indicates that the complexity, and in many cases even cell-specific modulation, of the cyclic nucleotide signalling pathway is related to inputs from other classic signal transduction pathways (Murphy, *et al.*, 1994; Spence, *et al.*, 1995). A possible site of interaction is at the level of phosphodiesterase (PDE) activity. In addition, there is the possibility of interaction between the cAMP and cGMP systems, where the most likely point of interaction would be the level of PDEs. However this is unlikely to have occurred in this study in rabbit pulmonary arteries, as all incubations were carried out in the presence of IBMX, a non-selective PDE inhibitor.

The main aim of this study was to assess the involvement of the cyclic nucleotides, cAMP and cGMP, in rabbit pulmonary arteries of various sizes. In addition to examining the effect of LVD secondary to coronary artery ligation, I also attempted to relate the intracellular cyclic nucleotide levels to the functional contractile ability of the pulmonary resistance arteries. ET-1 was shown to markedly inhibit forskolin-stimulated cAMP production in the main pulmonary arteries and pulmonary resistance arteries from both sham-operated and coronary ligated rabbits. However, despite apparent trends in

apparent trends in the levels of $[cAMP]_i$ and $[cGMP]_i$ in pulmonary arteries under basal and ET-1-stimulated conditions, no definite conclusions can be drawn from these results due to the lack of stastistical evidence. Thus the uncertainty in the role of these cyclic nucleotides in ET-1 mediated responses in rabbit pulmonary arteries remains.

Future studies would include analysis of G-proteins involved in ET-receptor activation. Components of the PIP_2 system would be investigated using various agents which interfere in this cascade such as activators or inhibitors of PLC or PKC; this second messenger system may have a greater involvement at basal level and / or stimulated ET-1 level in this rabbit model. With regard to future radioligand binding studies, saturation experiments would provide K_D and B_{max} values for rabbit pulmonary artery membranes and may reveal differences between sham-operated and coronary ligated rabbit preparations. Furthermore, additional competition studies using selective ET receptor antagonists would provide further information on ET-receptor subtypes present.

Chapter 8

Influence of nitric oxide on ET-receptor mediated responses in pulmonary resistance arteries of fetal and neonatal rabbits.

8.1 Introduction

As previously introduced in section 1.2 of chapter 1, the pulmonary circulation undergoes significant structural and functional alterations at birth (Haworth & Hislop, 1981; Hall & Haworth, 1986, 1987; Dunn *et al*, 1989). Vasodilation of the small pulmonary arteries and reduction in the pulmonary artery pressure (PAP) and pulmonary vascular resistance (PVR) occurs minutes after birth and continues to fall in the proceeding hours (Haworth & Hislop, 1981). The marked reduction in PAP and thus resistance is vital in order for the pulmonary circulation to deal with the 10 fold increase in blood flow (PBF) which occurs in the normal transition of the role of gas exchange from the placenta to the lungs. However in some newborns, this normal decrease in PVR and increase in PBF does not occur and results in persistent pulmonary hypertension of the newborn (PPHN) (Hageman *et al*, 1984; see section 1.3.2). Despite considerable study however the mechanisms involved in the pulmonary adaptation to extra-uterine life are incompletely understood.

Included in the categories of mediators which may play a role in the normal transition of the pulmonary circulation at birth and in the pathophysiology seen in PPHN are the peptide endothelin (ET) and the oxidant radical nitric oxide (NO). Both these endothelium-derived agents have marked functional effects on the fetal, neonatal and adult pulmonary circulation (e.g. Barnard *et al*, 1991; Perreault & DeMarte, 1991; Kinsella *et al*, 1994). Furthermore, both ET-1 and NO has also been reported to cause structural alterations of the developing and adult pulmonary vasculature (e.g. Roberts *et al*, 1995, Thomae *et al*, 1995; Janakidevi *et al*, 1992)

Acetylcholine (ACh)-induced vasorelaxations of arteries have been shown to be mediated by the release of the endothelium derived relaxing factor (EDRF), NO (Furchgott & Zawadski, 1980). Previous investigators have demonstrated alterations in ACh-induced relaxation's in pulmonary vasculature from fetus to newborn and during the initial days of life. EDRF activity has been shown to be absent or greatly reduced in the fetal pulmonary arteries (PA) of the of rabbit and sheep (Zubrow *et al*, 1989;

Abman, *et al.*, 1991). In porcine pulmonary arteries, the vasodilator response to ACh was negligible in the newborn and increased to a maximum at 3-10 days of life (Liu *et al*, 1992). Thus in addition to the developmental change in the endothelium-dependent response after birth, it may also change at birth when dramatic alterations in pulmonary haemodynamics occur.

In this chapter, I examined the vasodilator ability of ACh in fetal, neonatal and adult rabbit pulmonary arteries. Effect of developmental age on adrenergic stimulation was also investigated using NA. The majority of previous *in vitro* developmental studies on isolated pulmonary arteries have been on conductance vessels however, it is the pulmonary resistance arteries (PRAs) which are the important determinants of PVR *in vivo* (Staub, 1985). Hence the responsiveness of these small PRAs were examined throughout this study.

Previous reports show reduced endothelium- dependent relaxation's in newborn pulmonary vasculature compared with several days after birth (e.g. Liu *et al,* 1992). This may indicate diminished NO levels at birth. EDRF is inactivated by superoxide anions (Rubyani & Vanhoutte, 1986). In this chapter, I also examined the effect of superoxide dismutase (SOD), an inhibitor of superoxide anions (Rubyani & Vanhoutte, 1986), on ACh responses in PRAs from 0-24 hr old rabbits, so as to investigate the possibility influence of superoxide anions in the newborn.

Both ET-1 and NO influence the effects mediated by one another. ET production is regulated by inhibitory mechanisms such as NO. Agents that increase cyclic guanosine monophosphate (cGMP) concentrations, such as nitrovasodilators and superoxide dismutase (SOD), all prevent thrombin-induced ET production (Boulanger & Luscher, 1991; Saijonmaa, *et al.*, 1990). Endothelium- derived NO release therefore exerts a negative feedback on ET production via a cGMP- dependent mechanism (Luscher *et al,* 1992). Furthermore, ET can stimulate the release of NO when it binds to ET_B receptors of endothelial cells (Zellers, *et al.*, 1994).

Measurement of plasma ET-1 levels in newborn infants show elevated levels at birth, which then decrease by day 4 and 5 of life (Endo, *et al,* 1996; Malamitsi Puchner,

et al, 1993). Similar changes in plasma levels have also been reported in various animals, such as the pig (Levy, *et al,* 1995). Loesch and Burnstock (1995) showed that the endothelial cells of the main pulmonary artery in the newborn rat are rich in ET and nitric oxide synthase (NOS), suggesting a substantial involvement in the vasomotor control of the pulmonary circulation during the early stages of postnatal development. In this chapter I examined the effect of inhibition of nitric oxide synthase (NOS) activity on responsiveness of rabbit PRAs, at various developmental ages, to the non-selective $ET_{A/B}$ receptor agonist, ET-1, and the selective ET_B- receptor agonist, SXS6c (Williams *et al,* 1991).

8.2 Methods

Rabbit pulmonary resistance arteries

Fetal (2 days preterm) and neonatal New Zealand White rabbit pups were studied at 0-24 hrs, 4 days and 7 days after birth. Adult rabbits at ~ 3.5 kg body weight were also examined. The pregnant rabbits, fetal and neonatal pups were killed by sodium pentabarbitone as described in section 2.1.2. The lungs were promptly removed from the animals and small intralobar pulmonary resistance arteries (PRAs ~ 300μm i.d.) were dissected out according to the methods stated in section 2.2.3.2. These were mounted as ring preparations (~2mm long) on a wire myograph, bathed in Krebs solution at 37°C. Using the normalisation process explained in section 2.2.5, vessels were tensioned to an equivalent transmural pressure of ~ 15 mmHg. This pressure was chosen as it is similar to *in vivo* pressures of rabbit pulmonary arterioles. Preliminary studies in our laboratory showed that bubbling with 95% O2 inhibits responses to vasoconstrictors in rabbit PRAs so we bubbled with 3% O_2/ 5% CO_2 balance N_2 for fetal rabbit vessels and 16% O_2/ 5% CO_2 balance N_2 for all others. These gas mixtures were chosen as they are similar to those which the vessels would be exposed to *in utero* and *in vivo*.

Experimental protocol

Vessels were allowed to equilibrate for 1 hour prior to the addition of any drugs. The response of the PRAs to 50mM KCl was determined twice; once the contractile response had reached a plateau, the vessels were washed out at least 6 times with fresh Krebs solution. Vessels were then preconstricted with 1μM NA and 1μM ACh was added on top of the induced tone. In PRAs in separate studies, cumulative concentration-response curves (CCRCs) to NA (1nM-10μM) were carried out. Following 6 washes and at least 20 minutes "resting' time, vessels were preconstricted with 1-10μM 5-HT and ACh (1nM-10μM) was added cumulatively to the bath on top of the induced contraction. The effect of SOD on ACh CCRC was examined in 0-24 hour rabbit PRAs only. 50 U/ml was added to the bath 30 minutes prior to the addition of 10nM ET-1 to induce tone upon which ACh (1nM-30μM) was added.

Following washout and adequate time to return to baseline tension, CCRCs to ET-1 and SXS6c (1pM-0.3μM) were constructed either in the absence or presence of 100μM L-NAME which was preincubated with the vessels for 30 minutes prior to the cumulative addition of peptide.

Note

I investigated the possibility that the previous addition of drugs could have an affect on the responses to ET-receptor agonists. The preceding exposure of the isolated vessels to NA, 5-HT or ACh did not influence these responses. However the vasorelaxation studies involving ET-1 as a preconstricting agent and preincubation of the preparations with SOD, were carried out on a separate group of preparations, where subsequent ET-receptor mediated responses and the influence of L-NAME were not examined.

Data analysis

CCRCs are expressed either as a percentage of their own maximum contraction or as a percentage of the second reference contractile response to 50mM KCl. pEC_{10},pEC_{25} and pEC_{50} values (where appropriate) were calculated by computer interpolation from individual CCRCs and expressed as -log M concentration (refer to methods in section 2.5.2). Contractions to KCl were expressed as mg weight tension. Relaxation's induced by ACh were calculated as a percentage of the level of preconstriction to NA or 5-HT, as appropriate, in each preparation. Statistical comparison of the means of groups of data were made by Student's unpaired t-test or ANOVA; $P<0.05$ was considered statistically significant. Throughout, data are expressed as mean±SEM and *n /n* = number of ring preparations / number of animals..

8.3 Results

Contractile responses to 50mM KCl are shown in figure 8.1. PRAs from 7 day old and adult rabbits produced a significantly greater response than vessels from fetal and newborn rabbits. The average internal diameters (μM) and equivalent transmural pressures (mmHg) of PRAs from fetal and neonatal rabbits were as follows: fetal- 295.5±12.6 μM/ 12.8±0.6 mmHg; 0-24 hr- 329.0±14.3/ 11.8±0.8; 4 day- 343.6±16.5/ 10.5±0.3; 7 day- 325.5±21.6/ 12.6±0.9 (n= > 10 lungs in each case). Thus PRAs from animals at these age points exhibit similar internal diameters when set up at the same equivalent internal diameters.

Responses to NA and ACh

Figure 8.2A shows the response to 1μM NA in fetal, neonatal and adult rabbit PRAs. NA-evoked vasoconstriction was significantly greater in the 0-24 hr preparation than in fetal, 4 day, 7 day and adult rabbit PRAs. Rabbit PRAs produced a greater

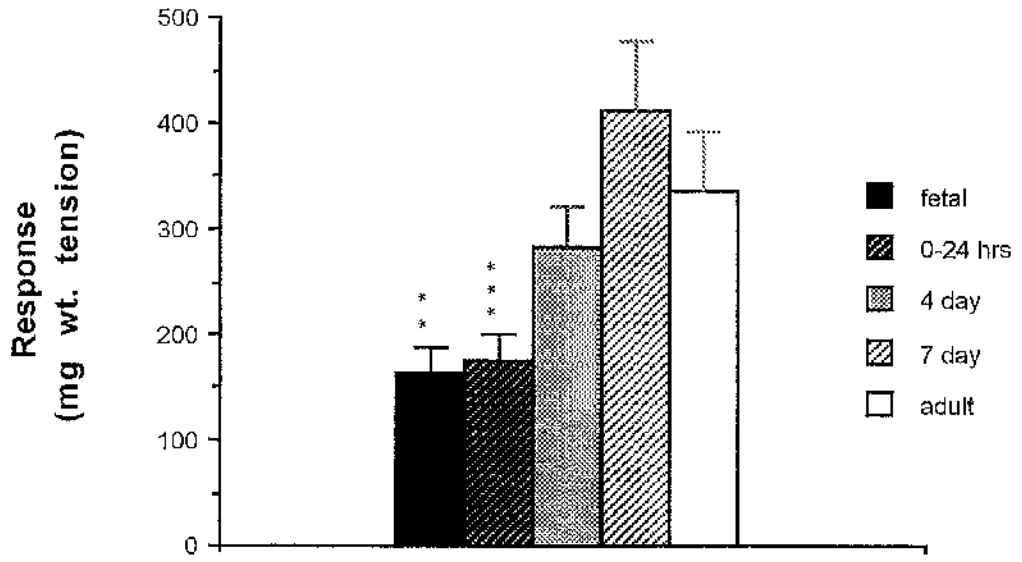


Figure 8.1. Contractile response to 50 mM KCl in PRAs from fetal and neonatal rabbits. n= >10 lungs for all preparations. Data are expressed as absolute contraction mg weight tension. Each column represents the mean ±SEM. Statistical comparisons were made using ANOVA followed by Tukey's post test; **p< 0.01, ***p<0.001 cf. 7 day response.

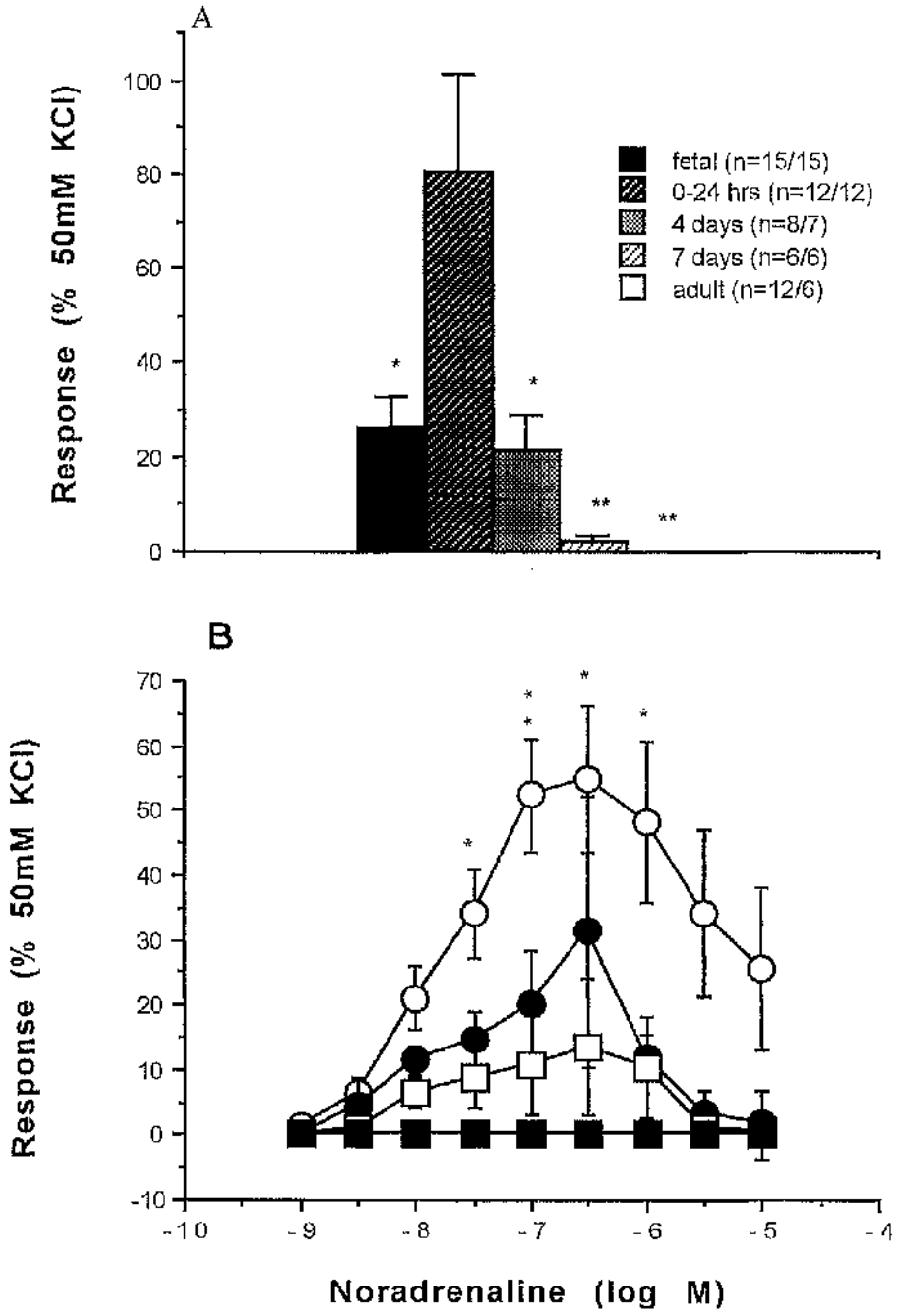


Figure 8.2 Responses to NA in rabbit PRAs. **A** Response to 1μM NA; *p<0.05, **p<0.01 all other age points cf. 0-24 hr. **B** NA CCRCs in 0-24 hr (○, n=8/8), 4 day (●, n=3/3), 7 day (❑, n=5/5) and adult (■, n=6/6) rabbit PRAs; *P<0.05, ** P<0.01 0-24 hr cf. 7 day. Data are expressed as reference contraction to 50mM KCl. Each point represents mean ±SEM. Statistical comparisons were made using ANOVA followed by Tukey's post test.

contractile response to NA following birth; in neonatal vessels, the ability of NA to evoke vasoconstriction progressively decreased during the first week after birth such that by adulthood no response was noted.

CCRCs to NA are shown in figure 8.2B. As can be seen, this resulted in a 'bell'-shaped response in vessels from all age points studied except in adult, where again no response was noted. The maximum response was noted at 0.3μM NA. Progressively greater NA concentrations produced a fall in tension toward or even slightly below baseline tension. Again the greatest magnitude of response was noted in 0-24 hour vessels; responses to 30nM-1μM NA were significantly greater in 0-24 hr compared with 7 day rabbit PRAs. (max. 54.9±11.3 cf. 13.4±10.4 %, $P<0.05$). NA had a similar sensitivity in all vessels examined. pEC_{50} values for NA are shown in table 8.1.

AGE	NA		ACh	
	pEC_{50}	n/n	pEC_{50}	n/n
0-24 hours	7.7±0.1	8/8	7.4±0.1*	7/7
4 days	7.4±0.2	3/3	7.8±0.1	6/6
7 days	7.9±0.1	5/5	7.8±0.1	6/6

Table 8.1 Sensitivity to NA and ACh in PRAs from 0-24 hour, 4 day and 7 day old rabbits. n/n= number of ring preparations from number of animals. Statistical comparisons were made by ANOVA followed by Tukey's post test. * $P<0.05$, 0-24 hr pEC_{50} vs. 4 day and 7 day.

Figure 8.3A shows the vasodilator effect of 1μM ACh in vessels preconstricted with 1μM NA. Since responses to 1μM NA were very small in 7 day old and non-existent in the adult PRAs, no ACh data is available for these age points. The greatest relaxation was observed in 4 day rabbit PRAs, where vessels relaxed to or below baseline tension. A substantial relaxation to baseline tension was also noted in fetal

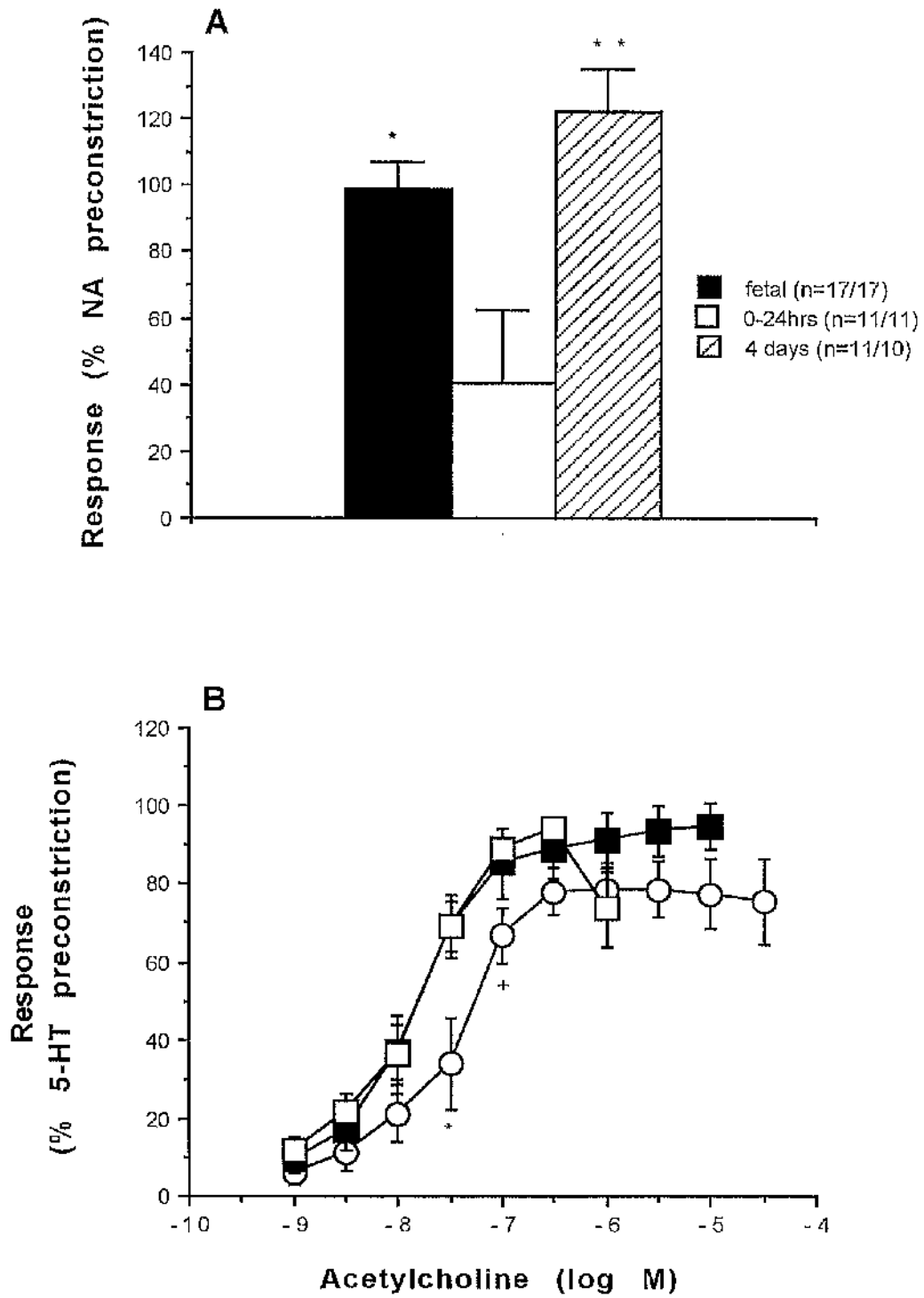


Figure 8.3 ACh-induced vasorelaxations of rabbit PRAs. **A** Response to 1μM ACh expressed as percentage of NA (1μM)- induced tone. **P*<0.05, ***P*<0.01 fetal and 4 day cf. 0-24 hr response. **B** ACh CCRCs expressed as a percentage of 5-HT (1-10μM)-induced tone. * *P*<0.05 0-24 hr (○, n=7/7) cf. 4 (■, n=6/6) and 7 day (❑, n=6/6); +*P*<0.05 0-24 hr cf. 7 day. Statistical comparisons were made using ANOVA.

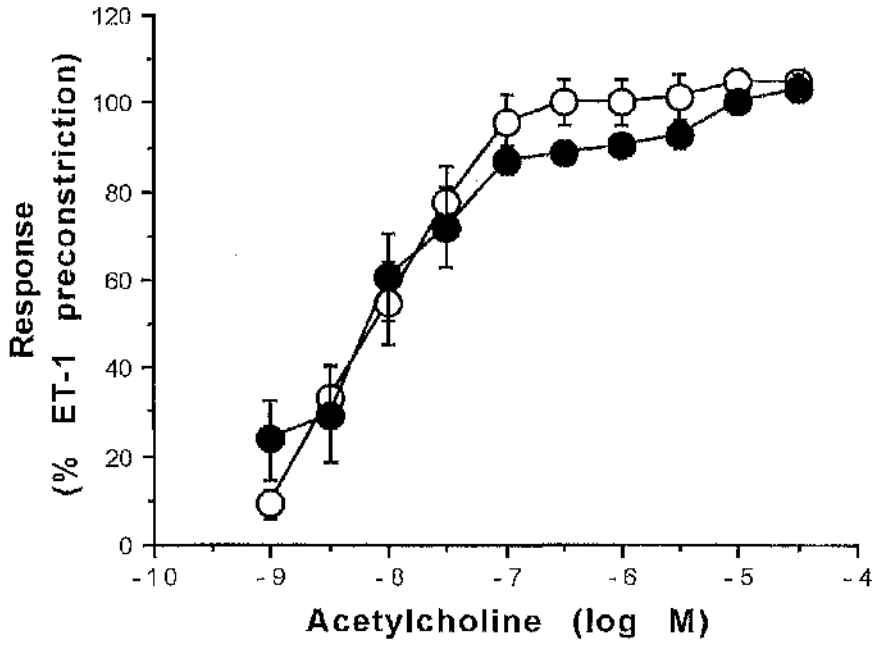


Figure 8.4 Effect of SOD on ACh- induced vasorelaxations of PRAs from 0-24 hour old rabbits. Control ACh CCRC (○, n-=5/5) and in the presence of 50 U/ml SOD (●, n=6/6). Data are expressed as percentage of ET-1- induced tone. Each point represents mean ± SEM.

vessels. The response at both these age points were significantly greater than that of 0-24 hour rabbit PRAs (~40% of NA preconstriction).

Cumulative responses to ACh in vessels preconstricted with 5-HT are shown in figure 8.3B. 5-HT induced tone was ~100% KCl response in 0-24 hr and 4 day vessels and ~ 60% KCl in PRAs from 7 day old rabbits. PRAs from 0-24 hour rabbits were the least sensitive to ACh (P<0.05 cf. 4 and 7 days; table 8.1). The magnitude of the

vasodilatory responses to 0.03 and 0.1μM ACh were significantly less in 0-24 hr compared with 4 and 7 day vessels (fig. 8.2B). Vasorelaxation of 0-24 hr and 4 day vessels reached a plateau at 0.3μM ACh, however an increasing contractile response was noted in 7 day vessels at and beyond this concentrations. ACh evoked a contractile response at all concentrations examined in adult rabbit PRAs.

The effect of SOD on ACh-induced relaxations in 0-24 hr rabbit PRAs is shown in figure 8.4. Tone was induced with 10nM ET-1; this caused a contraction of ~60% of the reference KCl response. ACh relaxed the vessels backed to baseline tension and the presence of 50u/ml SOD had no effect on sensitivity or magnitude of the response.

The above results demonstrate that the magnitude of the relaxatory response to 1μM ACh in 0-24 hour rabbit PRAs varied depending on the preconstricting agent; this is shown in table 8.2. A significantly greater vasorelaxation was seen when tone was induced with ET-1 compared to 5-HT. However, the contractile response to ET-1 was comparatively smaller than that noted to 5-HT.

Preconstricting agent	Induced tone % 50mM KCl)	Relaxatory response to 1μM ACh (% induced tone)
1μM NA	80.6±21.0	40.2±22.0
1-10μM 5-HT	99.0±10.1	78.7±6.6
10nM ET-1	63.5±4.6^{+}	100.0±5.3*

Table 8.2 Relaxatory response of 0-24 hour rabbit PRAs to 1μM ACh following preconstriction with NA, 5-HT or ET-1. Statistical comparisons were made by ANOVA followed by Tukey's post test: relaxatory response following ET-1 cf. 5-HT-induced tone, * $P<0.05$; ET-1 cf. 5-HT-induced tone, $^{+}$ $P<0.05$.

Effect of L-NAME on ET-1 and SXS6c induced responses

100μM L-NAME alone caused an increase in baseline tension in 9% of fetal, 28% of 0-24 hrs, 23% of 4 day and 38% of 7 day old rabbit PRAs tested. The magnitude of this response was very variable.

The response of fetal and neonatal PRAs to ET-1 in the presence and absence of 100μM L-NAME are shown in figures 8.5 -8.8. pEC_{50} values and maximum responses are shown in table 8.3. L-NAME had no effect on the sensitivity to ET-1 in fetal (figure 8.5A), 0-24 hour (figure 8.6A) or 4 day old (figure 8.7A) rabbit PRAs (table 8.3). In comparison, a significant increase in sensitivity was evident in vessels from 7 day old rabbits (figure 8.8A, table 8.3). The magnitude of the maximal ET-1-induced vasoconstriction was not affected by L-NAME in fetal preparations (figure 8.5A) however an increase by ~50 % was noted in 0-24 hr (figure 8.6B) and 7 day old rabbit PRAs (figure 8.8B). The greatest effect was seen in 4 day rabbit PRAs, where the maximum vasoconstriction was augmented by ~150% (figure 8.7B, table 8.3). L-NAME markedly increased the magnitude of the response at ET-1 concentrations greater than 3nM in 0-24 hour vessels. However in 4 and 7 day old rabbit PRAs, a significant increase in contractile response was seen from 0.1nM ET-1.

	ET-1 control			**ET-1+ L-NAME**		
	pEC_{50}	max. response	n/n	pEC_{50}	max. response	n/n
Fetal	8.7±0.2[a]	95.3±9.0	6/6	8.9±0.3	119.4±20.5	6/6
0-24 hours	8.8±0.2[a]	84.0±14.0	6/6	8.9±0.3	148.5±17.4*	7/7
4 days	8.6±0.1[a]	101.7±16.8	7/6	9.0±0.3	250.0±23.7***	6/6
7 days	8.0±0.2	92.7±11.1	6/6	8.9±0.2++	140.6±10.7*	6/6

Table 8.3 pEC_{50} values and maximum responses for ET-1 in the presence and absence of 100μM L-NAME in fetal and neonatal rabbit PRAs. Maximum response expressed as % contraction to 50mM KCl. Statistical comparisons were made by Students unpaired t-test: max. response control vs. +L-NAME * $P<0.05$, *** $P<0.001$; control pEC_{50} vs. +L-NAME ++ $P<0.01$;and ANOVA 7 day old control ET-1 vs. other age points [a] $P<0.05$. ET-1, endothelin-1; L-NAME, L-N^{ω}-nitro-L-arginine methylester.

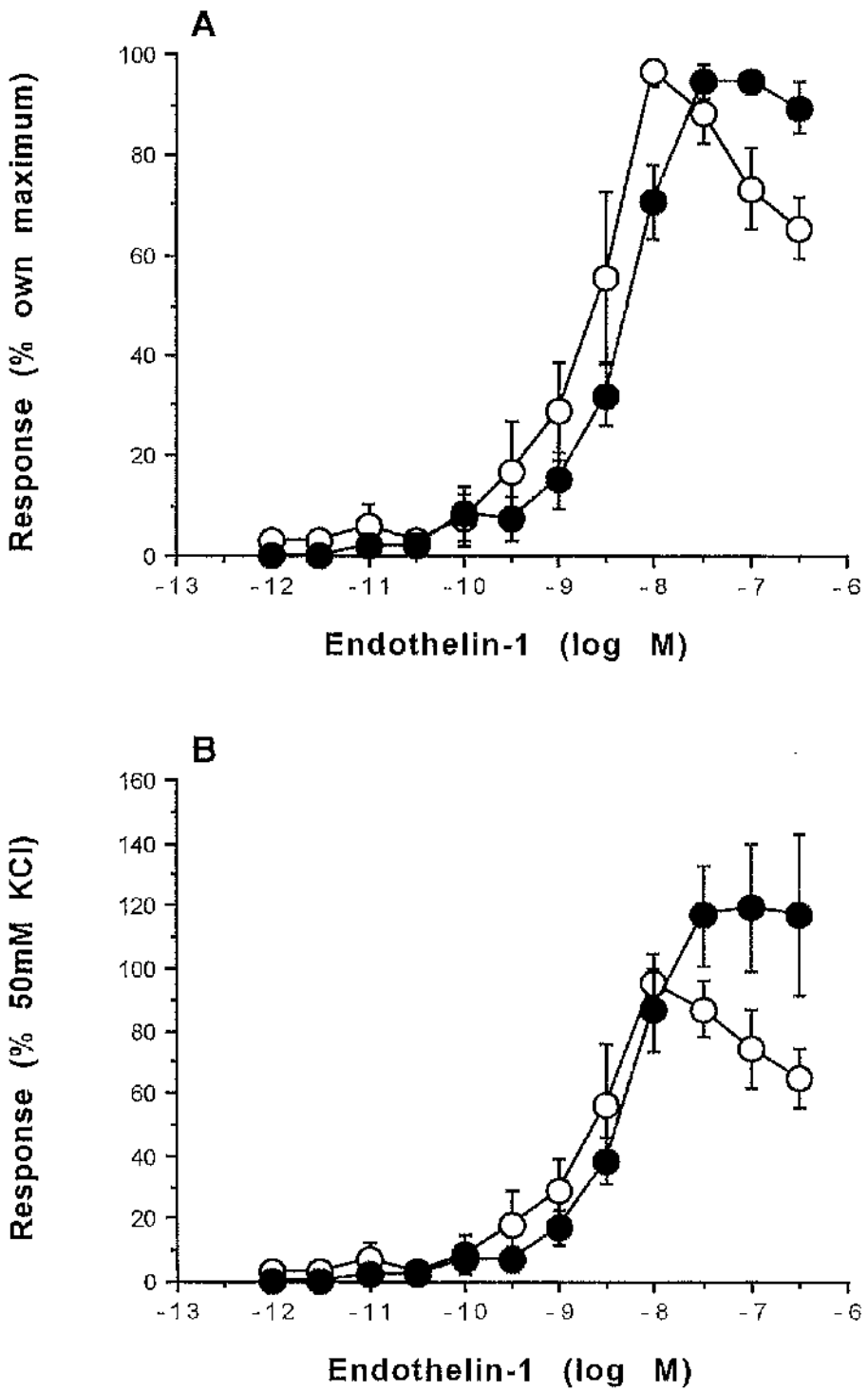


Figure 8.5 Effect of L-NAME (100μM) on responses to ET-1 in PRAs from fetal rabbits. ET-1 CCRC's ; control (○, n=6/6) and in the presence of L-NAME (●, n=6/6). **A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50mM KCl. Each point represents mean ± SEM.

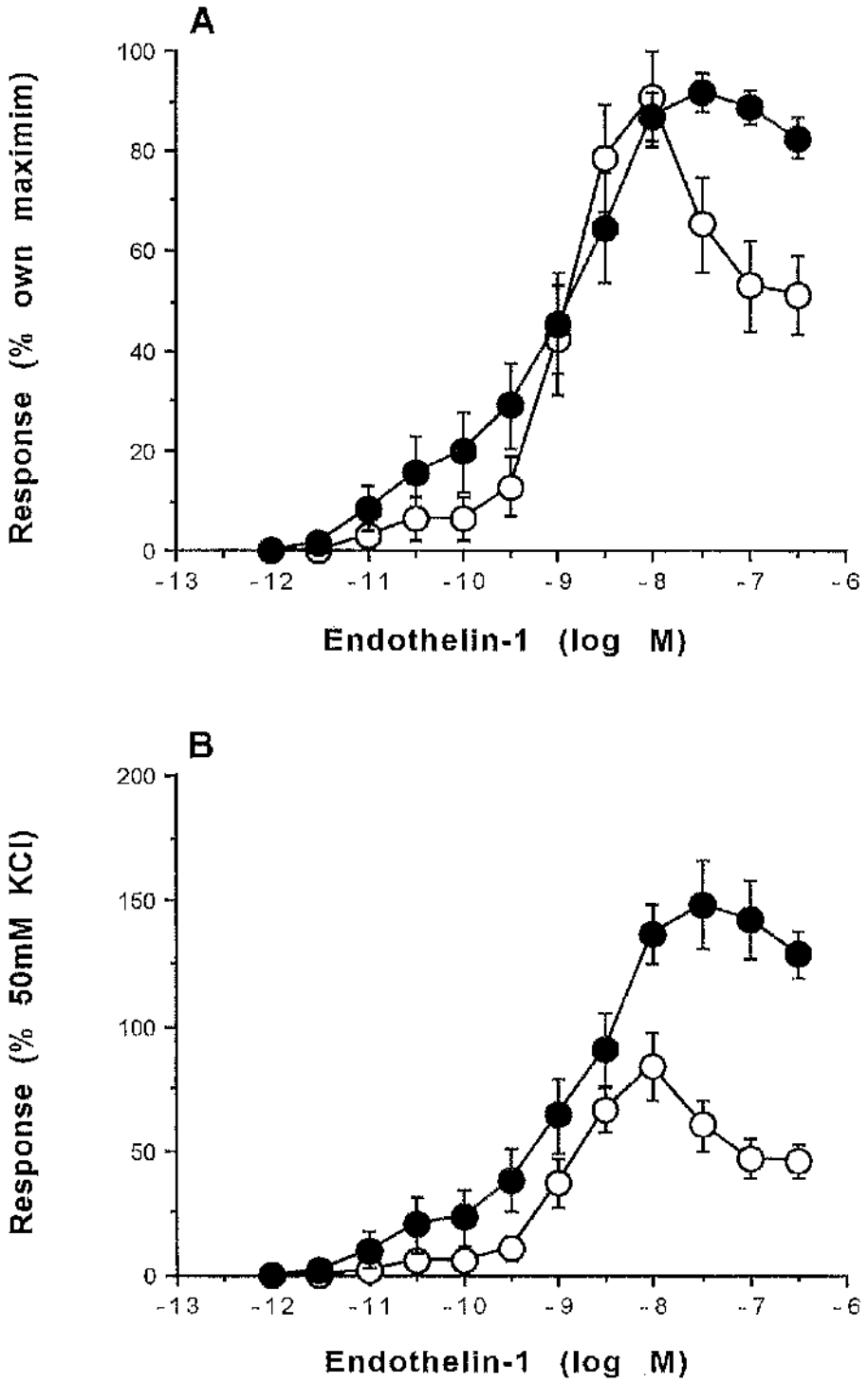


Figure 8.6 Effect of L-NAME (100μM) on responses to ET-1 in PRAs from 0-24 hr old rabbits. ET-1 CCRC's ; control (○, n=6/6) and in the presence of L-NAME (●, n=7/7). **A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50mM KCl. Each point represents mean ± SEM.

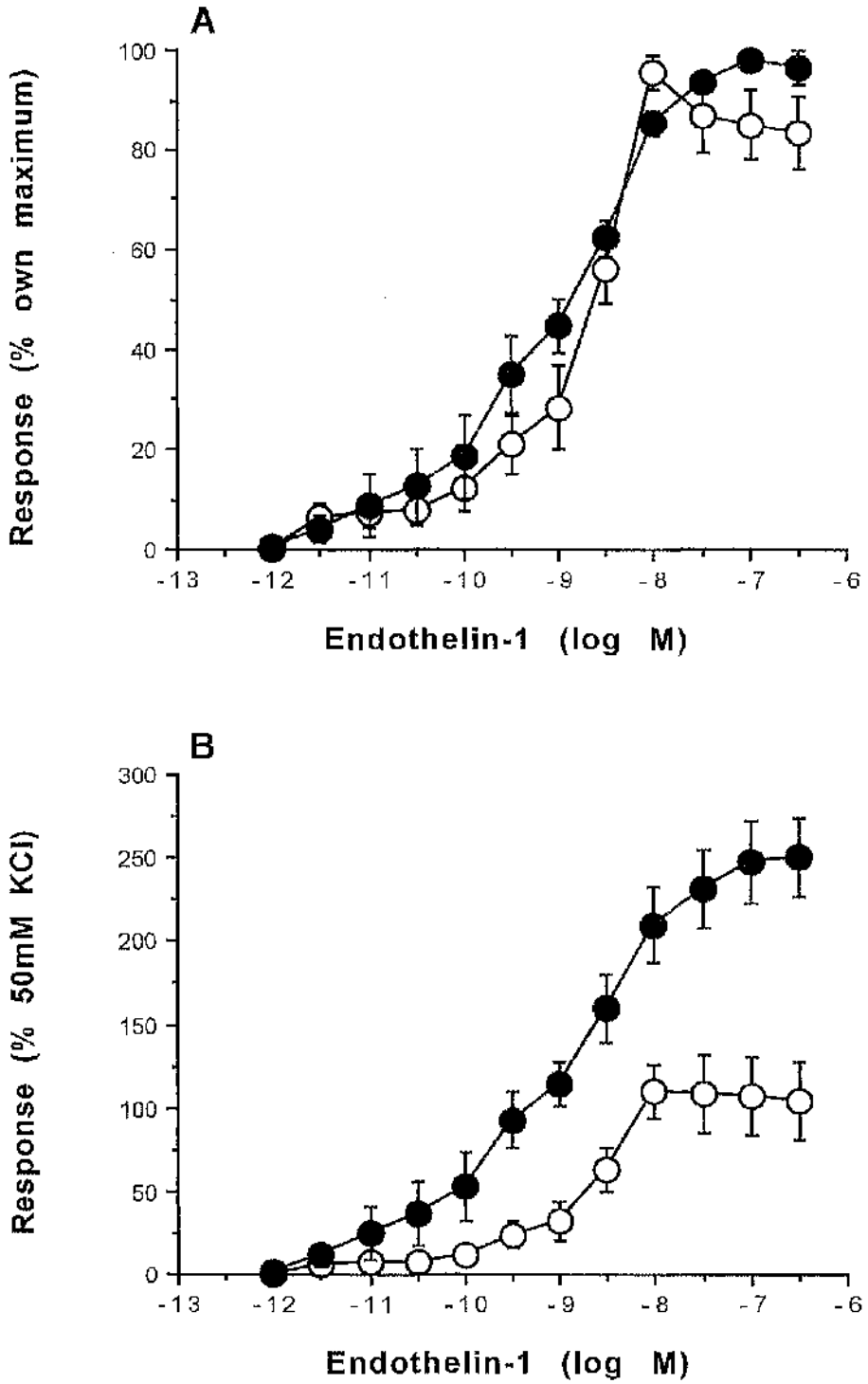


Figure 8.7 Effect of L-NAME (100μM) on responses to ET-1 in PRAs from 4 day old rabbits. ET-1 CCRC's ; control (○, n=7/6) and in the presence of L-NAME (●, n=6/6). **A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50mM KCl. Each point represents mean ± SEM.

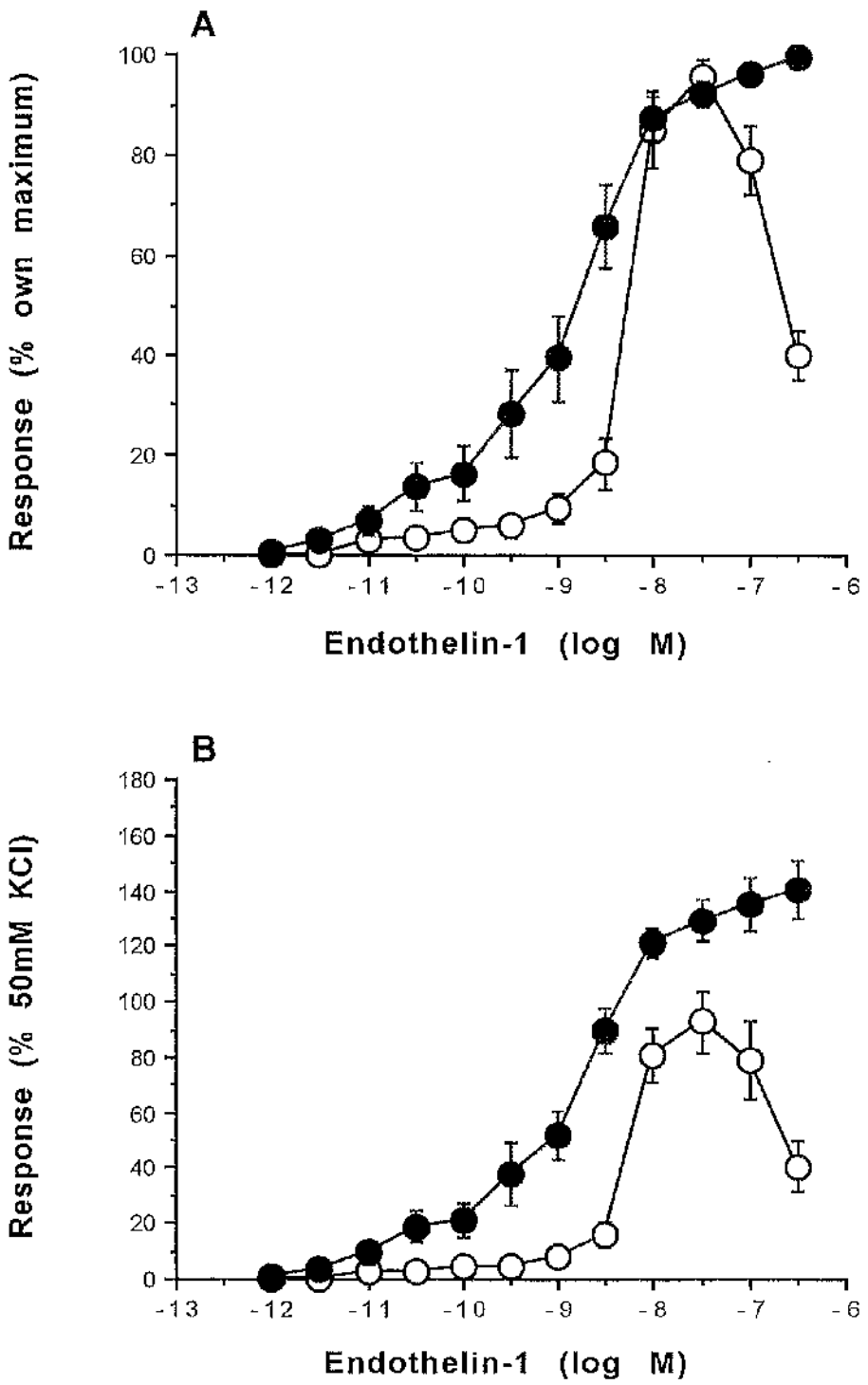


Figure 8.8 Effect of L-NAME (100μM) on responses to ET-1 in PRAs from 7 day old rabbits. ET-1 CCRC's ; control (○, n=6/6) and in the presence of L-NAME (●, n=6/6). **A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50mM KCl. Each point represents mean ± SEM.

The response of fetal and neonatal PRAs to SXS6c in the presence and absence of 100μM L-NAME are shown in figures 8.9.-8.12. pEC$_{50}$ values and maximum responses are shown in table 8.4. L-NAME had no significant effect on sensitivity or maximum contractile response to SXS6c in fetal (figure 8.9), 0-24 hour (figure 8.10) or 4 day old rabbit PRAs (figure 8.11, table 8.4). In 7 day rabbit vessels, a significant inhibition of SXS6c-induced response was noted (figure 8.12A, table 8.4). The rightward shift in the response was such that the maximum response occurred at 3nM in the presence of L-NAME compared with 30pM in control SXS6c CCRC. At this age, no influence on the magnitude of the response was noted from 1pM-0.1nM however, a marked augmentation was evident at concentrations above 1nM SXS6c (figure.8.12B, table 8.4).

	SXS6c control			**SXS6c+ L-NAME**		
	pEC$_{50}$	max. response	n/n	pEC$_{50}$	max. response	n/n
Fetal	8.6±0.3	39.5±12.2^{b}	6/6	8.9±0.4	105.2±51.1	4/4
0-24 hours	9.7±0.1^{a}	97.8±27.7	6/6	9.4±0.3	198.0+23.6	10/10
4 days	10.8±0.3^{aa}	86.1±8.9	6/6	10.9±0.3	97.5±29.1	6/6
7 days	11.1±0.2^{aa}	41.3±7.1^{bb}	6/6	9.6±0.2^{+++}	91.7±21.4 *	5/5

Table 8.4 pEC$_{50}$ values and maximum responses for SXS6c in the presence and absence of 100μM L-NAME in fetal and neonatal rabbit PRAs. Maximum response expressed as % contraction to 50mM KCl Statistical comparisons were made by Students unpaired t-test: max. response control vs. +L-NAME * P<0.05; control pEC$_{50}$ vs. +L-NAME ^{+++}P<0.001; and ANOVA followed by Tukey's post test: fetal control SXS6c pEC$_{50}$ vs. other age points ^{a}P<0.01, ^{aa}P<0.001; 4 day old control max. vs. other age points,. ^{b}P<0.05, ^{bb}P<0.01. SXS6c, sarafotoxin S6c; L-NAME, L-N$^{\omega}$-nitro-L-arginine methylester.

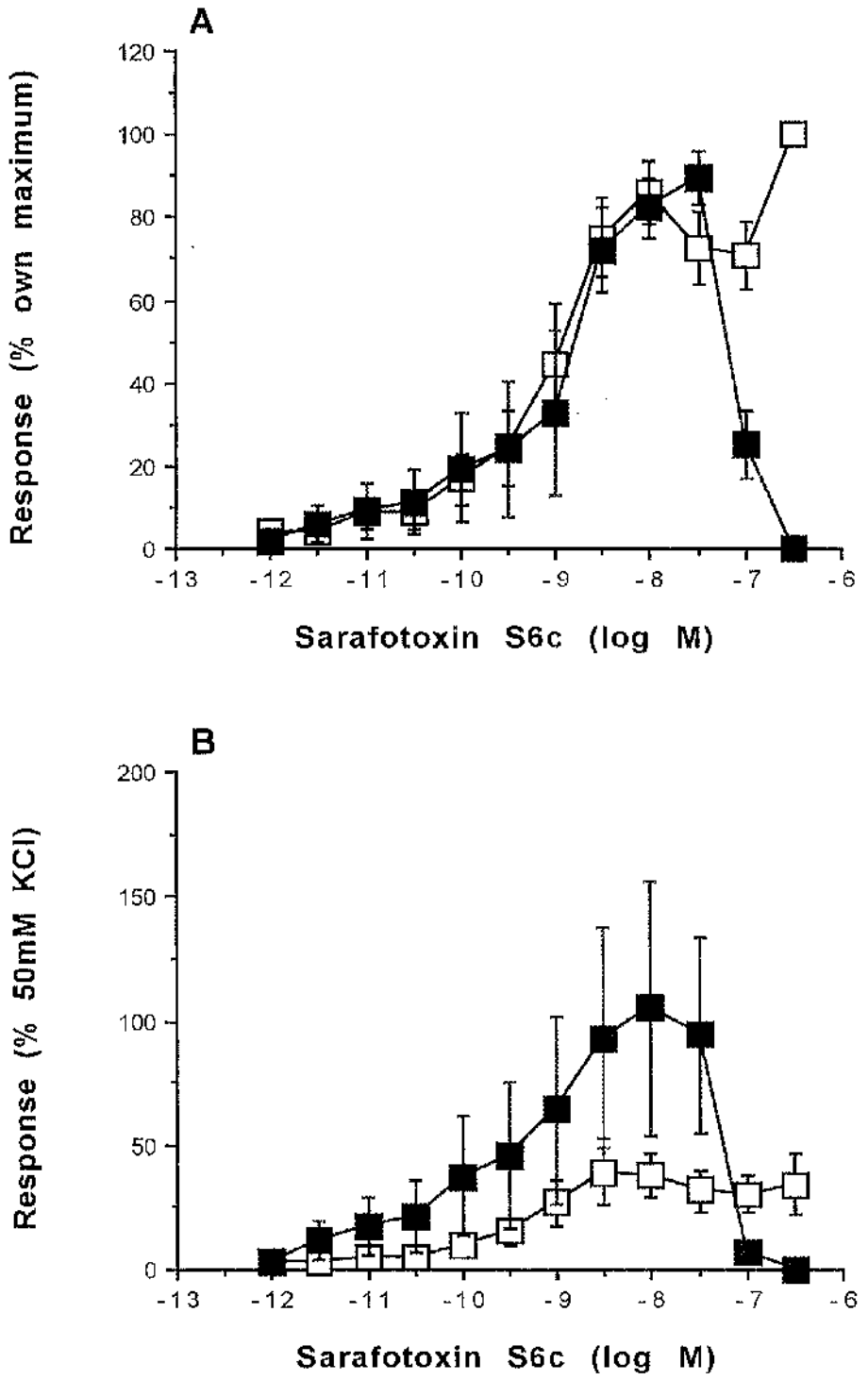


Figure 8.9 Effect of L-NAME (100μM) on responses to SXS6c in PRAs from fetal rabbits. SXS6c CCRC's ; control (□, n=6/6) and in the presence of L-NAME (■, n=4/4). **A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50mM KCl. Each point represents mean ± SEM.

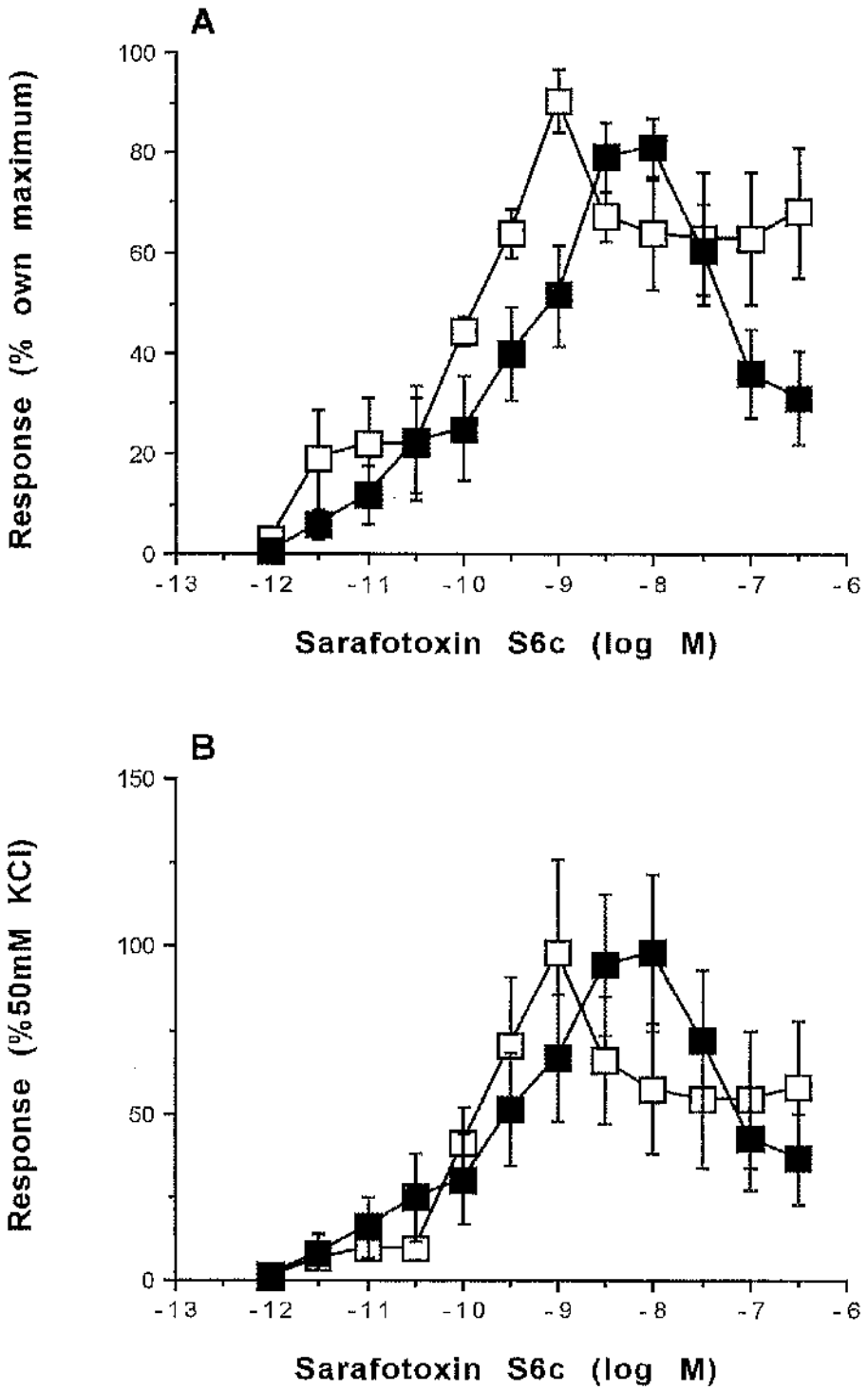


Figure 8.10 Effect of L-NAME (100μM) on responses to SXS6c in PRAs from 0-24 hr old rabbits. SXS6c CCRC's ; control (❑, n=6/6) and in the presence of L-NAME (■, n=10/10). **A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50mM KCl. Each point represents mean ± SEM.

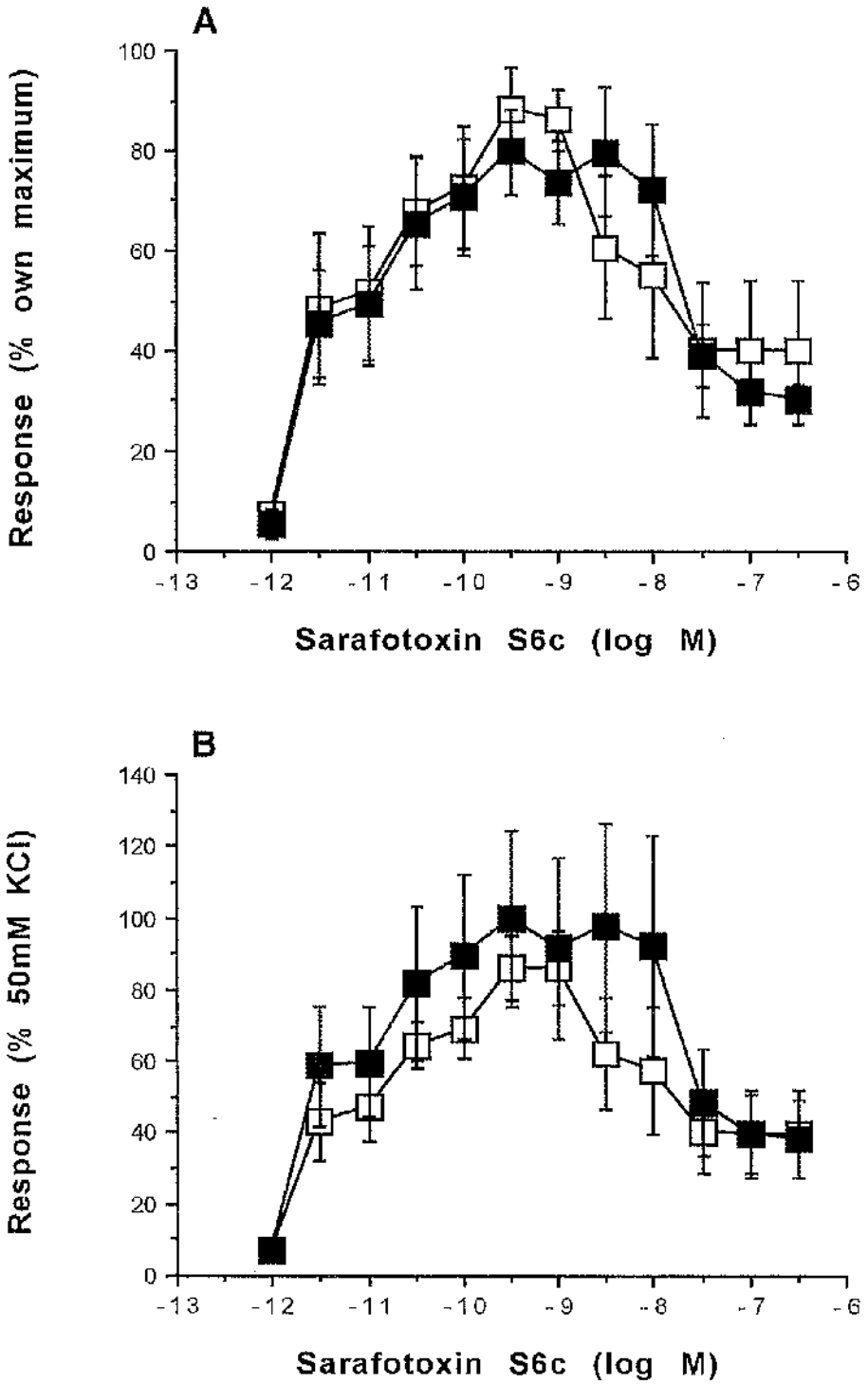


Figure 8.11 Effect of L-NAME (100μM) on responses to SXS6c in PRAs from 4 day old rabbits. SXS6c CCRC's ; control (□, n=5/5) and in the presence of L-NAME (■ n=6/6). **A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50mM KCl.

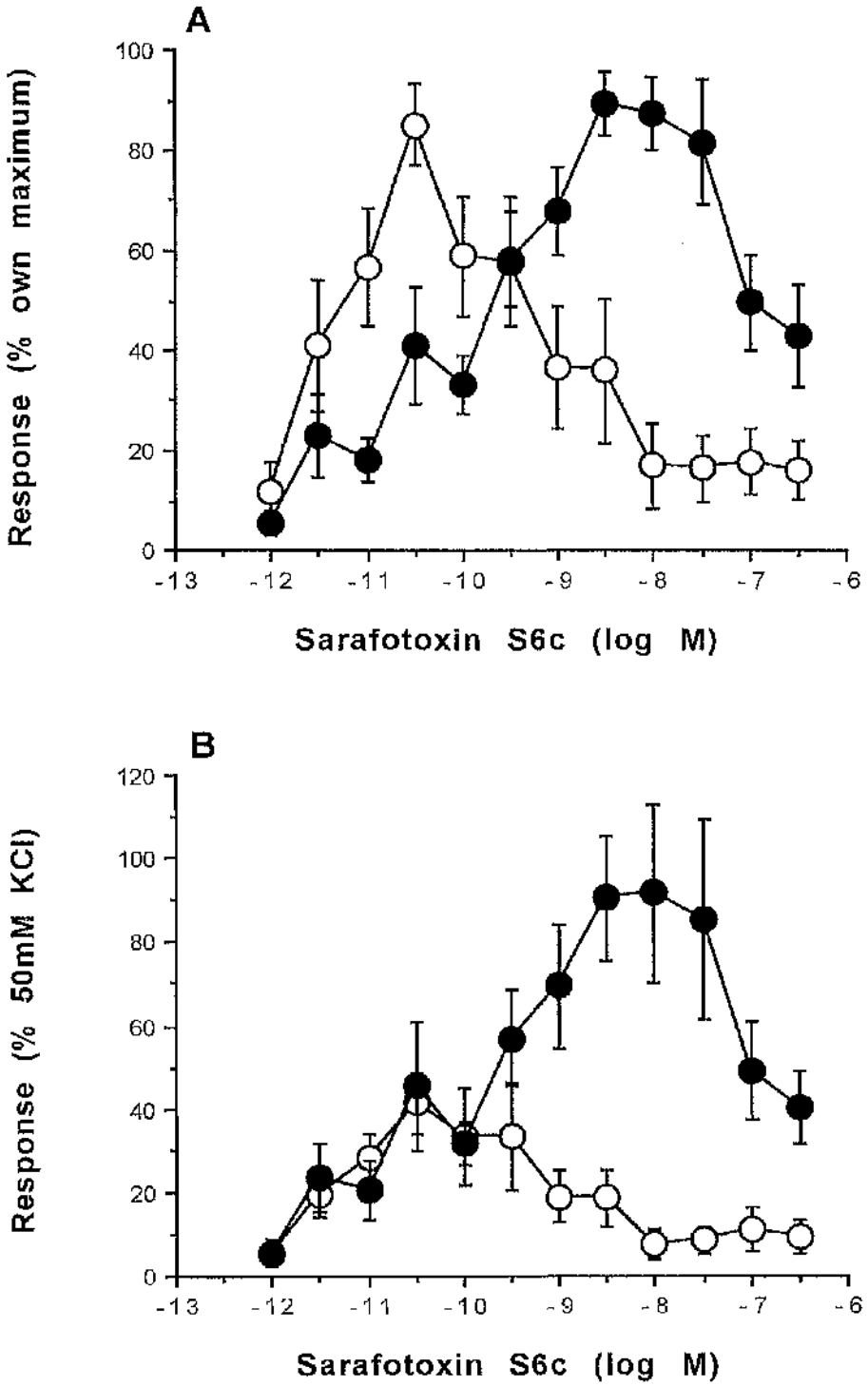


Figure 8.12 Effect of L-NAME (100μM) on responses to SXS6c in PRAs from 7 day old rabbits. SXS6c CCRC's ; control (○, n=6/6) and in the presence of L-NAME (●, n=5/5). **A** Data are expressed as percentage own maximum contraction. **B** Data are expressed as percentage reference contraction to 50mM KCl. Each point represents mean ± SEM.

8.4 Discussion

A vast amount of information is available on the reactivity of large diameter vessels (> 1 mm) from the neonatal lung (e.g. Davidson & Eldemerdash, 1990; Steinhorn *et al*, 1993; Liu *et al*, 1992). However, arteries with a diameter greater than 500μM are thought to be relatively unimportant in controlling vascular resistance (Andersson *et al*, 1985). Furthermore, marked structural and functional differences exit throughout the vasculature thus making extrapolation of data from larger vessels to smaller resistance arteries inappropriate (e.g. Andersson *et al*, 1985; Buckner *et al*, 1982; Somlyo *et al*, 1965). The results of this chapter demonstrate that PRAs (~300 μM ID) from fetal and neonatal rabbits differ in their responsiveness to the vasoactive compounds KCl, NA, 5-HT, ACh, ET-1 and SXS6c *in vitro*. Furthermore, the influence of NO on ET-receptor mediated responses also alters following birth and during the first week of extra-uterine life. Thus, these marked changes are seen when the pulmonary circulation is in a transitional state as it adapts to perform its vital function of gas exchange *in vivo* (Inselman & Mellins, 1976). These differences indicate that for a complete understanding of the postnatal adaptation of the pulmonary circulation, it is necessary to access responses at a level of vascular tree more important in controlling pulmonary resistance.

Part of the structural remodelling of the neonatal lung involves the extension of vascular smooth muscle into the alveolar region (Inselman & Mellins, 1976). The response to KCl, which is mediated through it's effect on the membrane potential (Burnstock *et al*, 1963) and is therefore receptor independent, indicates the integrity and mass of the smooth muscle present. The contractile response of PRAs to KCl significantly increased between fetus and birth to 7 days of life. The magnitude of the response at 7 days was similar to that observed in adult vessels. These findings suggests smooth muscle extension or maturation in pulmonary resistance arteries of the same anatomical location within the first week of extra-uterine life. A continued increase in the KCl-evoked response of small intrapulmonary arteries, of similar diameter to the

vessels examined here, have also been reported in fetal and neonatal lamb (Dunn *et al,* 1989). Furthermore, this study also demonstrated no change in KCl response over time in larger diameter intrapulmonary arteries (530-1000μM i.d.). This finding indicates further functional differences between vessels of different sizes in the transitional pulmonary circulation.

Responses to NA

Neonatal PRAs had similar sensitivity to NA but differed in magnitude of the induced vasoconstriction. The response to NA was very variable; all 0-24 hr rabbit PRAs examined responded however the percentage of responders then progressively decreased such that no adult PRAs studied evoked a response to adrenoceptor stimulation. Endogenous NA and adrenaline act on multiple adrenoceptor subtypes. Both α_1- and α_2-adrenoceptors mediate vasoconstriction, however endothelial α_2-adrenoceptors mediating vasodilation through the release of NO have been demonstrated in pulmonary vessels (Liu *et al,* 1991). The presence of α_1- and α_2-receptors have been demonstrated in isolated pulmonary vessel rings and in the pulmonary vascular bed of the rabbit (Docherty & Starke, 1981; MacLean *et al,* 1993a; 1993b). β-adrenoceptors, mediating vasodilation (Boe & Simonsson, 1980), are exclusively of the β_2-receptor subtype on the pulmonary vessels of the rabbit (O' Donnell & Wanstall, 1985). NA was shown to evoke a "bell"- shaped response in neonatal PRAs; a maximum was reached at 0.3μM and further increments in NA concentrations produced fall in tension toward baseline values. This suggests that low concentrations of NA act at α_1-adrenoceptors evoking a vasoconstrictor response; whereas β_2-receptors are activated by higher NA concentrations, producing a relaxatory response. α_2-adrenoceptors may also be involved in the vasorelaxations observed in the neonatal rabbit PRAs. NO mediates α_2-adrenoceptor agonist- induced pulmonary vasodilation (Liu *et al,* 1991; Pepke-Zaba *et al,* 1993). Further studies using selective agonists and antagonists would shed further light on this.

In rabbit PRAs, the ability of NA to evoke vasoconstriction was greatest 0-24 hours after birth, it then decreased during the first week of life and was non-existent in adulthood. There are many potential explanations for this. In the newborn pig, plasma ET-1 levels were shown to be greater at birth than at 3 days or later and a similar situation occurs in the human infant (Levy, *et al,* 1995; Endo, *et al,* 1996; Malamitsi Puchner, *et al,* 1993). Low concentrations of ET-1 potentiate the contractions to other vasoconstrictor hormones, such as NA (Tabuchi, *et al.,* 1989). The magnitude of the contractile response to NA was significantly greater in PRAs from 0-24 hr old rabbits compared to 4 day, 7 day and adult preparations. Thus NA evokes it greatest response at a time when plasma ET-1 levels have been shown to be elevated. This observation also implies that the functional population of contractile adrenoceptors on rabbit PRAs is at its greatest at birth and then begins to decline with increasing age. Alternatively, the greater magnitude of vasoconstrictions in the newborn could be related to a reduced level of agonist-induced NO level at birth compared to several days old, which is suggested by the reduced ACh-induced relaxation in 0-24 hr compared to 4 and 7 day vessels (this is discussed later).

NA was shown to be ineffective in small intrapulmonary arteries of adult rabbit however larger pulmonary arteries have been shown to readily contract in response to NA (Mac Lean *et al,* 1993a; 1993b). Hence there is a possible decrease in adrenergic activity with decreases in vessel diameter in the rabbit lung; indeed, this has also been demonstrated in the rabbit by Su and co-workers (1978). A significantly smaller magnitude of response to NA and adrenaline in smaller diameter pulmonary arteries compared with larger diameter vessels has also been reported in the lamb (Dunn *et al,* 1989). Differences in sensitivity to adrenergic agonists depending on vessel size has also been demonstrated in isolated PAs from guinea pig (Buckner *et al,* 1982).

Responses to ACh

Alterations in the response to ACh were also observed at different age points. The magnitude of the relaxatory response to ACh was significantly greater at 4 days compared to 0-24 hrs old when PRAs were preconstricted with NA. Similarly, on raising the tone with 5-HT, ACh-induced vasorelaxation was greater in the 4 and 7 day neonate compared to the newborn preparation, which was also significantly less sensitive to ACh. These results demonstrate a two phase change in the endothelium-dependent vasodilation response to ACh in rabbit PRAs, as has previously been shown in porcine intrapulmonary artery (Liu, *et al.*, 1992). The ACh response of rabbit PRAs increased after birth with maximal relaxations at 4 and 7 days of life but by adulthood, ACh was unable to evoke vasorelaxations. Similar findings have been reported in neonatal isolated pulmonary vessels of other species (Abman, *et al*, 1991; Steinhorn, *et al.*, 1993; Zellers & Vanhoutte, 1991; Levy *et al.*, 1995).

The possibility that the relatively reduced endothelium-dependent relaxation of the newborn was due to a reduced NO level was investigated using SOD. Several reactants in the plasma milieu cause inactivation of NO, including superoxide anions (Moncada, *et al.*, 1991). Rubanyi & Vanhoutte (1986) showed that EDRF half life was prolonged in the presence of SOD, a scavenger of superoxide anions. Other studies in our laboratory by Morecroft & MacLean (1996) demonstrated that the endothelium-dependent response to ACh in large pulmonary arteries of the newborn rabbit was restored in the presence of SOD. The preconstricting agent used in the study by Morecroft and MacLean was ET-1; I also employed this agent to induce tone in the smaller PRAs so that a direct comparison could be made between vessels of different diameters. However, in comparison to the findings of Morecroft, the PRAs relaxed to baseline tension in response to ACh alone and the presence of SOD had no affect. These findings suggest that the presence of superoxide anions inhibits vasorelaxations of larger diameter PAs but do not affect the smaller PRAs of the newborn rabbit. The fact that ACh- induced a 100% relaxation in PRAs but had negligible affect on larger PAs

demonstrates functional differences within the same species depending on vessel diameter.

ACh has been shown to cause a marked dilation of the pulmonary vascular bed of the fetal lamb (Dawes, *et al*, 1956). Fetal rabbit PRAs demonstrated a 100% relaxation to ACh. A substantial relaxation has also been shown in the larger pulmonary arteries of the fetal rabbit, also preconstricted with NA (Morecroft & MacLean, 1995a). In this previous study the magnitude of the relaxation was shown to depend on the vessel size; the response of the extra-lobal was significantly greater than that of the main pulmonary artery. Furthermore, the ACh-induced response of the fetal PRAs shown in this chapter is comparatively greater that the relaxation shown in the larger PAs. This suggests that the size of the fetal vessel studied influences the endothelium-dependent response, as a greater relaxation is evident with decreasing vessel size.

In comparison, other studies in isolated pulmonary arteries from fetal animals have shown diminished basal and stimulated release of endothelial NO in the near- term fetus relative to animals a few weeks older (Abman, *et al*, 1991; Steinhorn, *et al.*, 1993; Zellers & Vanhoutte, 1991). Although the results of this study show a comparatively smaller response of 0-24 hr rabbit PRAs compared to 4 and 7 days old, the relaxations observed in the newborn preparation were still marked. Previous studies however have demonstrated a negligible response to ACh at birth (Liu, *et al.*, 1992).

These conflicting findings may be may explained by differences in experimental conditions. Conditions *in vivo* and *in vitro* differ significantly and between different in *vitro* studies, the size of the vessel studied or the preconstricting agent used prior to evoking relaxatory responses varies. Indeed, I found that the magnitude of the ACh-induced relaxation of 0-24 hr rabbit PRAs depended upon the agent used to induce tone. A comparatively greater relaxation was noted following ET-1- induced tone compared with 5-HT preconstriction. However, the magnitude of the vasoconstriction to this latter compound was markedly greater than that of ET-1 and so the vessel had to relax from a greater level of tension. Former reports show that conflicting responses to ACh could be explained by the level of pre-existing tone. For example, Hyman & Kadowitz (1988;

1989) demonstrated, in the feline and rabbit pulmonary vascular bed, that ACh induces a pressor response under resting conditions but causes a depressor response under conditions of elevated tone. Perez-Vizcaino *et al* (1996) also demonstrated that differences in the relaxant effects of ACh, SNP and ATP in isolated intrapulmonary arteries of neonatal piglets, depended upon the vasoconstrictor used. Another factor which varies between the experimental conditions of different studies is the percentage of O_2 used to maintain the preparations. For example Liu *et al* (1992) and Steinhorn *et al* (1993), when investigating endothelium-dependent vasodilations in neonatal/adult pig and newborn/juvenile sheep, aerated the isolated PAs with gas mixture containing 95%/94% O_2; however this level of oxygenation far exceeds that the pulmonary arterial vasculature is exposed to *in vivo* . Whereas in the functional studies I conducted in this chapter and chapter 9 on this thesis, fetal and neonatal preparations were bubbled with gas mixture containing 3% and 16% O_2, respectively. Previous studies have indicated that the functional response to varies agonists varies depending on the pO_2 to which pulmonary vessels are exposed to. For example, Wang and Coceani (1992) showed that in isolated pulmonary resistance vessels from fetal lamb, indomethacin *per se* did not alter tone of vessels pre-equilibrated at low pO_2 (~22 mmHg), whereas it became a constrictor at high pO_2 (~71 mmHg).

Differing results may also be species related. For example, PRAs from the adult rat exhibit a relaxatory response to ACh (MacLean, *et al,* 1994a) whereas, as shown in this chapter, the ability of ACh to evoke relaxations is lost in the adult rabbit and a contractile response is noted. ACh- mediated vasoconstriction of small pulmonary vessels of the rabbit has previously been reported (Sada, *et al*, 1987). Catravas *et al* , (1984) also observed vasoconstrictor response to ACh in the rabbit pulmonary circulation. This phenomenon has also been shown in isolated pulmonary veins; in bovine vessels by Ignarro and colleges (1985) and in canine vessels by DeMay and Vanhoutte (1982).

Furthermore, in this chapter, the ACh- evoked response was altered from vasorelaxation, observed in fetal and neonate, to vasoconstriction, seen in adulthood and

this contractile response was evident at higher ACh concentrations in PRAs from 7 day old PRAs. Similarly, Steinhorn *et al* (1993) showed that preconstricted isolated intralobar pulmonary veins from juvenile sheep (5-6 weeks old) contracted to ACh and preparations from newborn animals relaxed to low ACh concentrations and then contracted to higher concentrations. These findings suggests an alteration in muscarinic receptor subtypes with developmental age. It has been shown in the central nervous system that distinct developmental time courses exist for muscarinic receptors (Coyle & Yamamura, 1976; Lee, *et al,* 1990). In addition, muscarinic receptor-mediated cellular responses develop over time (Lee, *et al,* 1990). Alternatively, the endothelium derived factor released in response to ACh from adult pulmonary resistance arteries may contractile in nature., e.g. ET, superoxide anions (Vanhoutte & Katusic, 1988).

Influence of NO on ET-receptor mediated responses

ET-1 and SXS6c were potent vasoconstrictors of fetal and neonatal rabbit PRAs. Vessels from rabbits at all age points demonstrated a marked contractile response to SXS6c, indicating the presence of a significant population of ET_B receptors mediating vasoconstriction. Similar findings have been documented for the large PA of the adult rabbit. (Panek, *et al.*, 1992; LaDouceur, *et al.*, 1993). My results in chapters 4 and 5 also indicate this to be the case also in the PRAs of the adult rabbit. Differences in sensitivity to these peptides were seen at different developmental ages, suggesting alterations of ET- receptor subtypes in the transitional pulmonary circulation. This is investigated further in chapter 9 (this thesis) using selective antagonists and is discussed more extensively.

Shaul *et al* (1993) measured NO production, as reflected by cGMP accumulation, in pulmonary artery segments of fetal and newborn lambs. They observed that pulmonary vascular NO production increased from late gestation until the fourth week after birth. Similarly, measurement of constitutive endothelial NOS mRNA in the rat showed that the highest levels were detected within 24 hrs after birth and this was

detected in endothelial cells lining small and medium sized blood vessels (Kawai *et al*, 1995). These finding suggests that pulmonary vascular NOS enzyme activity increases after birth. In this chapter, I investigated this possibility in the PRAs from fetal and neonatal rabbits using the NOS enzyme inhibitor L-NAME (Rees, *et al*, 1990).

L-NAME alone was shown to produce an increase in baseline tension of a variable magnitude in a proportion of the PRAs examined. The proportion of vessels exhibiting a response increased markedly between fetus and newborn and was greater at 7 days old. These findings could be due to an increased endogenous tone which is normally attenuated by NO. Alternatively they may suggest that an increase in basal NOS levels occurs following birth and in the first week of neonatal life in the rabbit. These results are also in agreement with previous studies in pig and sheep, where a diminished basal and stimulated release of NO from isolated pulmonary arteries of near-term fetus relative to animals only a few weeks older was reported (Abman, *et al*, 1991; Liu, *et al*, 1992; Steinhorn, *et al*, 1993; Zellers & Vanhoutte, 1991). Thus, the apparent increase in NO associated with birth may participate in the postnatal reduction of the pulmonary vascular resistance. Furthermore, the apparent increment from newborn to early neonate could aid the continued decrease in pulmonary vascular resistance which occurs during the days and weeks following birth (Rendas, *et al*, 1978).

Previous investigations suggest an active physiological role for both NO and ET-1 in the adaptation of the pulmonary circulation to extra-uterine life. Endo *et al* (1996) found an increase in serum NO metabolites and decrease in plasma ET-1 between birth and 5 days of age in healthy human neonates. In the newborn rat, endothelial cells of the pulmonary artery were shown, using immunocytochemistry techniques, to be rich in NOS and ET (Loesch & Burnstock, 1995). However to date there are few reports on the *in vitro* interaction between NO and ET in the pulmonary artery. Hence, in this chapter, I also investigated the possible influence of NO of ET-1 receptor -mediated responses in PRAs from fetal and neonatal rabbits using L-NAME.

In fetal rabbit PRAs the presence of L-NAME had no effect on sensitivity or magnitude of the maximal response of either ET-1- or SXS6c- evoked vasoconstrictions.

Vessels from newborn rabbits also demonstrated no change in sensitivity to ET-1 or SXS6c following NOS inhibition. The maximal contractile response to SXS6c was also unaffected whilst a marked augmentation of the ET-1- induced contractile response was noted following birth. Similar effects to those in the 0-24 hr preparation were shown in 4 day old rabbit vessels except the increase in ET-1-induced contractile response was substantially greater. The most pronounced effects of NOS inhibition on ET-receptor mediated responses were shown in PRAs from 7 day old rabbits. A marked augmentation was noted in both ET-1- and SXS6c- mediated vasoconstriction; the relative increase (compared with control maximum for each peptide) was greater in the SXS6c response. Furthermore, vessels from this postnatal age demonstrated a significantly increased sensitivity to ET-1, but a marked reduction in SXS6c potency following inhibition of NOS. These observation suggest that sensitivity to ET-1 is normally attenuated by NO in 7 day rabbit vessels. I am unable to explain, however, the apparent decrease in SXS6c sensitivity following blockade of NO in this preparation. In chapter 6 of this thesis I showed that L-NAME had a marked effect on ET-1 and SXS6c-induced responses in PRAs from coronary-ligated rabbits but was without effect in preparations from sham-operated animals. Other investigators have also reported that ET-1 may modulate its vasoconstrictor effect by the release of NO (De Nucci, *et al*, 1988; Rodman, *et al*, 1989). The NOS inhibitor L-NNA was shown to augment concentration-dependent contractions to ET-1 in isolated small (2-3 mm) porcine pulmonary veins but not arteries (Zellers, *et al*, 1994).

In comparison to neonatal vessels, the lack of effect of L-NAME on ET-receptor mediated responses of fetal PRAs provides further evidence for diminished NO levels in the pulmonary vasculature in fetal compared with neonatal life. This does not agree with the marked relaxatory response to ACh observed in these vessels however, there could be differential effects on control of basal NO (examined by L-NAME studies) and agonist-induced NO (studied via the use of ACh). This has also been suggested by Cremona *et al* (1994) after examining inhibition of NO release on vascular tone of isolated lungs of pig, sheep, dog and man. In addition, Mian and Martin (1995)

demonstrated in the rat aorta, that basal activity of NO was more sensitive to inactivation by superoxide anion than ACh-stimulated activity. Furthermore, the augmentation of ET-1- induced vasoconstriction in neonatal vessels indicates further the interaction of these endothelial derived agents in the transitional pulmonary circulation Due to the marked effects of NO and ET-1/ SXS6c on the PRAs, the balance of the NO/ET system in the adapting neonatal circulation would be crucial in determining pulmonary vascular resistance.

These observation suggests in particular that an increase in NO production is associated with birth. This may be basally released NO or NO released by agonist stimulation by ET-1 via the ET_{B1} receptor or ACh. Indeed, the sensitivity to SXS6c in this preparation progressively increased in PRAs from fetal to 7 day old rabbits (discussed in chapter 9). This result indicated an alteration in ET_B -receptor mediated responses and, taking the findings of this chapter into consideration also, a possibly increase in agonist-induced release of NO during the first week of life. Furthermore, the differential effect of L-NAME on SXS6c and ET-1 responses suggests a heterogeneous population of receptors, differentially influenced by NO. I also observed this phenomenon in adult rabbit PRAs (chapter 6 this thesis).

At the highest concentration of ET-1 and SXS6c, the response of the fetal and neonatal PRAs showed a dramatic "drop off " in tension. This is also evident in the SXS6c response in adult rabbit PRAs (chapter 4 this thesis). Endothelins are also known to mediate vasodilation via the release of NO from endothelial cells and this is thought to be mediated via ET_B receptor activation (Carville, *et al,* 1993, De Nucci, *et al.*, 1988; Eddahibi, *et al.*, 1991). At all age points, the presence of L-NAME caused the ET-1 CCRC to appear more biphasic in nature; the responses to higher ET-1 concentrations levelled off and the " drop off " in tension was less evident, particularly at 0-24 hours following birth. This may be indicative of the involvement of NO in the " drop off " in ET-1-evoked response. However, the fall in tension in the SXS6c CCRC at the higher concentration range is still observed in the presence of L-NAME. Although the actions of other mediators (e.g. PGI_2, EDHF) cannot be ruled out, this phenomenon may also be

due to desensitisation of the response as desensitisation of ET_B- receptor mediated responses has been demonstrated in the adult rabbit PA (LaDouceur, *et al.*, 1993) and swine pulmonary vein (Sudjarwo, *et al.*, 1993).

In this chapter I attempted to assess the vascular reactivity of rabbit PRAs at a time when marked alterations are occurring in the pulmonary circulation as it adapts to extra- uterine life. In summary, the contractile responses to KCl increased in magnitude with increasing age, implying smooth muscle extension or maturation in pulmonary resistance arteries of the same anatomical location at this time. Contractile responses to NA were smaller in fetal rabbit PRAs compared to newborn vessels. This may relate to a reduced basal NO level in the fetus, as suggested by the relatively lesser effect of L-NAME in fetal compared to newborn vessels. However, an augmented agonist-induced NO level is indicated before birth by the greater vasorelaxatory response to ACh in fetal than in newborn PRAs. Vasoconstrictions to receptor- mediated agents NA and 5-HT were comparatively greater in the newborn compared with 7 day old vessels. This may be related to the apparent increased level of NO in the 7 day vessels compared with 0-24 hr rabbit PRAs, which is indicated by (1) the increased vasodilator response to ACh and (2) the more pronounced effect of L-NAME on ET-receptor mediate responses in PRAs from 7 day old rabbits.

Chapter 9

Endothelin receptor subtypes in pulmonary resistance arteries from fetal and neonatal rabbits

9.1 Introduction

Endothelin-1 (ET-1) is one of the important endothelial-derived products that has been suggested to play an important role in the transition from *in utero* to postnatal pulmonary circulation (Zielger *et al.*, 1995). Usaki *et al* (1990) showed evidence of increased maternal plasma levels of ET-1 during labour and the presence of large amounts of ET-1 in the amniotic fluid. In the newborn pig, the concentration of plasma ET-1 was higher at birth than at 3 days or later (Levy *et al*, 1995). Loesch and Burnstock (1995) showed that the endothelial cells of the main pulmonary artery in the newborn rat are rich in ET (and NOS), suggesting a substantial involvement in the vasomotor control of the pulmonary circulation during the early stages of postnatal development. The many effects of ET on the pulmonary circulation suggests that it has a role in the transition from fetal to neonatal circulation (also see section 1.5.2).

ETs have also been implicated in many pathophysiologic conditions including pulmonary hypertension (PHT). The pathophysiological state of persistent pulmonary hypertension of the newborn (PPHN) can arise when, at birth, the normal decrease in PVR and increase in PBF does not occur. This condition results in substantial morbidity and mortality in more than 1 in 1000 newborn infants (Reece *et al*, 1987; Hageman *et al*, 1984). Elevated circulating ET-1 levels have been reported in patients with both primary and secondary PHT and in infants with PPHN (Stewart *et al*, 1991; Rosenberg et *al.*, 1993). Kumar *et al* (1996) have also shown a significant elevation in PPHN and a positive correlation with disease severity, suggesting that ET-1 may serve as a marker of the disease severity in these infants and in the development of PPHN. Selective inhibition of pulmonary ET-receptors may therefore effect pulmonary vasodilation.

In order to fully understand the possible role of endothelin (ET) in the normal adaptation of the pulmonary circulation at birth and the pathophysiological state of PPHN, it is important to classify which ET receptors are present in the pulmonary vasculature at different developmental ages. Two subtypes of mammalian (ET) receptor have been cloned and sequenced. The first was denoted ET_A and demonstrates

selectivity for endothelin-1 (ET-1) over ET-3 (Arai *et al.*, 1990). The other receptor, ET_B, is non-isopeptide selective (Sakurai *et al.*, 1990). Whilst both receptors have been shown to mediate contraction, the ET_B receptor may also mediate vasorelaxation via endothelial release of nitric oxide (Masaki *et al*, 1991). The following nomenclature was suggested to distinguish them: ET_{B1} for ET_B dilator and ET_{B2} for ET_B constrictor receptor (Sokolovsky *et al*, 1992; Warner *et al*, 1993). The ET receptors are discussed in chapter 1, section 1.4.5.

However, the types of ET receptor appear to vary with their localisation and between species. For example, vasoconstriction is evoked via ET_A receptor activation in the rat, dog and pig large pulmonary arteries (Douglas *et al*, 1993, MacLean *et al*, 1994, Nakamiche *et al*, 1992). However, in the rabbit large pulmonary artery ET_B receptors mediate vasoconstriction (La Douceur *et al*, 1993; Fukuroda *et al*, 1994; Hay *et al.*, 1996). My results from chapters 4 and 5 suggest that vasoconstrictor ET_B receptors also predominate in the small pulmonary resistance arteries of the rabbit. In human pulmonary resistance arteries ET-1-evoked vasoconstriction also appear to be mediated via stimulation of ET_B receptors (Chapter 5 this thesis; McCulloch *et al*, 1996). Studies have also demonstrated varied responses between *in vivo* and *in vitro* preparations. For example, ET_B receptor activation has also been shown to have no effect at rest but evoke selective pulmonary vasodilation during pulmonary hypertension in intact newborn lambs (Wong *et al.*, 1995). A vasodilator effect was also reported in neonatal pig pulmonary circulation (Pinheiro *et al*, 1993).

In analysing the results from studies *in vivo* or intact organ preparations *in vitro*, a role of parenchymal or vascular agents cannot be distinguished. Furthermore, the majority of *in vitro* studies on isolated pulmonary arteries have been on conductance vessels. However, as previously mentioned, it is the pulmonary resistance arteries (PRAs) which are the important determinants of pulmonary vascular resistance, hypoxic-induced vasoconstriction and pulmonary hypertension *in vivo* (Staub, 1985). Hence, I examined the responsiveness of PRAs to ET-1 from the fetal and neonatal rabbit.

In chapter 8 I investigated the reactivity of rabbit PRAs at various developmental ages. The effect of L-NAME on ET-receptor mediated responses was examined and discussed. In this chapter I investigated the ET receptor-subtype(s) responsible for vasoconstriction of PRAs before and after birth. Functional responses to ET-receptor stimulation in rabbit PRAs from fetal and neonatal rabbits at 3 age points during the first week of life were investigated using ET-1 and sarafotoxin S6c (SXS6c; an ET_B-selective agonist) (Williams *et al,* 1991). The ET receptor antagonists used to characterise the endothelin receptors were the ET_A -selective antagonist FR139317 (Sogabe *et al.,* 1992), BQ788, a potent and selective ET_B receptor antagonist (Ishikawa *et al,* 1994) and the non-selective ET_A/ET_B receptor antagonist SB209670 (Ohlstein *et al,* 1994a; 1994b).

9.2 Methods

Rabbit pulmonary resistance arteries

Fetal (2 days preterm) and neonatal New Zealand White rabbit pups were studied at 0-24 hrs, 4 days and 7 days after birth. The pregnant rabbits and fetal and neonatal pups were killed by sodium pentabarbitone as described in section 2.1.2. The lungs were promptly removed from the animals and small intralobar pulmonary resistance arteries (PRAs ~ 300μm i.d.) were dissected out according to the methods stated in section 2.2.3.2. These were mounted as ring preparations (~2mm long) on a wire myograph, bathed in Krebs solution at 37°C (see section 2.2.5). Using the normalisation process explained in section 2.2.5, vessels were tensioned to an equivalent transmural pressure of ~ 15 mmHg. This pressure was chosen as it is similar to *in vivo* pressures of rabbit pulmonary arterioles. Preliminary studies in our laboratory showed that bubbling with 95% O2 inhibits responses to vasoconstrictors in rabbit PRAs so we bubbled with 3% O_2/ 5% CO_2 balance N_2 for fetal rabbit vessels and 16% O_2/ 5% CO_2 balance N_2 for all

others. These gas mixtures were chosen as they are similar to those which the vessels would be exposed to *in utero* and *in vivo*.

Experimental protocol

Vessels were allowed to equilibrate for 1 hour prior to the addition of any drugs. The response of the PRAs to 50mM KCl was determined twice; once the contractile response had reached a plateau, the vessels were washed out at least 6 times with fresh Krebs solution. Cumulative concentration-response curves (CCRCs) were then constructed to ET-1 or SXS6c (1pM - 0.3μM) in PRAs following either a 45 minute "rest period" or 45 minute incubation period with the selected concentration of an ET receptor antagonist. The antagonists used were FR139317 (at 1μM), BQ788 (at 1μM) and SB209670 (at 0.1 and 1μM; examined in 0-24 hr and 4 day old preparations only).

In control SXS6c experiments, 0.1μM ET-1 was added to the bath at the end of the SXS6c CCRC, before washing, so as to estimate the SXS6c response as a percentage of response to a maximal ET-1 concentration.

Note

Control responses to ET-1 and SXS6c were carried out, whenever possible, in each lung preparation. Due to limitation in equipment and time it was not always possible to run a control CCRC to the peptide whilst studying the different antagonists. However the control curves in this study have been updated with each group of experiments . No differences were found in the control ET-1 or SXS6c responses when tested throughout the period of investigation, thus the data for the control curves which are shown have been pooled over many protocols. Unfortunately due to time constraints it was not possible to study a range of antagonists concentrations in each tissue. The concentrations which were studied, were chosen due to their pA_2/pK_B values in other vascular preparations.

Data analysis

Results are expressed graphically as percentage of their own maximum contraction, or percentage of reference contraction to second application of 50mM KCl. pEC_{10}, pEC_{25} and pEC_{50} values (where appropriate) were calculated according to the methods stated in section 2.5.2, and expressed as -log M concentration. Where ever possible, pK_B values were estimated for single stated concentration of antagonist according to the methods stated in section 2.5.3. Statistical comparison of the means of groups of data were made by Student's unpaired t test; $p<0.05$ was considered statistically significant. Throughout, data are expressed as mean±SEM and *n /n* = number of ring preparations / number of animals.

9.3 Results

The average internal diameters (μm) and equivalent transmural pressures (mmHg) of PRAs from fetal and neonatal rabbits examined in this chapter were as follows: fetal- 334.1±12.6 μm / 13.0±0.8 mmHg; 0-24 hr- 323.2±55.4 / 12.2±0.6; 4 day- 333.9±15.6 / 13.0±0.6; 7 day- 315.8±26.9 / 12.5±0.9 (n= > 15 preparations in each case). Thus PRAs from animals at these age points exhibited similar internal diameters when set up at the same equivalent internal diameters.

Responses to ET-1 and SXS6c

Both ET receptor agonists, ET-1 and SXS6c, were potent vasoconstrictors of rabbit pulmonary resistance arteries at all age points studied. Figures 9.1-9.4 show the response to both peptides at the various ages and data for pEC_{10}, pEC_{25} and pEC_{50} values are summarised in table 9.1. ET-1 evoked a phasic response in fetal and neonatal preparations. The first component had a gradual slope up to 0.1nM in fetal and 0-24 hr vessels and 1nM ET-1 in 4 and 7 day old vessels. A markedly steeper component up to

Developmental Age	ET-1				SXS6c			
	n/n	pEC_{10}	pEC_{25}	pEC_{50}	n/n	pEC_{10}	pEC_{25}	pEC_{50}
Fetal	6/6	9.5±0.5	9.2±0.3	8.7±0.2[a]	6/6	9.6±0.4	9.5±0.3	8.6±0.3
0-24 hours	6/6	9.7±0.3	9.2±0.2[a**]	8.8±0.2[a**]	6/6	10.4±0.1	10.1±0.1	9.7±0.1 [b]
4 days	7/6	10.0±0.3[a***]	9.3±0.2[aa***]	8.6±0.1[a***]	6/6	11.8±0.2[bbcc]	11.3±0.2 [bbcc]	10.7±0.3 [bbc]
7 days	6/6	8.9±0.2 [a***]	8.5±0.1***	8.0±0.2 ***	6/6	11.7±0.1 [bbcc]	11.5±0.1 [bbcc]	11.1±0.2 [bbcc]

Table 9.1 Sensitivity to ET-1 and SXS6c in PRAs from fetal and neonatal rabbits.

Statistical comparisons were made by ANOVA followed by Tukey's post test: ET-1 vs. SXS6c at same age point ** $p<0.01$, ***$p<0.001$; 7 day old ET-1 vs. other age points [a] $p<0.05$, [aa]$p<0.01$; fetal SXS6c vs. other age points [b] $p<0.01$, [bb] $p<0.001$; 0-24 hour old SXS6c vs. other age points [c] $p<0.01$, [cc] $p<0.001$. Values are mean ± SEM. ET-1, endothelin-1; sarafotoxin S6c, SXS6c; n/n, number of ring preparations / number of animals.

10-30nM ET-1 was then observed, with a fall in the contraction at higher concentrations. In a substantial percentage of the experiments, ET-1 and SXS6c elicited phasic changes in tone of variable amplitude and synchronisation, which persisted at times beyond the tonic phase of the response.

Similar sensitivity to ET-1 was noted in the PRAs from fetal, 0-24 hour and 4 day old rabbits, which in turn, were all significantly more sensitive than vessels from 7 day old animals. 4 day vessels were markedly more sensitive to ET-1 than 7 day rabbit PRAs at all levels, i.e. pEC_{10}, pEC_{25} and pEC_{50} (table 9.1). The values for the magnitude of the maximal contractile responses are shown on table 9.2. ET-1 evoked a similar maximum response at all age points studied (~93% of KCl response).

	Endothelin-1	n/n	Sarafotoxin S6	n/n
Fetal	95.3±9	6/6	39.5±13.2 **	6/6
0-24 hours	84±14	6/6	97.8±27.7	6/6
4 days	101.7±16.8	7/6	86.1±8.9	6/6
7 days	92.7±11.1	6/6	41.3±7.1**	6/6

Table 9.2 Maximal contractile responses to ET-1 and SXS6c in PRAs from fetal and neonatal rabbits. Data are expressed as a percentage of reference contraction to second application of 50mM KCl. Statistical comparisons were made by Students' unpaired t-test: ET-1 vs. SXS6c at same age point, ** $p<0.01$. Values are mean ± SEM. ET-1, endothelin-1; sarafotoxin S6c, SXS6c; n/n, number of ring preparations / number of animals.

SXS6c also evoked a marked contractile response. According to pEC_{50} values, the order of potency for SXS6c was 7 days = 4 days > 0-24 hours > fetal (table 9.1). Like ET-1, SXS6c also produced a phasic response with an eventual fall in the contraction. The concentration at which this drop off of the maximum response occurred varied with age, being noted over 0.1nM in fetal PRAs and then progressively lower concentrations with increasing age. Maximal contractions to SXS6c were similar in 0-24

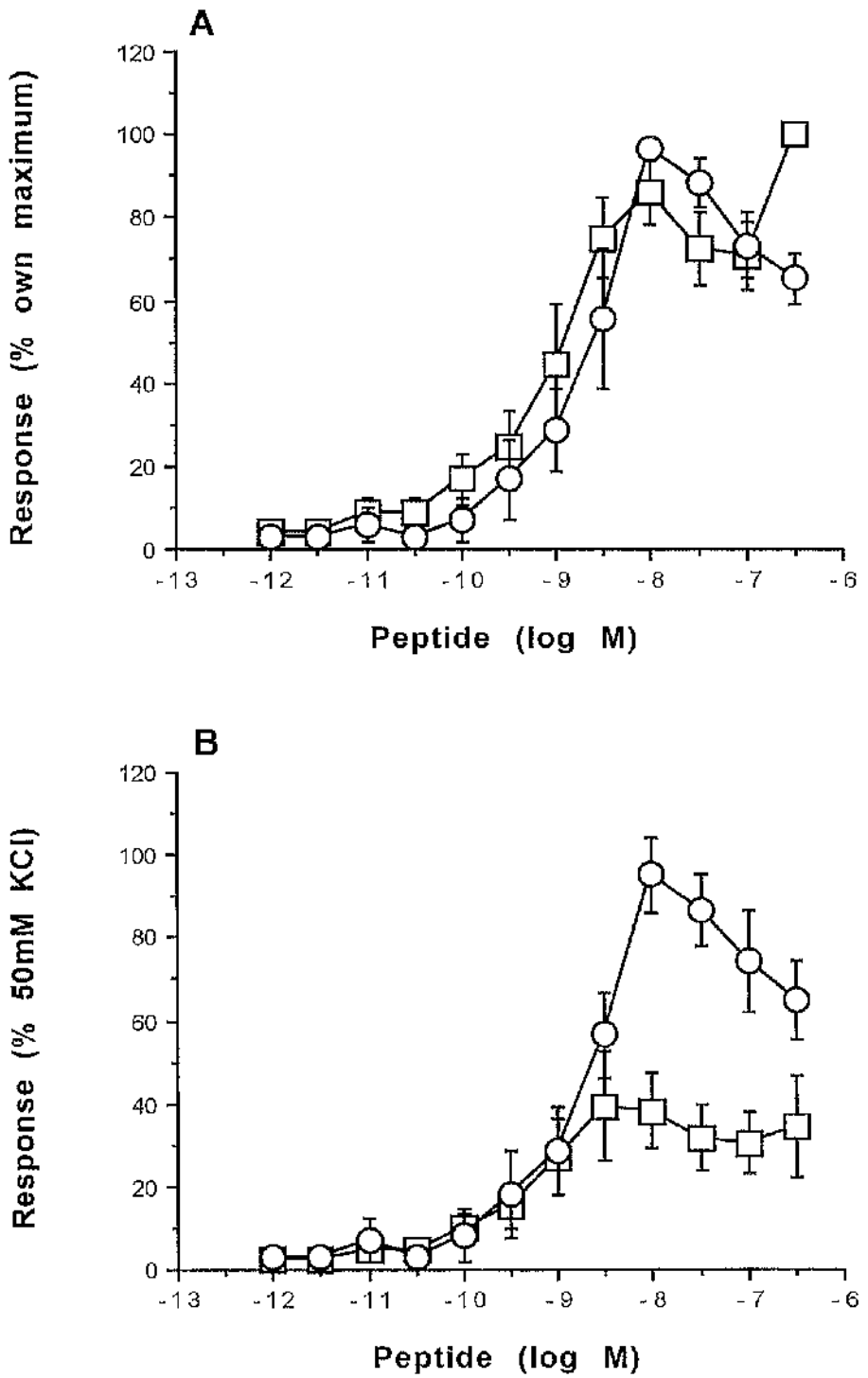


Figure 9.1 Responses to ET-1 and SXS6c in PRAs from fetal rabbits. CCRC's to ET-1 (○, n=6/6) and SXS6c (□, n=6/6). **A** Data are expressed as a percentage own maximum contraction. **B** Data are expressed as a percentage reference contraction to 50mM KCl. Each point represents mean ± s.e. mean.

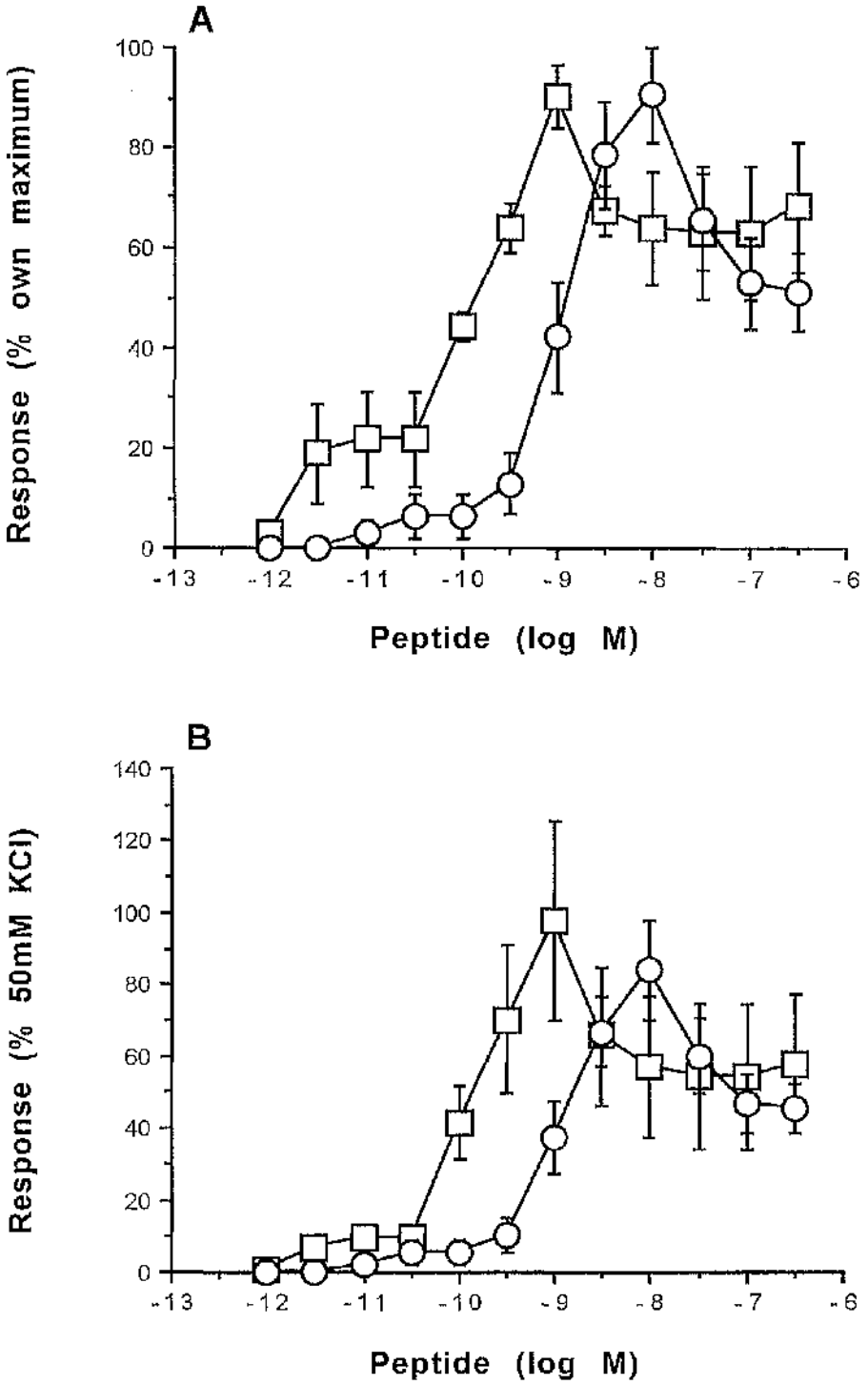


Figure 9.2 Responses to ET-1 and SXS6c in PRAs from 0-24 hours old rabbits. CCRC's to ET-1 (○, n=6/6) and SXS6c (❑, n=6/6). **A** Data are expressed as a percentage own maximum contraction. **B** Data are expressed as a percentage reference contraction to 50mM KCl. Each point represents mean ± s.e. mean.

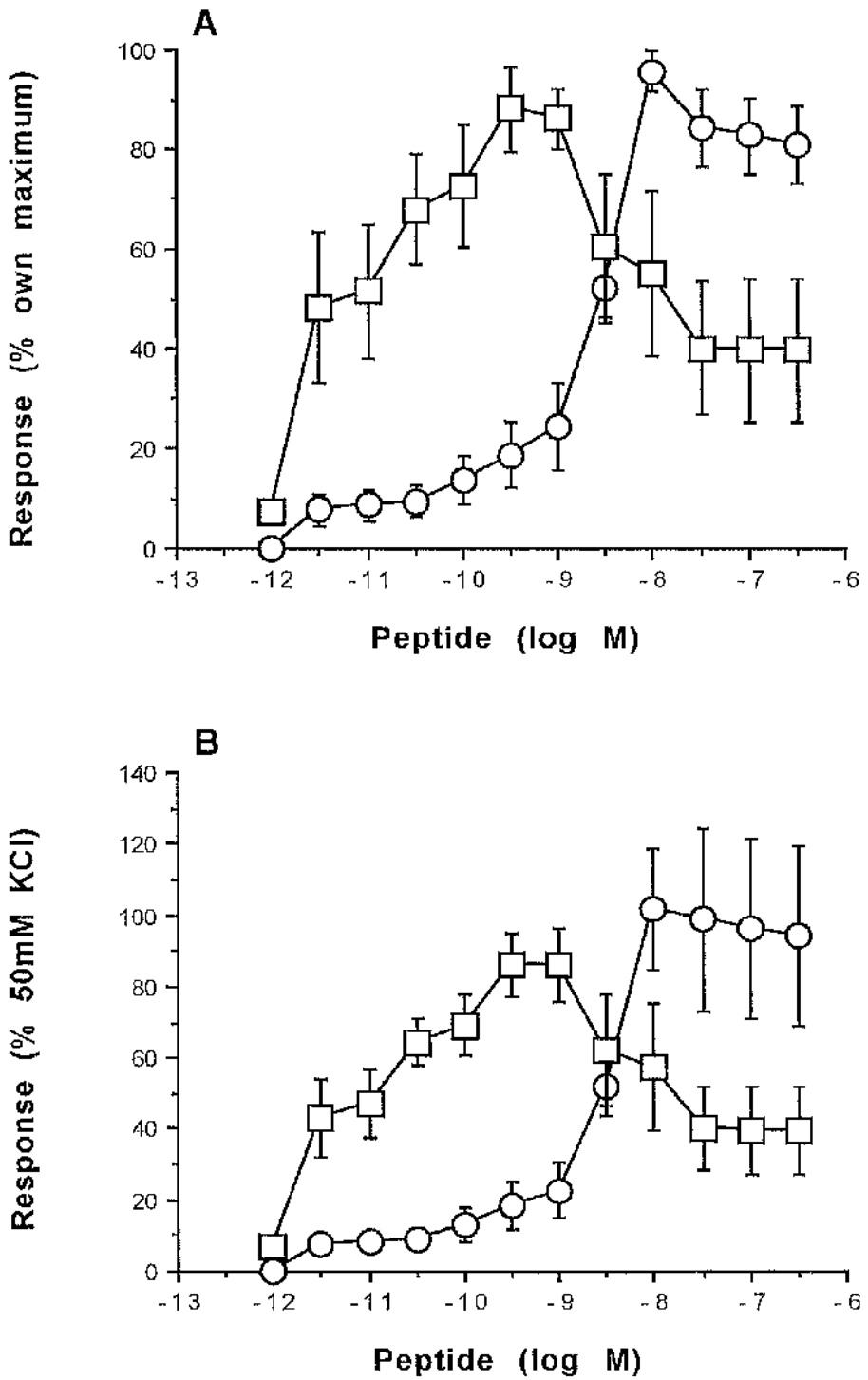


Figure 9.3 Responses to ET-1 and SXS6c in PRAs from 4 day old rabbits. CCRC's to ET-1 (○, n=7/6) and SXS6c (❑, n=6/6). **A** Data are expressed as a percentage own maximum contraction. **B** Data are expressed as a percentage reference contraction to 50mM KCl. Each point represents mean ± s.e. mean.

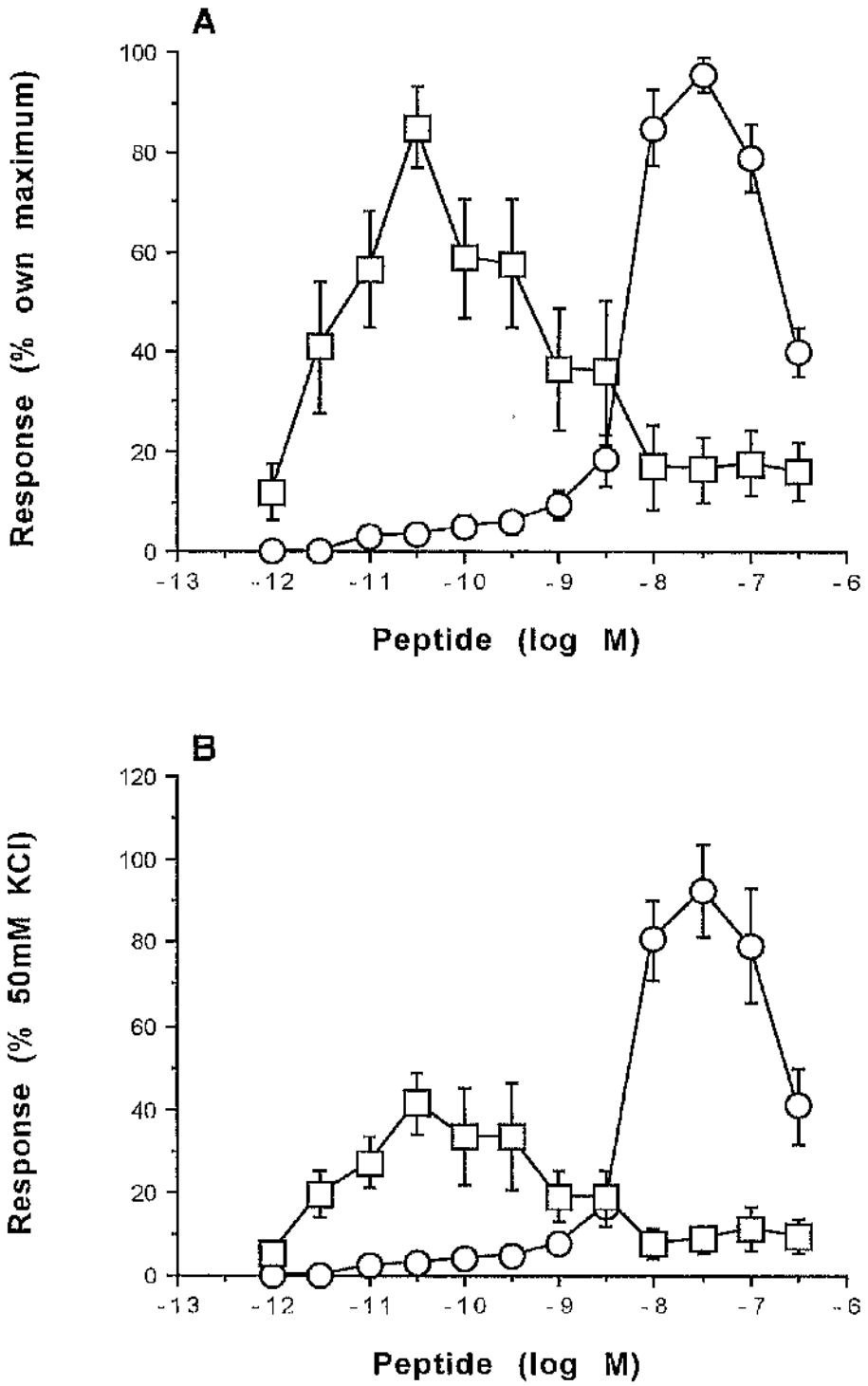


Figure 9.4 Responses to ET-1 and SXS6c in PRAs from 7 day old rabbits. CCRC's to ET-1 (○, n=6/6) and SXS6c (□, n=6/6). **A** Data are expressed as a percentage own maximum contraction. **B** Data are expressed as a percentage reference contraction to 50mM KCl. Each point represents mean ± s.e. mean.

hour and 4 day old vessels (~90% of KCl response, table 9.2) and were significantly greater than that observed in fetal and 7 day old rabbit PRAs (~40%). The maximum contractile response to SXS6c when expressed as a percentage of the response to 0.1μM ET-1 in the same preparation was similar at all age points, being ~ 57% (47-62%). In summary, the potency of SXS6c increased markedly with developmental age and the concentration at which the desensitisation occurred markedly decreased with developmental age. This indicates a marked alteration in the ET_B- receptor mediated response during the first week of life.

ET-1 and SXS6c were equipotent in the fetal PRAs (figure 9.1A) however the magnitude of the SXS6c maximum response was markedly smaller (figure 9.1B, table 9.2). In comparison, SXS6c was significantly more potent than ET-1 at 0-24 hours following birth (figure 9.2A) and this greater potency was even more pronounced at 4 (figure 9.3A) and 7 days old (figure 9.4A). The magnitude of the SXS6c maximum response was similar to that noted to ET-1 in the 0-24 hour (figure 9.2B) and 4 day old preparation (figure 9.3B) but as was seen in the fetus, was significantly smaller than the ET-1 maximum by 7 days following birth (figure 9.4B; table 9.2).

Effect of antagonists on ET receptor mediated responses

FR139317 vs. ET-1

pEC_{25} and pEC_{50} values for ET-1 in the presence and absence of FR139317 are summarised in table 9.3. Figures 9.5-9.9 illustrate the effect of this antagonist on ET-1-induced responses at the different age points. The selective ET_A receptor antagonist FR139317 failed to inhibit the ET-1-induced vasoconstriction in fetal PRAs (figure 9.5A). In comparison, ET-1 responses in PRAs from 0-24 hour (figure 9.6A) and 4 day old (figure 9.7A) rabbits were significantly inhibited by this antagonist. The estimated pK_b was 6.41±0.16 for 0-24 hour PRAs but this value and pEC values could not be calculated for 4 day results since the responses in the presence of the antagonist did not

	Fetal		0-24 hours		4 days		7 days	
	pEC_{25}	pEC_{50}	pEC_{25}	pEC_{50}	pEC_{25}	pEC_{50}	pEC_{25}	pEC_{50}
Endothelin-1 (n/n)	9.2±0.3 (6/6)	8.1±0.2	9.2±0.2 (6/6)	8.8±0.2	9.3±0.2 (7/6)	8.6±0.1	8.5±0.1 (6/6)	8.0±0.2
ET-1 + FR (1μM) (n/n)	8.7±0.7 (6/6)	8.4±0.3	8.6±0.1* (6/6)	8.2±0.2 *	nc (6/6)		8.4±0.3 (6/6)	7.7±0.3
ET-1 + BQ (1μM) (n/n)	nc (6/6)		8.3±0.1** (6/6)	8.0±0.1**	8.6±0.0** (6/6)	8.2±0.04**	8.9±0.2 (7/7)	8.3±0.2
ET-1 +SB (1μM) (n/n)	ns		nc (4/4)		nc (5/5)		ns	
ET-1 + SB (0.1μM) (n/n)	ns		7.8±0.1*** (5/5)	7.5±0.1***	8.2±0.2** (6/6)	7.6±0.2***	ns	

Table 9.3 Sensitivity of fetal and neonatal rabbit PRAs to ET-1 in the presence and absence of selective antagonists.

Statistical comparisons were made by ANOVA followed by Tukey's post test: control ET-1 vs. presence of antagonist *$p<0.05$, ** $p<0.01$, ***$p<0.001$. Values are mean ± SEM. ET-1, endothelin-1; n/n, number of ring preparations / number of animals; ns, not studied; nc, not calculated.

FR = FR139317 ET_A receptor antagonist; BQ = BQ788 ET_B receptor antagonist; SB= SB209670 non-selective $ET_{A/B}$ receptor antagonist

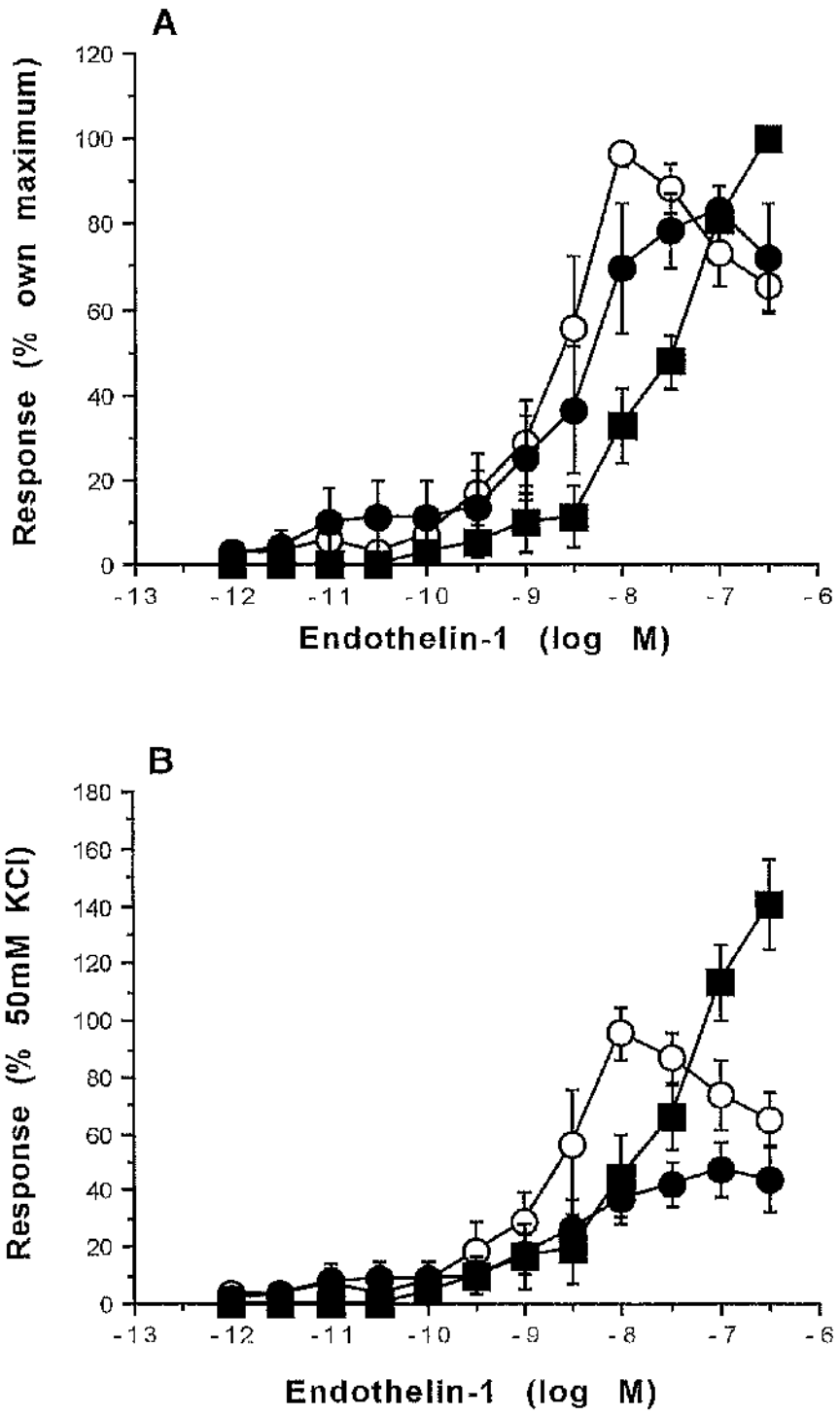


Figure 9.5 Responses to ET-1 in fetal rabbit PRAs: effect of selective antagonists. CCRC's to ET-1 (○, n=6/6), in the presence of 1μM FR139317 (● n=6/6) and in the presence of 1μM BQ788 (■, n=6/6). **A** Data are expressed as a percentage own maximum contraction. **B** Data are expressed as a percentage reference contraction to 50mM KCl. Each point represents mean ± s.e. mean.

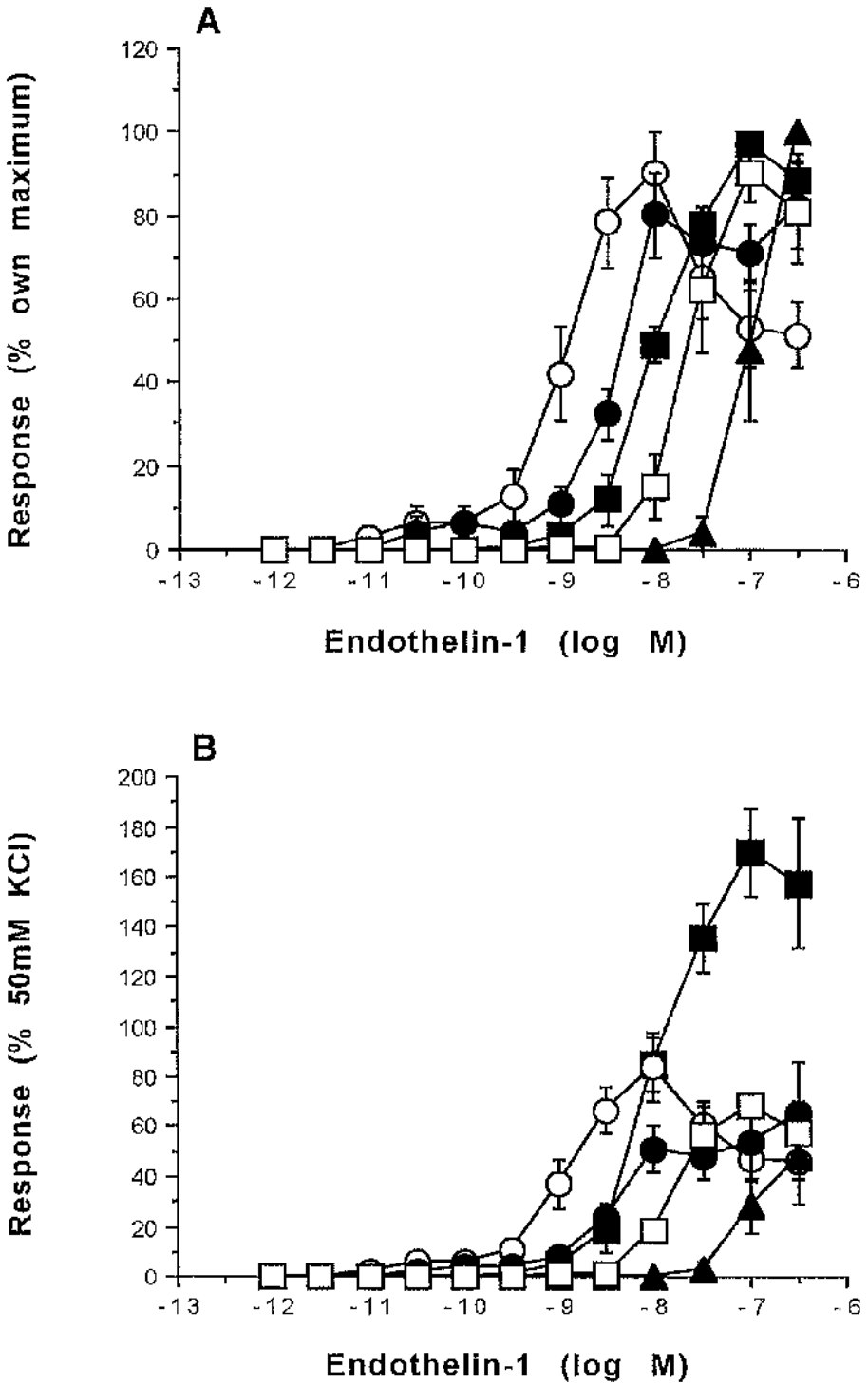


Figure 9.6 Responses to ET-1 in 0-24 hr old rabbit PRAs: effect of selective antagonists. CCRC's to ET-1 (○, n=6/6), in the presence of 1μM FR139317 (●, n=6/6), 1μM BQ788 (■, n=6/6), 0.1μM SB209670 (❑, n=5/5), and 1μM SB209670 (▲, n=4/4). **A** Data are expressed as a percentage own maximum contraction. **B** Data are expressed as a percentage reference contraction to 50mM KCl.

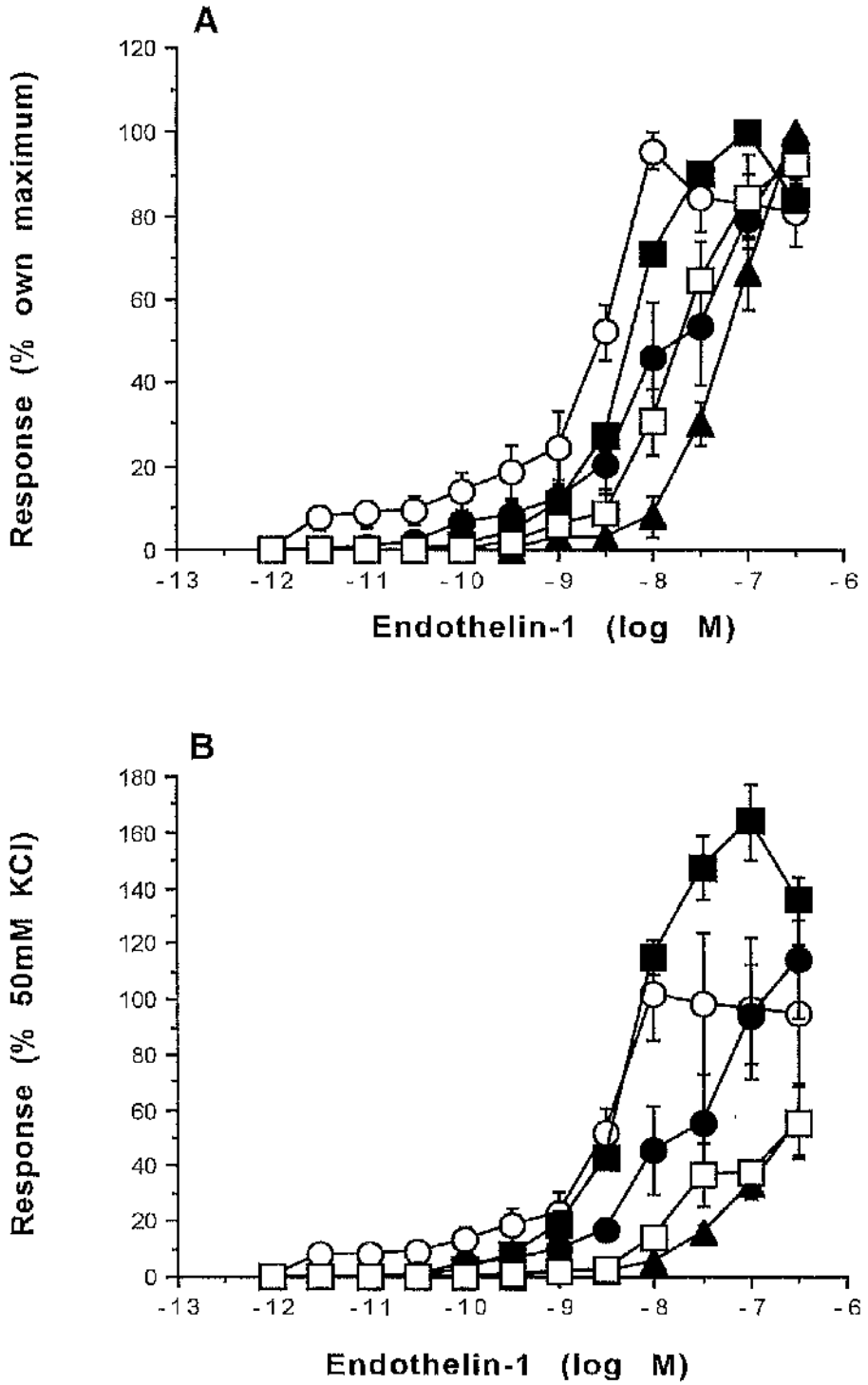


Figure 9.7 Responses to ET-1 in 4 day old rabbit PRAs: effect of selective antagonists. CCRC's to ET-1 (○, n=6/6), in the presence of 1μM FR139317 (●, n=6/6), 1μM BQ788 (■, n=6/6), 0.1μM SB209670 (❑, n=6/6), and 1μM SB209670 (▲, n=5/5). **A** Data are expressed as a percentage own maximum contraction. **B** Data are expressed as a percentage reference contraction to 50mM KCl. Each point represents mean±SEM.

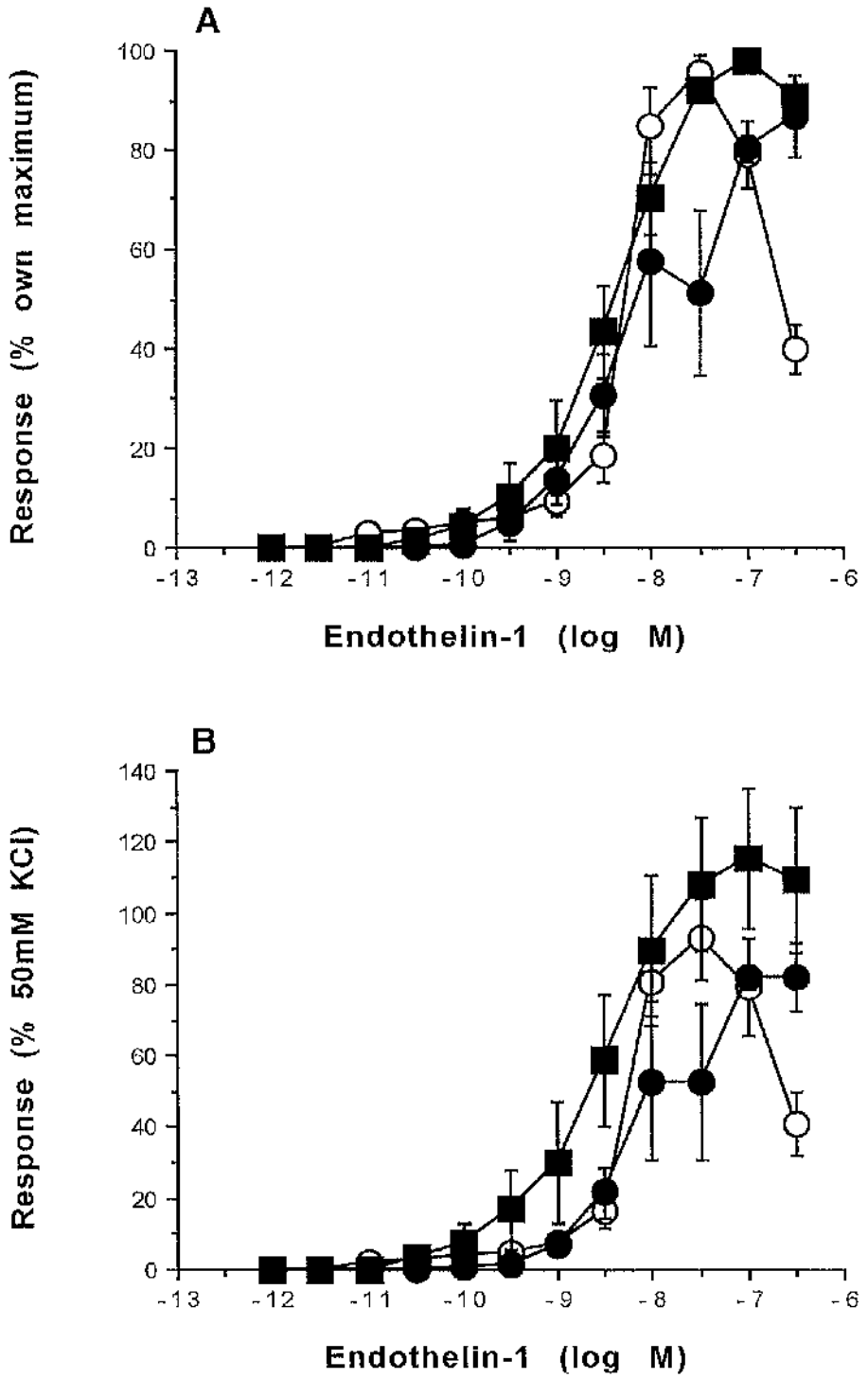


Figure 9.8 Responses to ET-1 in 7 day old rabbit PRAs: effect of selective antagonists. CCRC's to ET-1 (○, n=6/6), in the presence of 1μM FR139317 (● n=6/6) and in the presence of 1μM BQ788 (■, n=7/7). **A** Data are expressed as a percentage own maximum contraction. **B** Data are expressed as a percentage reference contraction to 50mM KCl. Each point represents mean ± s.e. mean.

reach a maximum. In 7 day old rabbit PRAs, inhibition was not evident at the level of pEC values which were calculated. However as can be seen from figure 9.8A, FR139317 caused a shift in the steep component of the ET-1- induced CCRC, which occurred at concentrations above 3nM, with an estimated pK_B value of 6.76±0.2. This is further illustrated by the reduction in the response to 30nM ET-1 from 92.7±11.1% to 52.8±21.8%.

Furthermore, FR139317 produced a marked reduction in the maximum ET-1 response of the fetal PRAs (control 95.3±9.0 vs. 47.4±9.9, $P<0.01$). The magnitude of the maximal contraction to ET-1 was not significantly altered in PRAs from 0-24 hr and 7 day old rabbits. In the presence of FR139317, the ET-1 response in 4 day old preparations did not reach a maximum within the concentration range of peptide studied.

BQ788 vs. ET-1

Figures 9.5-9.8 show the effect of 1μM BQ788 on ET-1- induced vasoconstriction. pEC_{25} and pEC_{50} values and maximal contractile responses for ET-1 in the presence and absence of BQ788 are illustrated in tables 9.3 and 9.4, respectively. This selective ET_B receptor antagonist significantly inhibited ET-1 responses in PRAs from fetal, 0-24 hour and 4 day old rabbits (figure 9.5A, 9.6A, 9.7A; table 9.3). A significant increase in pEC_{10} value was also noted in the vessels from 0-24 hr ($P<0.01$) and 4 day old rabbits ($P<0.05$). The estimated pK_B value was 6.71±0.13 for 0-24 hour rabbit PRAs ant this was significantly greater than the value of 6.23±0.08 calculated for 4 day old PRAs ($P<0.05$). A value for pK_B and pEC values could not be calculated for fetal vessel responses since a maximum was not reached within the ET-1 concentration range studied. However the rightward shift in the response in fetal PRAs was demonstrated further by the significant decrease in the magnitude of the control maximum contraction, attained at 10nM ET-1, from 95.3±9.0% to 44.9±14.4% in the presence of BQ788 ($P<0.05$; figure 9.5B). BQ788 failed to inhibit ET-1 CCRCs of 7 day old rabbit PRAs and did not significantly alter the maximum contractile response (figure

9.8; table 9.3, 9.4). In comparison, this selective ET_B receptor produced marked increase (~70%) in the magnitude of the maximum contraction 0-24 hr and 4 day preparations (figure 9.6B, 9.7B; table 9.4).

	ET-1	n/n	ET-1+ 1μM BQ788	n/n
0-24 hours	84.0±14.0	6/6	169.6±17.7 **	6/6
4 days	101.7±16.8	7/6	163.9±13.4 *	6/6
7 days	92.7±11.1	6/6	115.4±19.9	7/7

Table 9.4 Maximal responses to ET-1 in the presence and absence of BQ788 in PRAs from neonatal rabbits. Data are expressed as a percentage of reference contraction to second application of 50mM KCl. Statistical comparisons were made by Students' unpaired t- test: ET-1 vs. ET-1+ BQ at same age point; * $P<0.05$,** $P<0.01$. Values are mean ± SEM. ET-1, endothelin-1; n/n, number of ring preparations / number of animals.

BQ788 vs. SXS6c

Figures 9.9-9.12 show the effect of 1μM BQ788 on SXS6c induced vasoconstrictions. pEC_{25} and pEC_{50} values for SXS6c in the presence and absence of BQ788, which could be calculated, are illustrated in table 9.5. BQ788 produced a dramatic rightward shift in the SXS6c- induced responses at all age points studied. Inhibition was most pronounced in the neonate PRA response. BQ788 abolished responses to lower SXS6c concentrations; the threshold concentration was shifted from 1pM to 10nM in 0-24 hr vessels (figure 9.10A) and ~1nM in 4 day old rabbit PRAs (figure 9.11A). This was further illustrated by the significant decrease in the pEC_{10} value in 4 day PRA SXS6c response ($P<0.01$). The maximum SXS6c- induced vasoconstriction in 4 day rabbit PRAs was not altered in the presence of BQ788 (figure 9.11B). SXS6c plus BQ788 response failed to reach a maximum within the concentration range examined in vessels from fetal (figure 9.9B), 0-24 hr (figure 9.10B)

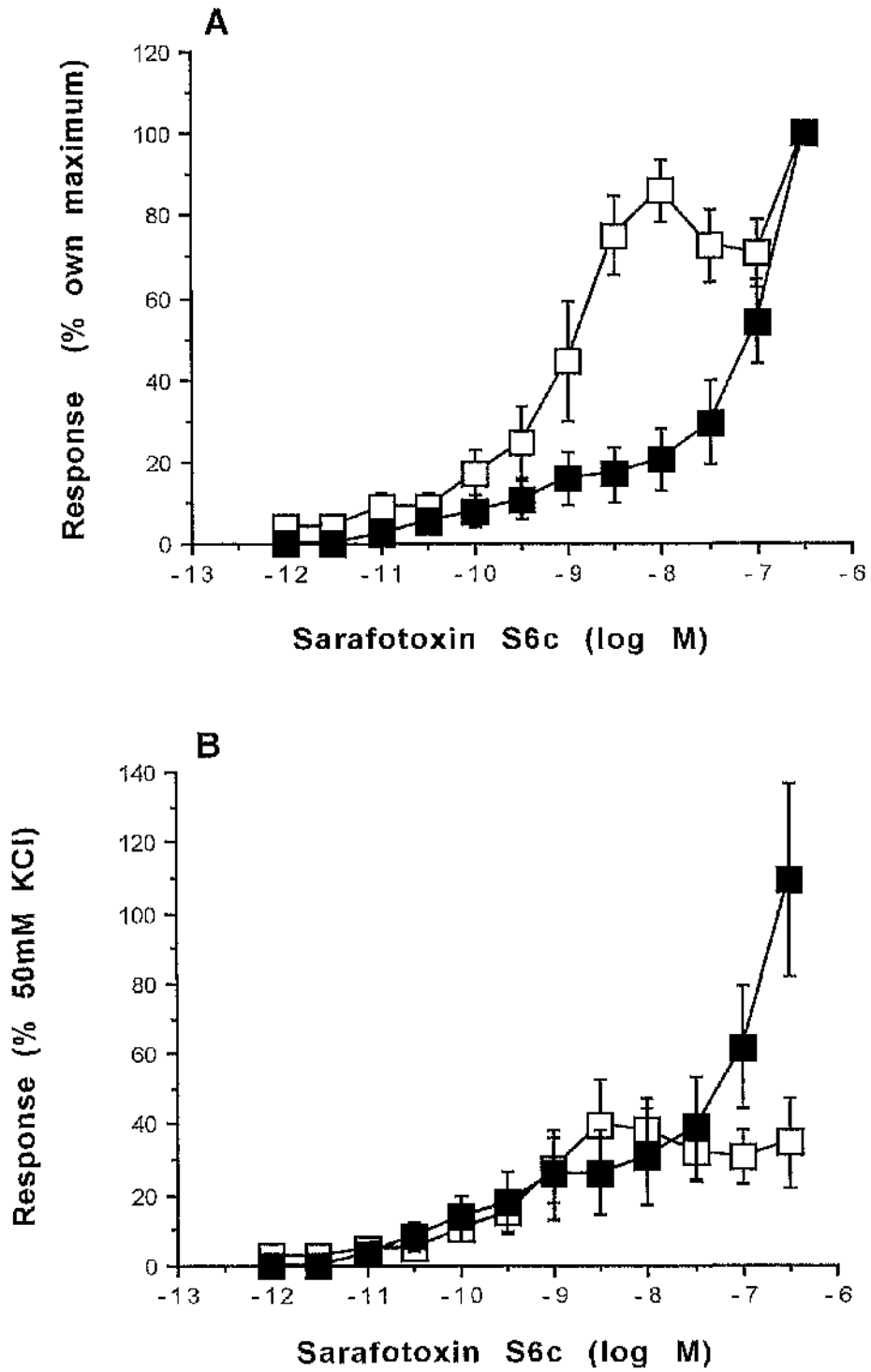


Figure 9.9 Responses to SXS6c in PRAs from fetal rabbits: effect of BQ788 CCRC's to SXS6c (□, n=6/6) and in the presence of 1μM BQ788 (■, n=7/7). **A** Data are expressed as a percentage own maximum contraction. **B** Data are expressed as a percentage reference contraction to 50mM KCl. Each point represents mean ± s.e. mean.

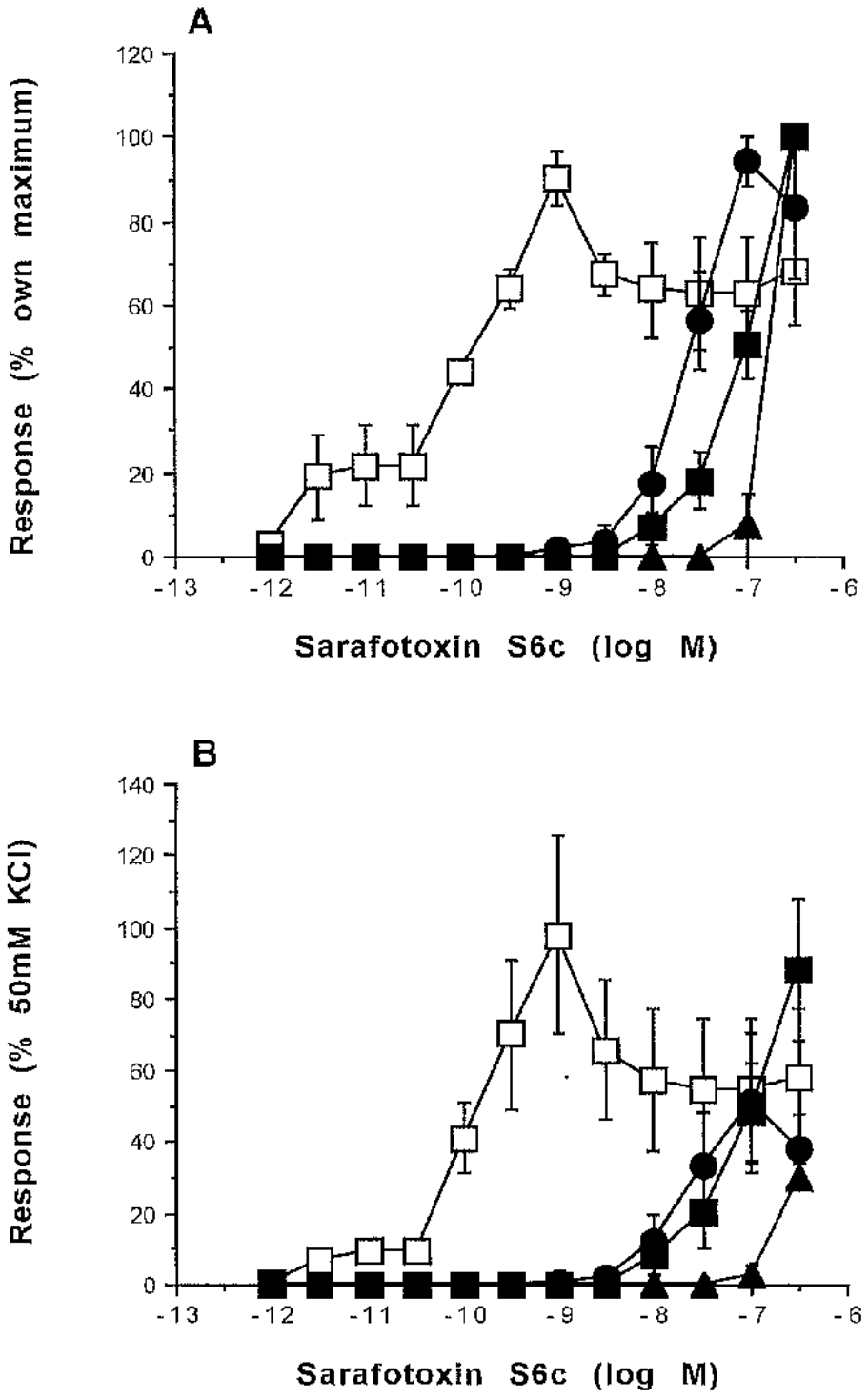


Figure 9.10 Responses to SXS6c in 0-24 hr old rabbit PRAs: effect of selective antagonists. CCRC's to SXS6c (□, n=6/6), in the presence of 1μM BQ788 (■, n=5/5), 0.1μM SB209670 (● n=3/3), and 1μM SB209670 (▲, n=2/2). **A** Data are expressed as a percentage own maximum contraction. **B** Data are expressed as a percentage reference contraction to 50mM KCl. Each point represents mean ± s.e.m.

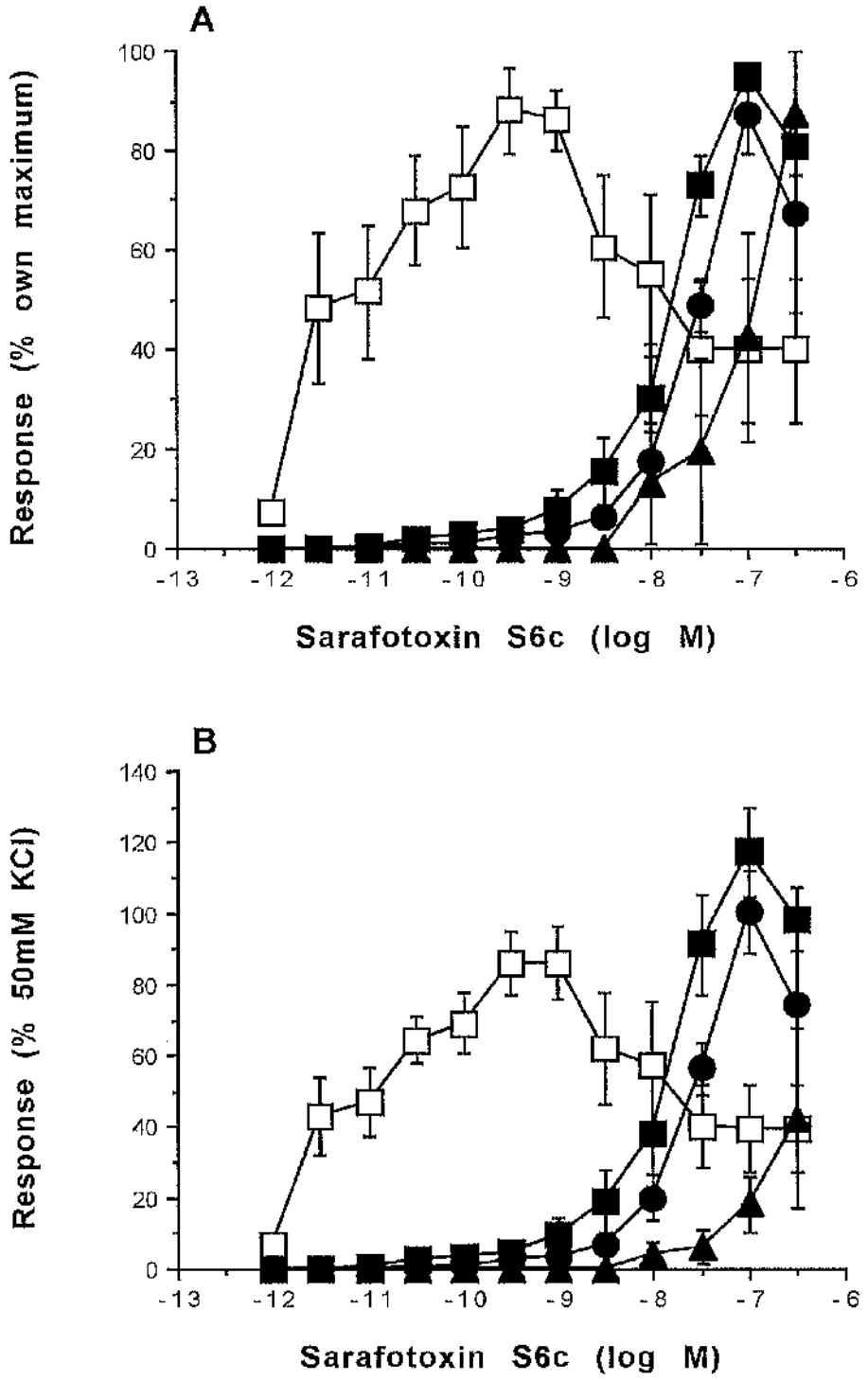


Figure 9.11 Responses to SXS6c in 4 day old rabbit PRAs: effect of selective antagonists. CCRC's to SXS6c (□, n=6/6), in the presence of 1μM BQ788 (■, n=8/7), 0.1μM SB209670 (● n=5/5), and 1μM SB209670 (▲, n=4/4). **A** Data are expressed as a percentage own maximum contraction. **B** Data are expressed as a percentage reference contraction to 50mM KCl. Each point represents mean ± s.e.m.

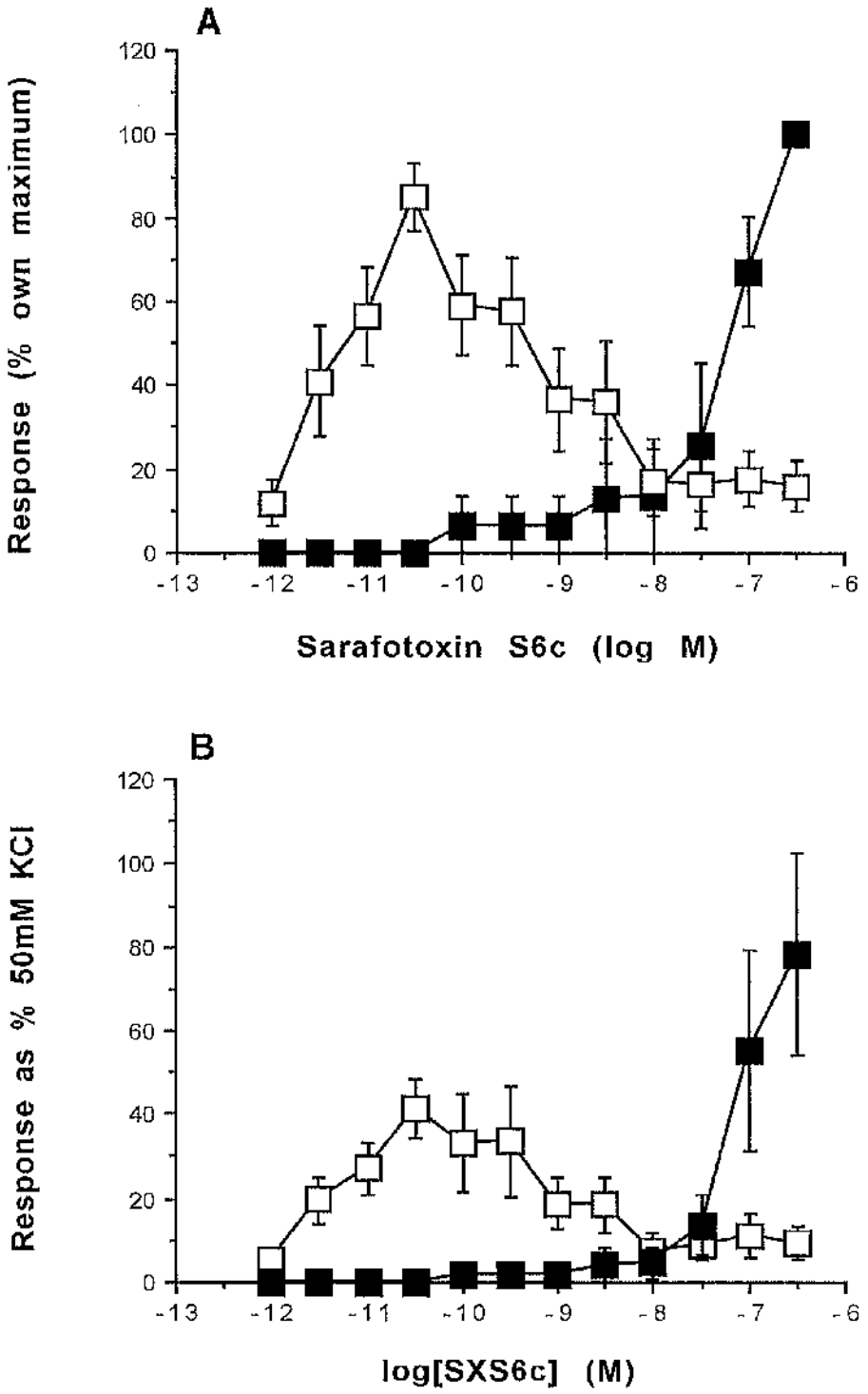


Figure 9.12 Responses to SXS6c in PRAs from 7 day old rabbits: effect of BQ788 CCRC's to SXS6c (□, n=6/6) and in the presence of 1μM BQ788 (■, n=4/4). **A** Data are expressed as a percentage own maximum contraction. **B** Data are expressed as a percentage reference contraction to 50mM KCl. Each point represents mean ± s.e. mean.

	Fetal		0-24 hours		4 days		7 days	
	pEC_{25}	pEC_{50}	pEC_{25}	pEC_{50}	pEC_{25}	pEC_{50}	pEC_{25}	pEC_{50}
Sarafotoxin S6c (n/n)	9.5±0.3 (6/6)	8.6±0.3	10.1±0.1 (6/6)	9.7±0.1	11.3±0.2 (6/6)	10.7±0.3	11.5±0.1 (6/6)	11.1±0.2
SXS6c + 1μM BQ (n/n)	nc (7/7)		nc (5/5)		8.2±0.2*** (8/7)	7.8±0.1***	nc (4/4)	
SXS6c + 1μM SB (n/n)	ns		nc		nc (4/4)		ns	
SXS6c + 0.1μM SB (n/n)	ns		7.9±0.1*** (3/3)	7.5±0.1***	7.8±0.1*** (5/5)	7.5±0.1***	ns	

Table 9.5 Sensitivity of fetal and neonatal rabbit PRAs to SXS6c in the presence and absence of selective antagonists.

Statistical comparisons were made by ANOVA followed by Tukey's post test: control SXS6c vs. presence of antagonist *p<0.05, ** p<0.01, ***p<0.001. Values are mean ± SEM. Sarafotoxin S6c, SXS6c; n/n, number of ring preparations / number of animals; ns, not studied; nc not calculated.

BQ = BQ788 ET_B receptor antagonist; SB= SB209670 non-selective $ET_{A/B}$ receptor antagonist.

and 7 day animals (figure 9.12B), thus preventing calculation of pK_B and pEC values. An estimated pK_B value of 8.7±0.12 was calculated for PRAs from 4 day old rabbits.

SB209670 vs. ET-1

The effect 0.1 and 1μM SB209670 on ET-1-induced responses in 0-24 hour and 4 day old rabbit PRAs are shown in figures 9.6 and 9.7, respectively. pEC_{25} and pEC_{50} values which could be calculated are summarised in table 9.3. SB209670 caused a very significant inhibition of the entire ET-1 CCRC in PRAs from 0-24 hr and 4 day old rabbits. This inhibition was concentration dependent; 0.1μM SB209670 produced an ~ 11 fold shift in the ET-1 pEC_{50} and a greater shift was apparent in the presence of 1μM antagonist. Threshold ET-1 concentration evoking vasoconstriction was shifted from 10pM to 0.3-1nM by 0.1μM SB209670 and to 1-30nM by 1μM SB209670 (figures 9.6A and 9.7A). This pronounced inhibition was further demonstrated by the marked shift in the response at the level of the pEC_{10} value; ET-1 control vs. ET-1 + 0.1μM SB209670, $P<0.01$. The maximal contractile response to ET-1 was not altered by 0.1μM SB209670 in 0-24 hr or 4 day rabbit vessels. ET-1 response failed to attain a maximum in the presence of 1μM SB209670 at both neonatal ages (figures 9.6B and 9.7B), thus estimations of pEC and pK_B values unfortunately could not be made. However calculated pKB values for 0.1μM SB 209670 against ET-1 responses in 0-24 hour and 4 day rabbit PRAs were 8.3±0.1 and 8.0±0.1, respectively.

SB209670 vs. SXS6c

Figure 9.11 shows the effect 0.1 and 1μM SB209670 on SXS6c-induced responses in 4 day old rabbit PRAs and pEC_{25} and pEC_{50} values are summarised in table 9.5. Preliminary data for PRAs from newborn rabbits are also shown in figures 9.10. Contractile responses to SXS6c were almost completely abolished by SB209670 in PRAs from 4 day old rabbits The threshold concentration evoking vasoconstriction was

shifted from 1pM to 1nM SXS6c by 0.1μM SB209670 and to 10nM SXS6c in the presence of 1μM antagonist (figure 9.11A; control ET-1 pEC_{10} vs. ET-1+ 0.1μM SB209670 pEC_{10}, $P<0.001$). An even greater inhibition was noted in SXS6c- evoked responses compared to ET-1- induced vasoconstrictions. 0.1μM SB209670 caused an ~30 fold shift in the SXS6c pEC_{50} and an even greater shift was seen in the presence of 1μM SB209670 (figure 9.11A). The maximal contractile response was not reached within the peptide concentration range studied when 1μM SB209670 was present, thus agonist potencies in the presence of this antagonist could not be estimated. The maximum vasoconstriction was not significantly altered from control SXS6c maximum by 0.1μM SB209670. pK_B values for 0.1μM SB209670 against SXS6c of 9.2±0.1 and 10.2±0.2 were calculated for 0-24 hr and 4 day PRAs, respectively. Preliminary data indicates a similar degree of inhibition of SXS6c-induced response in PRAs from 0-24 hr rabbits (figure 9.10, table 9.5).

9.4 Discussion

The results of this chapter demonstrate a marked alteration in ET- receptor mediated vasoconstriction in rabbit PRAs between fetal life and birth and during the first week of neonatal life. In parallel with the changes in functional responses to ET-1 and SXS6c at different developmental ages, variations in the effectiveness of selective antagonists were also exhibited. In particular, a hypersensitivity to ET_B-receptor mediated vasoconstriction is evident within 0-24 hours following birth.

ET-1 and SXS6c were potent vasoconstrictors. Both these peptides contracted all vessels in a concentration- dependent manner and a phasic response was elicited at all age points studied. The phasic nature of the cumulative response to ET-1 in fetal rabbit PRAs is similar to that previously described in isolated small PRAs (~169μm i.d.) from fetal sheep (Wang & Coccani, 1992). This may suggest the involvement of different ET-receptor subtypes or indeed may indicate receptor desensitisation. The potency of these peptides varied with developmental age. ET-1 was least potent in PRAs from 7 day old

rabbits whereas sensitivity to SXS6c progressively increased in PRAs from fetal to 7 day old rabbits. In chapter 4, I studied the effect of ET-1 and SXS6c in adult rabbit PRAs and this provided pEC_{50} values of ~7.9 for ET-1 and ~8.6 for SXS6c. Hence PRAs from 7 day neonatal and adult rabbits are similar in sensitivity to ET-1, whereas sensitivity to SXS6c in the 7 day old rabbit is markedly greater than that observed in the adult rabbit PRAs. It therefore appears that sensitivity to ET-1 decreases over the first week of life and is increased compared to that observed in the adult. An attenuation of the sensitivity to SXS6c from ~11.1 at 7 days to that existing in adult vessels, ~8.6, must occur at a later age. Measurements of plasma ET-1 in newborn infants demonstrated that high levels exist on day one postpartum but by day 4 and 5 of life these levels have decreased (MalamitsiPuchner, *et al*, 1993; Endo, *et al*, 1996). Similar alterations in plasma levels have also been demonstrated in various animals, such as the newborn pig (Levy, *et al*, 1995; Noguchi, *et al*, 1997). Hence the hypersensitivity to ET_B receptor stimulation indicated by the results of this chapter appears to exist at a time when plasma ET-1 levels have been shown to be elevated. This increased sensitivity may also be due to a comparatively greater number of ET receptors present on the pulmonary vasculature in the newborn compared to adult lung as Hislop *et al* (1995) have demonstrated a reduction in [I^{125}]ET-1 binding in isolated porcine pulmonary arteries between birth and adulthood. Giaid *et al* (1991) reported that immunoreactivity for ET-1 was substantially decreased in adult human lung compared with the developing fetal lung.

ET-1 and SXS6c were equipotent in their ability to evoke vasoconstrictions in fetal rabbit PRAs. ET-1 has also been demonstrated as a potent vasoconstrictor in isolated small pulmonary arteries and veins from term fetal lambs (Wang & Coceani, 1992). Also, in a previous *in vivo* study, Ivy *et al* (1994) showed that big ET-1 induced a rise in total pulmonary resistance of chronically prepared late gestation fetal lambs. The lung synthetic system for ET-1 has been reported to develop early in gestation, is found in several cell constituents including the endothelium, and is more active in the fetus than in the adult (Hemsen, *et al*, 1990; Giaid, *et al*, 1991). Furthermore, formation

of this peptide is accelerated by hypoxia, at least in the adult (Shirakami *et al*, 1991). These previous reports coupled with the results in this chapter, showing ET-1 to be a potent vasoconstrictor of fetal PRAs, are in accord with the possibility of this peptide contributing directly to the elevated vascular tone of the fetus. However, this concept is in apparent contrast with other studies *in vivo* and the perfused organ *in vitro*, in which ET-1 was shown to be primarily a dilator of the perinatal circulation. In unventilated fetal lungs of the sheep, injection of ET-1 into the pulmonary artery was shown to dilate both large and small pulmonary arteries with no effect on pulmonary veins (Todd & Cassin, 1992). Other previous *in vivo* studies have also demonstrated that the action of ET-1 is influenced by the perinatal age and thus, the tone of the vasculature. In 1 day old piglet lungs, injection of ET-1 caused vasodilation of the pulmonary arteries, and by 7 days old, the dilatation was transitory and followed by vasoconstriction (Perreault & DeMarte, 1993). In fetal sheep, infusion of ET-1 decreases the PVR, but after the onset of ventilation, the vasodilator response decreases and infusions of high concentrations of ET-1 cause vasoconstriction (Cassin, *et al.*, 1992). ET-1 did not change pulmonary artery pressure in lambs (<1 week old) but markedly increased pulmonary artery pressure in 6-12 months old sheep (Wong, *et al*, 1994b).

Theoretically, several possibilities could explain the apparent inconsistencies in findings. The contractile tone *in vitro* may be inadequate for expression of receptors mediating vasodilation (Cassin, *et al*, 1992). In this chapter I did not examine the effect of level of intrinsic tone per se on ET-1-evoked responses. However, ET-1 and SXS6c evoked potent contractile response at all developmental age points studied. Furthermore, in isolated small PRAs from the fetal lamb lung, even on preconstricting vessels no vasodilator response was unmasked and only further contractions were noted (Wang & Coceani, 1992). Alternatively, the target for ET-1 vasodilation could be confined to a segment of the pulmonary vasculature which is excluded in this isolated *in vitro* preparation. Nevertheless, vessels of the order of magnitude examined in this study contribute significantly to the PVR *in vivo* (Staub, 1985) and have also been shown to be the major site of resistance to blood flow in isolated perfused fetal lamb lung (Todd &

Cassin, 1992). Hence these vessels would be expected to also function as a target for any vasodilator agent. Yet another possibility is that these isolated vessels lack a viable endothelium and thus would be unable to produce dilator agents, such as NO and PGI_2, in response to ET-1 (De Nucci, *et al*, 1988). However, considering the results of chapter 8 in this thesis, this explanation seems unlikely as isolated PRAs, from animals at the same developmental ages and dissected from the same anatomical location, were shown to vasodilate in response to the endothelium-dependent agent ACh and indeed, L-NAME was shown to enhance ET-receptor mediated responses. The most plausible explanation is the idea that ET-1 exerts a vasodilator effect in the intact organ both directly and through the release of vasoactive agent(s) from the parenchyma. Under appropriate conditions, any such agent could override the direct constrictor effect of ET-1 on the vasculature. Considering that ET-1 is viewed as a local mediator, being formed and acting within the vessel wall (see introduction, chapter 1), then the results of *in vitro* studies, such as those described in this chapter, may provide a better reflection of the situation *in vivo*.

ET-1 caused similar maximal responses at all age points studied (~94% response to KCl) but the magnitude of the maximum contractile response to SXS6c was significantly greater at 0-24 hours and 4 days (~92%) compared to fetal and 7 days (~40%) after birth. These functional differences are most probably not related to alterations in the muscularisation of the arteries with postnatal age as previous studies show that the rapid decreases in pulmonary pressure, which occurs following birth, to be accompanied by a rapid decrease in degree of muscularisation of pulmonary vessels. Michel *et al.* (1991) reported a reduction in percentage muscle thickness and peripheral muscularization between <4 days and >2 weeks old in lungs from lambs. In newborn piglet lungs a rapid decrease in arterial medial wall thickness to one-half fetal level by 1 day of age with a further decrease by 3 days of age, with adult wall thickness being reached by 7 days old was reported (Haworth & Hislop, 1982). A more likely explanation for the variation in the maximal contractile responses to SXS6c shown in this chapter is an alteration in ET_B receptor between 0-24 hrs and 4 days after birth.

Hypersensitivity to ET_B receptor stimulation following birth was indicated by the notably greater potency of SXS6c compared to ET-1 in neonatal PRAs: vessels were approximately 10, 20 and 30 times more sensitive to SXS6c compared to ET-1 in 0-24 hour, 4 day and 7 day old preparations, respectively. These results are indicative of a predominant role of vasoconstrictor ET_B receptors during the first week of life. Other investigators have shown the predominance of this receptor in the larger pulmonary artery of the adult rabbit (La Douceur, *et al*, 1993; Fukuroda, *et al.*, 1994a; Hay, *et al.*, 1996). Furthermore, results of chapter 4 in this thesis also indicate this to be case in adult rabbit small PRAs.

Further evidence for the importance of ET_B receptor was provided by the observed effects of the selective antagonists. At all age points studied SXS6c- induced vasoconstrictions were significantly inhibited by the selective ET_B receptor antagonist BQ788, thus indicating that this peptide is indeed evoking its responses via the activation of ET_B receptors. BQ788 had an estimated pK_B value of ~8.7 against SXS6c in the 4 day old rabbit PRAs. However in adult rabbit PRAs, I calculated a pK_B value of 6.8 for BQ788 vs. SXS6c (chapter 5, this thesis). Hay and co-workers (1996) also showed that BQ788 has a pK_B value of 6.2 against SXS6c responses in larger rabbit PA. Hence this antagonist appears to be extremely potent against SXS6c-induced vasoconstrictions in neonatal PRAs. ET_B receptors also appear to be involved in ET-1-induced vasoconstrictions up to 4 days of age as BQ788 inhibited ET-1-evoked responses. The estimated pK_B was significantly greater in 0-24 hr (~6.7) compared with 4 day old rabbit PRAs (~6.2). This suggests that BQ788 is more potent against ET-1-evoked vasoconstrictions 0-24 hrs after birth and provides further evidence for hypersensitivity to ET_B receptor stimulation at this time. On the other hand, this may also reflect structural change in the receptor which could occur with the structural alterations in the pulmonary vasculature following birth. Haworth and Hislop (1981) reported a progressive loss in pulmonary arterial smooth muscle in the neonatal pig and, in the adult and developing bovine pulmonary arterial media, Frid *et al* (1994) identified phenotypic alterations of distinct smooth muscle cell populations with development.

BQ788 had no significant effect on the magnitude of the maximum responses to SXS6c at 4 days. In comparison a significant augmentation in the ET-1 maximum contraction was noted; this being most dramatic in newborn rabbit vessels. BQ788 had no effect on maximal contractile responses to ET-1 in PRAs from 7 day old rabbits and I also showed a similar lack of effect in response of PRAs from adult rabbits (chapter 5 this thesis). However a marked augmentation of ~70 % was evident 0-24 hrs and 4 days after birth. The reason for this is unknown but may possibly be due to the blockade of ET clearance receptors which have been suggested to be ET_B in nature. The ET_B selective antagonist BQ788 was shown to reduce the uptake of radiolabeled ET by 75% in the rat lung (Fukuroda, *et al*, 1994b). The significant increase in the magnitude of the response may also be due to BQ788 inhibition of endothelial ET_B receptors mediating vasorelaxations in PRAs at 0-24 hours and 4 days after birth.. Moreover, as the 7 day PRA SXS6c maximum response was not altered by BQ788, this may indicate that these receptors may decrease or disappear by this age. I did not examine these perinatal rabbit vessels for the ET-receptor mediated vasodilations however, as previously discussed, numerous studies in other species indicate a role for this ET_B receptor subtype in the perinatal pulmonary vasculature. Furthermore, in keeping with this latter postulate, Perreault and DeMarte (1993) demonstrated that ET-1 induced response altered from a pulmonary vasodilator response in 1 day old piglets to a vasoconstrictor response by 7 days of life.

Despite the apparent predominance of contractile ET_B receptors, BQ788 failed to inhibit ET-1 evoked responses in the 7 day old rabbit vessels. I also showed that ET-1-induced vasoconstrictions of adult rabbit PRAs were resistant to BQ788 (chapter 5 this thesis). Hay *et al* (1996) have also reported this phenomenon in the larger rabbit pulmonary artery. These authors concluded that the ET_B receptors in the rabbit large pulmonary arteries are insensitive to BQ788. It would appear, therefore, that on the basis of both agonist and antagonist interactions, that the ET_B receptor in the large pulmonary artery of adult rabbits and small pulmonary resistance arteries of 7 day old and adult rabbits are pharmacologically similar in terms of insensitivity to BQ788.

Evidence for a significant role of ET_A receptors was provided by the inhibitory effect of FR139317 on ET-1 -induced vasoconstriction of PRAs from 0-24 hour and 4 day old rabbits. A pK_B of ~6.4 was estimated for the newborn ET-1 responses. This is similar to values obtained for FR139317 in typical ET_A receptor preparations. e.g. guinea-pig pulmonary artery, pA_2 6.65 (Cardell, *et al.*, 1993). FR139317 did not significantly inhibit the fetal and 7 day rabbit PRA responses however a rightward shift was apparent at the higher ET-1 concentrations. Furthermore, the presence of FR139317 caused a significant reduction in ET-1 maximum vasoconstrictions in fetal vessels. This is indicative of ET-1 acting via ET_A receptors at high concentrations but at another receptor at low concentrations. A similar effect of ET_A receptor blockade has been observed in human PRAs and larger rabbit PAs where ET_A and ET_B receptors coexist (McCulloch, *et al,* 1996; LaDouceur *et al,* 1993). Vasoconstriction to ET-1 may follow binding to ET_A as well as ET_B receptors in newborn piglets also (Perreault & Baribeau, 1995). Hislop *et al* (1995) demonstrated, using an *in vitro* autoradiographic technique, the presence of both ET_A and ET_B receptor subtypes in medial smooth muscle cells in pig lungs from birth to adulthood. However, the majority of binding sites in the pulmonary arteries were shown to be ET_A in nature.

Infusion of the ET_A- selective antagonist BQ 123 increased PBF and decreased PVR in fetal sheep *in utero* and in the ovine fetal pulmonary circulation, indicating a role of ET_A receptor activation in maintaining basal fetal vascular tone (Wong, *et al,* 1994a; Ivy, *et al,* 1994). Also in these previous studies, where agonists were administered by intrapulmonary arterial injections, specific ET_B receptor activation produced a marked pulmonary vasodilation. However our results showed SXS6c as being a potent vasoconstrictor in fetal rabbit PRAs. Furthermore ET-1-evoked responses were significantly inhibited by the presence of BQ788. These results indicate the predominance of ET_B receptors which are contractile in nature in the fetal rabbit. However as previously stated I did not examine these vessels for ET-receptor mediated relaxatory responses. The contrast in findings compared with these previous studies may be due to differences in species or with experimental technique, i.e. intact lungs *in utero*

with intrapulmonary arterial injections, where parenchymal influences are possible, compared with isolated PRAs. This is discussed earlier in this chapter.

The involvement of both ET_A and ET_B receptors in ET-1-induced vasoconstrictions was demonstrated further by the near abolition of the responses in the presence of the non-selective ET_A/ET_B receptor antagonist SB209670 in PRAs from 0-24 hour and 4 day old rabbits. A greater inhibition was noted in the presence of this dual antagonist compared to either FR139317 or BQ788 alone at the same concentration. For example, (1μM) FR139317 and BQ788 resulted in a 6 and 8 fold increase respectively, in the ET-1 pEC_{50} in 0-24 hour old rabbit PRAs, whereas (0.1μM) SB209670 caused a significantly greater 13 fold shift. Synergy between ET_A and ET_B receptors has been reported in the larger rabbit pulmonary artery and human bronchi where administration of both an ET_A and ET_B receptor antagonist is required to inhibit responses to ET-1 (Fukuroda, *et al*, 1994c; 1996). My results in chapter 5 showed that in adult rabbit PRAs, SB209670 only antagonises ET-1 induced vasoconstrictions at the higher concentration range; this further indicates an alteration in the distribution of ET receptor subtypes with developmental age. Furthermore, SB209670 was even more potent at inhibiting SXS6c- evoked responses compared with ET-1- induced responses in neonatal PRAs. This greater potency as an ET_B receptor antagonist when SXS6c is the agonist rather than ET-1 has also been reported in large pulmonary artery of the adult rabbit (Ohlstein, *et al*, 1994a). Indeed, the results of chapter 5 in this thesis also suggest this to be the case also in small PRAs from adult rabbits.

Neither FR139317 or BQ788 were able to inhibit ET-1-induced responses in PRAs from 7 day old rabbits. I also observed this in adult rabbit PRAs (chapter 5 in this thesis). Other investigators have also found the necessity to block both ET_A and ET_B receptors in larger rabbit PA (LaDouceur,*et al*, 1993; Fukuroda, *et al*, 1994c) and human bronchi (Fukuroda, *et al*, 1996) in order to antagonise responses to ET-1 fully. In comparison, ET-1 induced vasoconstriction was effectively antagonised by inhibition of ET_B receptors alone in the fetal to 4 day old rabbit PRAs and by blockade of ET_A receptors alone in 0-24 hours to 4 day old rabbits. Thus in the newborn, it appears only

necessary to block either receptor subtype in order to inhibit ET-1 mediated responses whereas with increasing age, dual blockade is necessary. Therefore it may be that interaction or synergy between ET receptors develops in this preparation. In addition, the finding that FR139317 is effective against ET-1-induced responses in PRAs from 0-24 hour and 4 day old rabbits but not against those of 7 day and adult rabbit PRAs may indicate that as the influence of ET_B receptors increases, that of the ET_A receptors decreases.

The role of ET in the pathogenesis of PPHN is uncertain, but the circulating level of ET, which is normally high at birth, remains elevated in this condition (Rosenberg, *et al*, 1993). This has also been demonstrated in animal models of PPHN. In piglets exposed to hypoxia from birth, plasma ET-1 was significantly greater than normal for age (Noguchi, *et al*, 1997). In examining the possible role of ET-1 in PPHN, in addition to circulating ET-1 levels, it would appear that the balance of ET-receptor subtypes in the pulmonary vasculature would also determine the overall functional response to ET-1, thus affecting pulmonary vascular resistance. For example, a reduction or lack of endothelial ET_B receptors mediating vasodilation would presumably heighten responses to endogenous ET-1. This latter possibility is demonstrated by previous studies. In the newborn piglet lung a transient postnatal increase in endothelial ET_B receptor binding is evident at 3 days of age (Hislop, *et al*, 1995). However, these receptors are not evident in newborn piglets when pulmonary hypertension had been induced by prolonged exposure to hypoxia (Noguchi, *et al*, 1997). Ivy *et al* (1996) reported that chronic intrauterine pulmonary hypertension, induced by partial ligation of ductus arteriosus in late gestation fetal lambs, altered ET receptor activity in the ovine fetal lung. In particular, this caused a loss of ET_B receptor-mediated vasodilation, as indicated by the abolition of ET-3-induced increase in PBF. The lungs of rats made pulmonary hypertensive by monocrotaline injection showed a reduction in ET_B receptor mRNA (Miyauchi, *et al*., 1993). The results of this chapter can not rule out the existence of endothelial ET_B receptors mediating vasodilation in normal rabbit fetal and newborn PRAs. Their possible existence may be masked by the apparent predominance of

contractile ET_B receptors. Indeed, as previously discussed, the presence of BQ788 caused a marked increase in the maximum contractile response to ET-1 at 0-24 hrs and 4 days of age and this antagonist also inhibits the endothelial ET_B receptor which mediates endothelium-dependent relaxation (Douglas, *et al.*, 1995). Hence a reduction in ET_B vasodilator receptors might impair the vasodilation directly by reducing the release of endothelial derived NO or indirectly by reducing the clearance of ET from the circulation (Fukuroda, *et al*, 1994b). A lack of endothelial ET_B receptors in pulmonary vasculature in condition of PPHN may therefore contribute to the abnormal maintenance of a high pulmonary arterial pressure after birth.

A similar effect would be expected if an increase in relative number of functional contractile ET_A or ET_B receptors was to exist in this condition, as this would heighten responses to endogenous ET-1. In the newborn sheep, PHT and increased pulmonary blood flow altered the response of ET-1 from pulmonary vasodilation to vasoconstriction (Wong, *et al*, 1995b). Also shown in this study was a lack of effect of the ET_B receptor agonist 4-Ala ET-1 whereas the selective ET_A receptor antagonist BQ123 significantly reduced PVR. In a fetal lamb model of intrauterine pulmonary hypertension, the response to ET-1 infusion was altered to a predominantly vasoconstrictive response. Also, the vasodilator response to BQ123, the ET_A receptor selective antagonist, was greater following a longer period of ductus arteriosus ligation and thus greater pulmonary hypertension (Ivy, *et al.*, 1996). This suggests a progressive ET_A receptor-mediated vasoconstriction in this model. More recently these investigators demonstrated in this fetal lamb PHT model, that chronic intrauterine ET_A receptor blockade with BQ123 decreased pulmonary artery pressure *in utero*, decreased right ventricular hypertrophy and distal muscularisation of small pulmonary arteries, and increased the fall in pulmonary vascular resistance at birth (Ivy, *et al.*, 1997). Hence these previous studies indicate an involvement of ET_A receptors in the vasoconstrictor response, as does the inhibitory effect of FR139317 in PRAs from 0-24 hr and 4 day old rabbits which is demonstrated in this chapter.

In conclusion, the results of this study indicate a rapid alteration in ET_B receptor mediated contraction in rabbit PRAs during the first week of life. There is a significant population of ET_B receptors mediating vasoconstriction which coexist alongside ET_A receptors in fetal and neonatal rabbit PRAs. The contribution of these receptor subtypes to the overall ET -induced responses varies with developmental age and a marked hypersensitivity to ET_B receptor stimulation is apparent in newborn rabbit PRAs. Hence alterations in the ET_B receptor subtypes present on the vasculature may alter the haemodynamic effects of ET-1 in the pulmonary circulation and contribute to it's potential roles in (1) maintenance of elevated pulmonary vascular tone in utero, and/or (2) control of PVR as the pulmonary circulation adapts to extra-uterine life and/ or (3) the pathophysiological state of PPHN.

Chapter 10

General Discussion

10. General Discussion

I have extensively discussed the results of all studies in the relevant chapters. In this final chapter, I would like to draw on these findings, discuss them with respect to one another, speculate on the significance of the combined results and propose future studies. I examined the vascular reactivity of rabbit pulmonary resistance arteries under two different transitional states, from fetal to neonatal life and secondary to left ventricular dysfunction (LVD). This enabled me to look at changes induced by both active (oxygen) and passive (left atrial pressure) influences. Several features are common to both situations, including structural and functional alterations and in particular, the involvement of the endothelial-derived factors ET-1 and nitric oxide (NO).

Before discussing the vascular reactivity of isolated PRAs from the rabbit coronary ligation model of left ventricular dysfunction, I attempted to characterise particular parameters in this model which are related to the condition of pulmonary hypertension (PHT). As discussed in chapter 3, following coronary artery ligation, rabbits exhibited left ventricular hypertrophy, right ventricular hypertrophy, augmentation in lung weight and pulmonary vascular remodelling. Previous *in vivo* studies in this model have demonstrated a 44% increase in pulmonary artery pressure in 8 week coronary-ligated rabbits compared to sham-operated controls (Deuchar, *et al.*, 1997). My own findings, together with these *in vivo* studies, demonstrate the existence of pulmonary hypertension (PHT) secondary to LVD in this model. The possibility that heart failure may be the commonest cause of PHT is often overlooked. Heart failure following various forms of LVD affects 1-2% of the entire population and associated PHT is common (Trell, 1973; Rabinovitch, *et al.*, 1978). Thus the rabbit coronary ligation model may act as an ideal model to examine pulmonary vascular changes occurring in this condition.

It has been proposed that the potent peptide ET-1 plays an important role in various pathological conditions including heart disease and PHT, of various aetiologies.

In addition, there is currently much interest in the development of ET receptor antagonists for the treatment of LVD-related conditions. In light of this, I focused the functional studies on ET-receptor mediated responses in PRAs. The main findings, discussed primarily in chapters 4 and 5, demonstrate the role of contractile ET_{B2}-like receptors in mediating vasoconstriction in adult rabbit PRAs. This is consistent with previous findings in rabbit isolated larger pulmonary arteries (e.g. Warner, *et al.*, 1993; La Douceur, *et al.*, 1993; Hay, *et al.*, 1996) and binding studies of membrane preparations of rabbit pulmonary arteries (Fukuroda, *et al.*, 1994b). On comparing responses from preparations from 8, 16 and 32 week procedure animals, it was apparent that ET-receptor mediated responses were altered by the duration of coronary artery ligation. In PRAs from 8 week coronary-ligated rabbits, no change was observed in ET-1 or SXS6c responses, whereas sensitivity to both these peptides (at physiological and pathophysiological concentration ranges) was significantly reduced in vessels from 16 week LVD animals. Also, a marked attenuation in the maximal vasoconstrictor response to SXS6c was noted in PRAs from the 16 week LVD group compared to age-matched stock and sham-operated preparations. In PRAs from 8 week LVD animals, the magnitude of ET-3-mediated vasoconstriction was significantly reduced compared to sham-operated rabbit PRAs. Whilst in PRAs from 32 week ligated rabbits, no change in ET-1 responses but augmented sensitivity to SXS6c was noted. As these alterations occurred between 1pM-1nM, this suggests that ET-1 may be important in *in vivo* conditions, as in certain pathologies when plasma ET-1 levels equivalent to these concentrations have been observed (Cody, *et al.*, 1992; Kiowski, *et al.*, 1995). It should be noted that in cohorts of animals used for some of the studies, the ejection fraction of the 16 week ligated rabbits was markedly lower that of 8 week ligated animals, however a progression of this attenuation was not extended to the 32 week LVD animals. Hence the rabbits ligated for a longer duration do not appear to represent a more severe form of LVD but rather are the "survivors" from this group; this is probably on the basis that LVD was not as severe in the first instance. The findings in the 8 and 16 week experimental groups may indicate a reduced contribution of constrictor ET_B receptors or

greater involvement of endothelial ET_B receptors, mediating vasodilation. In the studies of chapter 4 I was unable to demonstrate ET-receptor mediated vasorelaxations in preconstricted PRAs, however the existence of these receptors in this preparation cannot be ruled out as they may possible be masked by the prevalent vasoconstrictor ET_B receptor subtype, which was implied by the agonist potency profile of SXS6c > ET-1 = ET-3 shown in this preparation (chapter 4). The finding that LVD had differential influences on responses to ET-receptor agonists suggests that ET_A and ET_B receptors may be differentially regulated in pathological conditions. Previous reports examining the role of ET-1 in heart failure are consistent with this. For example, blunted vasoconstriction to ET-1 with concomitant augmentation of vasoconstriction to SXS6c has been shown in forearm bloodflow measurements of heart failure patients (Love, *et al.*, 1996) and in coronary vessels of a dog model of experimental congestive heart failure (Cannan, *et al.*, 1996). These studies may indicate a greater involvement of vasoconstrictor ET_B receptors in the systemic and coronary vasculature secondary to LVD.

The studies employing the ET-receptor antagonists, discussed in chapter 5, show that the pharmacology of the ET-1 receptor in adult rabbit PRAs is extremely complex. There may be a quiescent ET_A receptor which synergises with a vasoconstrictor ET_B receptor which is BQ788 sensitive (SB209670 insensitive) and mediates responses to lower concentrations of ET-1. There is also a BQ788-insensitive receptor which is sensitive to SB209670 and mediates responses to higher ET-1 concentrations. These conclusions are indicated by my observations noted in 8 week sham-operated preparations. These were as follows: insensitivity to the ET_A receptor selective antagonist FR139317, which if anything tended to augment the ET-1 induced response; sensitivity to BQ788 at low ET-1 concentrations and the sensitivity of the responses to higher ET-1 concentrations to SB209670.

In both sham-operated and coronary-ligated rabbit PRAs, FR139317 tended to potentiate ET-1-induced responses at higher concentrations and this tendency was also observed at lower ET-1 concentrations in the LVD vessels. This

finding may indicate the presence of a putative inhibitory ET_A receptor. Evidence for such a receptor has been reported in several preparations, including rat fundic strip and PRAs (Gray & Clozel, 1993a; McCulloch & MacLean, 1996). Clearly what would be of use to examine this possibility further would be a selective ET_A receptor agonist, however as mentioned in chapter 1 such a compound has remained elusive. In 8 week coronary ligated rabbit PRAs, BQ788 and SB209670 also tended to potentiate responses to lower ET-1 concentrations. This suggests that after 8 weeks of coronary artery ligation, the BQ788-sensitive ET_B receptor mediated response is now potentiated by both BQ788 and SB209670. These findings and the tendency of FR139317 to potentiate responses to lower ET-1 concentrations, may indicate the presence of inhibitory ET_B and ET_A receptors which may be upregulated in the vessels from LVD rabbits. This maybe a physiological compensatory mechanism in response to the early elevation in pulmonary pressure to maintain responses to ET-1 constant, particularly at the lower, physiological relevant, concentration range.

Results of several of my studies suggest the involvement of a heterogeneous population of ET-1 receptors in PRAs from the LVD model. This is indicated by (1) the biphasic nature of the cumulative concentration responses to ET-1 (discussed in chapter 4), (2) the differential effects of selective antagonists on the two components of the ET-1 cumulative concentration response curve (chapter 5), and (3) the two receptor sites demonstrated in competition radioligand binding studies using ET-1 on pulmonary artery membrane preparations. Regarding this latter finding, which is discussed in chapter 7, the first receptor population displayed ultrahigh affinity with the second also demonstrating high affinity in the nanomolar range. This may relate to a previous binding assay study which demonstrated high and low-affinity ET_B receptors in brain tissue (Sokolovsky, *et al.*, 1992). An essential and extremely interesting future study would be to confirm the presence of two distinct ET receptor populations by conventional protein purification and molecular cloning. Also future radioligand binding studies would include further competition analysis using selective ET-receptor antagonists to provide further insight into the receptor subtypes involved. However, I

think that first and foremost, saturation studies in this preparation would be essential to obtain B_{max} and K_D values and to answer the intriguing question of whether a difference in the number of ET-receptors exists between control and LVD preparations. Indeed such a possibility may be related to the apparent differences in the effect of the selective antagonists between control and experimental groups which were shown. From the binding studies which I did perform, the calculated K_i for site one ($\sim 6x10^{-14}M$) is much lower than previously reported values for ET_A and ET_B receptors and such high affinity receptors were not evident in the functional studies performed. This population therefore appears to be of receptor subtype(s) which is not typical of an ET_A or ET_B receptor and which does not appear to mediate functional responses. Consistent with this is the findings of other investigators, discussed in chapters 5 and 7, who have also suggested the role of other receptor subtypes which do not fit current classification of ET_A and ET_B receptors (e.g. Sumner, *et al.*, 1992). This demonstrates the growing inadequacy of the current cloned ET receptors in accounting for many ET-receptor mediated responses which have been shown in this thesis and by other investigators.

Again on examining the vascular reactivity of PRAs from the second model, i.e., the transitional pulmonary vasculature from *in utero* to *in vivo*, I focused on ET-receptor mediated responses and the influence of developmental age. Primarily, a hypersensitivity to ET_B receptor mediated vasoconstriction was evident within 0-24 hours of birth. As discussed in chapter 9, PRAs from fetal to 4 day old rabbits are equisensitive to ET-1 and are 4-8 times more sensitive to this peptide than 7 day old and adult rabbit vessels, in which ET-1 is equipotent. Regarding the selective ET_B receptor agonist, sensitivity to SXS6c progressively increases in PRAs from fetal to 7 day old rabbits, and these have markedly greater sensitivity to SXS6c than PRAs from the adult. It therefore appears that sensitivity to ET-1 decreases over the first week of life and is increased compared to that observed in the adult. An attenuation of SXS6c sensitivity to that observed in the adult must occur at a later age. The hypersensitivity to ET_B receptor stimulation demonstrated in this preparation appears to exist at a time when plasma ET-1 levels have been shown to be elevated in the human neonate and animal models

(Endo, *et al.*, 1996; Noguchi, *et al.*, 1997) and when a comparatively greater number of ET receptors on the pulmonary vasculature have been demonstrated (Hislop, *et al.*, 1995). Maximal vasoconstrictor responses to SXS6c are markedly greater in PRAs from 0-24 hour and 4 day old rabbits. These functional differences are unlikely to be related to alterations in the muscularisation of arteries as previous studies show the rapid decrease in pulmonary pressure which occurs following birth, to be accompanied by a rapid decrease in the degree of arterial muscularisation (Haworth & Hislop, 1981; Michel, *et al.*, 1991). A more likely explanation is a structural or functional alteration in the ET_B receptor with change in the vascular smooth muscle phenotype which occurs at this time (Frid, *et al.*, 1994). The findings of my own studies and those performed by other investigators in other species are of great importance, as an understanding of the role of ET-1 in the normal adaptation of the pulmonary circulation to extrauterine life is essential before attempting to examine alterations in pathophysiologic conditions. In examining the possible influence of ET-1 in persistent pulmonary hypertension of the newborn (PPHN), the potent effects of ET-1 demonstrated in the pulmonary vasculature indicates that the balance of ET-receptor subtypes would determine the overall functional response. To hypothesise, a lack or reduction of endothelial ET_B receptors mediating vasodilation would presumably heighten the vasoconstrictor response, and this is observed in newborn piglets with hypoxic-induced PHT (Hislop, *et al.*, 1995). Similarly, an increase in functional contractility mediated by ET_A or ET_B receptors, would only add to the abnormal maintenance of a high pulmonary arterial pressure after birth which occurs in the pathological state.

In chapter 9, the effect of selective ET-receptor antagonists in the perinatal preparations are discussed. Interesting differences were noted between different developmental ages. The presence of BQ788 caused a marked increase in the ET-1 response in PRAs from 0-24 hour and 4 day old rabbits. However, this was not evident in vessels from animals at other age points studied and further demonstrates an alteration in receptor subtype with age. This reason for this augmentation is unknown but may be due to blockade of endothelial ET_B receptors mediating vasodilation via the release of

NO and/or activation of K^+ channels, or inhibition of ET clearance receptors. Future studies in this model would have to include an examination of the possible existence of ET-receptor mediated vasorelaxations. In addition, activation of ET_B receptors has also been shown to release adrenomedullin (Jougasaki, *et al.*, 1997), a recently discovered peptide which can produce pulmonary vasorelaxation (Shirai, *et al.*, 1997). This may represent an additional paracrine-autocrine role of the ET_B receptor subtype.

Since similar preparations were examined from both the models studies, interesting comparisons can be made between them. Despite the apparent predominance of vasoconstrictor ET_B receptors, BQ788 was unable to antagonise ET-1 mediated vasoconstrictions in 7 day old (chapter 9) and adult rabbit (chapter 5) PRAs and this has also been demonstrated in the larger pulmonary artery of this species (Hay, *et al.*, 1996). Ineffectiveness of FR139317 in inhibiting responses to ET-1 was also demonstrated in PRAs from 7 day old and adult rabbits. However, FR139317 had a significant inhibitory effect on ET-1 responses in the same preparation from newborn to 4 day old rabbits and BQ788 markedly antagonises ET-1 responses in PRAs from fetal to 4 day old rabbits (chapter 9). Also in neonatal preparations, a markedly greater inhibition was seen with the non-selective antagonist SB209670 compared with the effect of either ET_A receptor inhibition (using FR139317) or ET_B receptor inhibition (using BQ788) alone. Thus, it appears that in the fetus and in newborns younger than 7 days old, antagonism of either ET_A or ET_B receptor alone is sufficient to inhibit ET-1 responses and inhibition of both subtypes has a synergistic effect. However in PRAs from the older animal, interaction between the two subtypes seem to occur such that inhibition of either subtype alone is now insufficient to inhibit the response. The concept of receptor "crosstalk" has previously been proposed to explain the observation that dual inhibition of ET_A and ET_B receptors is required to inhibit ET-1 induced vasoconstrictions in various preparations, including the rabbit larger pulmonary artery (Fukuroda, *et al*, 1994c; 1996; Clozel & Gray, 1995). The mechanism for the plausible receptor crosstalk is not fully understood, however interaction at the signal transduction systems between ET_A and ET_B receptors has been suggested (Fukuroda, *et al.*, 1996; Ozaki, *et al.*, 1997).

Comparing the results of the studies with antagonists in the two models (chapters 5 and 9), the findings suggest that this "crosstalk" does not develop in rabbit PRAs until approximately 7 days after birth. One could suggest that this is manifested by a change in the smooth muscle phenotype particular to rabbit PRAs.

The altered ET-receptor mediated responses demonstrated here in the rabbit LVD model and with developmental age as well as by other investigators in other models, may also be due to an alteration in the clearance of ET-1. Numerous reports indicate the additional role of ET_B receptors as clearance receptors. The pulmonary circulation has been shown to be the major site of ET-1 clearance in several animal models, including the rabbit (Rimmar & Gillis, *et al.*, 1989), and ET_B receptors are exclusively responsible for pulmonary ET-1 removal in the dog *in vivo* (Dupius, *et al.*, 1996). Plasma ET-1 levels are elevated in heart failure and PHT of various aetiologies including neonatal and secondary to LVD (Noguchi, *et al.*, 1997; Cody, *et al.*, 1992). Thus it is possible that reduced clearance of this peptide contributes to the hyperendothelinemia of these pathophysiologies. Hence, it is plausible that alterations in the expression or function of this ET_B receptor subtype may be involved in the changes in ET-receptor mediated responses observed in the perinatal pulmonary vasculature and secondary to LVD. As stated, in both these states marked structural alterations occur in the vascular smooth muscle , particularly at the level of the pulmonary arterial system I examined functionally. Thus a structural or functional change in the receptor may occur with the alteration in vascular smooth muscle. Plasticity of vascular smooth muscle expressing ET_B receptors in other vascular regions, such as the cerebral circulation, has recently been shown in rat and man (Norel, *et al.*, 1996; Moller, *et al.*, 1997; Edvinsson, *et al.*, 1997). A similar phenomenon may exist in the pulmonary vasculature and may relate to the functional results observed.

In the rabbit LVD model, a similar contractile response to KCl was noted in PRAs from all stock/sham-operated and coronary-ligated rabbis (chapter 3), indicating that the alterations in functional responses were not related to changes the integrity or contractility of the vascular smooth muscle. Whilst in fetal and neonatal rabbit PRAs,

discussed in chapter 8, vasoconstrictor responses to KCl increased in magnitude with developmental age. This has also been shown in similar vessels from fetal and neonatal lambs (Dunn, *et al.*, 1989) and indicates smooth muscle extension or maturation in PRAs of the same anatomical location during the first week of extra-uterine life. Also in chapter 8, contractile responses to NA were shown to be smaller in fetal rabbit PRAs compared to newborn vessels. This may relate to a reduced basal NO level in the fetus, as suggested by the relatively lesser effect of L-NAME in fetal compared to newborn vessels. However from examining ACh-induced relaxatory responses in preparations from the various developmental ages, an augmented agonist-induced NO level was indicated before birth by greater endothelium-dependent relaxations observed in fetal compared with newborn PRAs. Vasoconstrictions to receptor-mediated agents NA and 5-HT were comparatively greater in the newborn compared with 7 day old vessels. This may be related to the apparent increased level of NO in the 7 day vessels compared with 0-24 hr rabbit PRAs, which was indicated by the more pronounced effect of L-NAME on ET-receptor mediate responses in PRAs from 7 day old rabbits and the increased vasodilator response to ACh. Such an increase in NO would participate in the postnatal reduction of the pulmonary vascular resistance. Alternatively, these augmented contractile responses may reflect increased local ET-1 levels in the newborn as this peptide has previously been shown to potentiate the vasoconstrictor response to other agents (Tabuchi, *et al.*, 1989) and elevated plasma ET-1 levels are reported in the first few days of life, in both human neonate and piglets (Endo, *et al.*, 1996; Levy, *et al.*, 1995).

Again by drawing on the findings in the two different models I examined, and focusing on the difference in developmental age, i.e. fetal, neonatal and adult, intriguing differences in the vascular reactivity of rabbit PRAs are apparent with this variable. For example, in the studies of chapter 3 in adult PRAs, NA was found to be without effect and ACh evoked a contractile response. Whereas in fetal and neonatal PRAs, NA was able to evoke a contractile response, albeit small in magnitude, and ACh induced a concentration-dependent relaxatory response (chapter 8). Moreover a transition in the

functional effect of ACh in this preparation was apparent with vasoconstriction being observed to higher ACh concentrations in 7 day old rabbit PRAs, thus similar to situation in the adult preparation. These intriguing findings may be related to alteration in receptor subtypes present on the vasculature with developmental age and, as previously discussed may be manifested through age-related changes in vascular smooth muscle phenotype. In addition, in both models I examined the influence of L-NAME on ET-1 and SXS6c-induced responses. Not only were the nature of the CCRCs to these peptides influenced by developmental age, but L-NAME had differential effects on these responses depending on this variable. No significant effect on response to either peptide was evident in fetal rabbit PRAs, whereas the ET-1 response was markedly augmented in newborn rabbit vessels. As discussed in chapter 8, this augmentation was heightened in 4 day old rabbit preparations and was greatest by 7 days after birth. In comparison from the studies of chapter 6 in adult PRAs, the ability of L-NAME (NO) to modulate ET-receptor mediated responses was only pronounced in LVD animals. This comparatively greater role of NO on functional responses in the neonatal PRAs is consistent with its putative role in the rapid and progressive reduction of pulmonary vascular resistance following birth, as it takes on its low pressure and low resistance characteristics.

Habib *et al* (1994) demonstrated an upregulation of NOS in heart failure patients. In the rabbit coronary-ligation model, L-NAME markedly potentiated ET-1- and SXS6c-induced responses in PRAs from LVD rabbits. As discussed in chapter 6, this suggests an increase in NO production associated with the development of PHT in these animals. This may be basally released NO as a greater percentage of PRAs from coronary-ligated rabbits demonstrated an increase in tone from baseline tension in response to L-NAME alone compared to age matched sham-operated animals.

Alternatively, the greater NO production in PRAs from 8 week LVD animals could be NO released via stimulation of endothelial ET_B receptors. Although such receptors could not be shown to induce a vasorelaxations in this preparation, an upregulation of endothelial ET_B receptors in 8 week LVD animals is indicated by

several ET receptor antagonist results in isolated PRAs (discussed in chapter 5). Also, PRAs from LVD animals exhibited decreased sensitivity to SNP compared to vessels from sham-operated animals. From these findings with L-NAME (chapter 6) and SNP (chapter 3) in this model and those of others in man (Needleman & Johnson, 1973), it may be postulated when there is increased stimulation of NO, the vascular smooth muscle compensates with reduced sensitivity to NO, thus explaining the attenuated SNP response observed in LVD animals. A previous study shows that the reverse case may hold when eNOS is inhibited, such that pulmonary vascular smooth muscle becomes more sensitive to SNP in chronic L-NAME treated rats (MacLean & MacMillan, 1993). This increase in NO may act as a compensatory mechanism in the early stages of increased pulmonary arterial pressure. However these findings remain controversial as other studies implicate decreased NO in pathological conditions, e.g. an impairment of NO production was reported in isolated pulmonary arteries from patients with chronic obstructive lung disease (Dinh-Xuan, *et al.*, 1993). In addition, in isolated aorta from a chronic heart failure rat model, stimulated NO release was unaffected but basal NO levels were shown to be decreased compared with control preparations (Teerlink, *et al.*, 1994b). These latter findings may indicate a lack of NO as being partly responsible for the manifestation of the pathological state. However the differential conclusions, that is decreased NO implicated in manifestation or increased NO level with possible compensatory role, are most probably related to differences in species, the pathological state, the vascular bed involved and the nature of the models.

With regard to my own findings, an increased NO production may also account for the attenuated vasoconstriction to sumatriptan and 5-HT in PRAs from 8 week coronary artery ligated animals compared to sham-operated rabbit vessels which was demonstrated in chapter 3. However, as discussed this may also be due to an alteration in 5-HT receptors, in particular 5-HT_{1D} receptors, in vessels from LVD animals. Further studies using selective antagonists are required to elucidate the receptor subtypes involved and to examine further the effect of PHT secondary to LVD. A previous *in vivo* study using this rabbit coronary ligation model demonstrated an increased pulmonary

pressor response to i.v. administered 5-HT in 8 week ligated rabbits when compared to sham-operated animals (Deuchar, *et al.*, 1997). Differing results between *in vitro* and *in vivo* studies of 5-HT have also been shown in the human pulmonary vasculature (Raffestin, *et al.*, 1985; Harris, *et al.*, 1960). When analysing the results of *in vivo* studies a role of parenchymal and/or vascular agents cannot distinguished, and the overall effect will represent the sum of the responses throughout the pulmonary vasculature; hence will show the balancing between the vasoconstrictor and vasodilator effects of 5-HT. Moreover, in contrast to a previous report in larger pulmonary artery of the rabbit where a 5-HT_{2A} type receptor appears to mainly involved in the vasoconstriction (Morecroft & MacLean, 1995b), a definite role of 5-HT_1-like receptors was indicated by my own studies in the PRAs of this species. Again comparing these current findings with those previously reported in the larger vessel, these smaller muscular arteries have a comparatively greater sensitivity to 5-HT. These findings indicate differences in 5-HT responses depending on vessel size, as has been previously demonstrated in rat pulmonary arteries (MacLean *et al.*, 1996b).

Another aim of this project was to research the role of intracellular cyclic nucleotides in rabbit PRAs and examine the possible effect of LVD using the rabbit model, under both basal and ET-1-stimulated conditions. Unfortunately due to the variability noted between tissues in the individual groups and thus lack of statistical evidence, no definite conclusions can be discussed from the findings shown in chapter 7. Measurement of intracellular cyclic nucleotide levels showed that basal levels and ET-1-induced levels of cGMP tended to be greater in arteries from coronary-ligated compared to sham-operated rabbits. Basal and forskolin-stimulated cAMP levels also tended to be greater in LVD rabbit preparations and ET-1 inhibited forskolin-stimulated cAMP in vessels from all animals. Further studies are required to elucidate if indeed $[cAMP]_i$ or $[cGMP]_i$ is altered in the LVD state and if it is involved in the mechanism of action of ET-receptor mediated responses in this preparation. Future studies would include examination of G-proteins involved and measurement of IP_3 levels to assess the involvement of the PIP_2 system. A study examining the possible interaction of ET_A and

ET_B receptors at the intracellular level and possible alterations secondary to LVD and with developmental age would prove most interesting. The results of such a study would be intriguing, particularly if taken in context with the findings of the studies with ET-receptor antagonists discussed in chapter 5, which may suggest the occurrence of receptor "crosstalk" in adult rabbit PRAs.

The animal models I used allowed me to examine my particular interests more extensively however, obviously all these studies have been performed with their relation to the situation in man in mind. Thus it is crucial to compare the responses in animal models with those in similar preparations obtained from human tissue. In chapter 5, examination of human PRAs demonstrated a similar sensitivity to ET-1 and also exhibit a biphasic response to this peptide, as observed in rabbit vessels of similar diameter. Due to the infrequency of available human tissue, only limited studies with ET receptor antagonists could be carried out, And I again I focused on those compounds examined in the animal models. The response to lower ET-1 concentration appeared to be mediated by vasoconstrictor ET_B receptors, as indicated by the inhibitory effect of BQ788 in this range. Whilst previous studies in this preparation demonstrated the involvement of ET_A receptors in vasoconstrictor responses to higher ET-1 concentrations (McCulloch & MacLean, 1995). In my own studies discussed in chapter 5, the non-selective antagonist SB209670 is the first compound shown to successfully inhibit the entire ET-1 response in this preparation. It is not yet clear if a similar situation occurs in human pulmonary hypertensive states. Of immense interest for future studies, would be to examine ET-receptor mediated responses in pulmonary arteries from patients with PHT. This would assess any changes in the vascular reactivity to ET-1, and would resolve possible alterations in the ET-receptor subtypes present which may occur. As is suggested in chapters 5 for rabbit PRAs, the difference in effectiveness of different antagonists may be explained by a heterogeneous population of ET_B receptors, which are diverse in terms of there sensitivity to particular antagonists. Obviously support for this hypothesis requires molecular biological evidence of the nature and location of ET-receptor subtypes on the pulmonary vasculature.

The ever growing literature from animal models of PHT indicates that alterations of ET-receptor mediated vascular function in heart failure may depend on the experimental model, the duration and the severity of the disease, and the type of preparation studied. These rapidly accumulating studies imply the possible use of ET receptor antagonists as a novel therapy in the treatment of PHT of various aetiologies. However the suitability of a non-selective antagonist or one specific for ET_A receptors remains controversial, as success in ameliorating the features of this condition has been reported with both types (e.g. Kiowski, *et al.*, 1995; Sakai, *et al.*, 1996). The contribution of ET-receptor subtypes to the overall response varies with developmental age and a marked hypersensitivity to vasoconstrictor ET_B receptor stimulation is apparent in newborn rabbit PRAs. In addition, the results of antagonists studies in the rabbit model of LVD highlights the concern that the effectiveness of ET-receptor antagonism therapeutically will depend on the level of endothelial ET_B receptor stimulation and on the relative selectivity for endothelial and smooth muscle ET_B receptor subtypes.

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