Morphology and Reactivity of Vasa Vasorum:

Mechanisms and Functional Implications

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ABSTRACT

Walls of conduit blood vessels are nourished by oxygen diffusion from luminal blood and vasa vasorum. The vasa vasorum form a network of microvessels in the adventitia and outer media of conduit blood vessels. Obstruction of flow through vasa vasorum is implicated in the pathogenesis of certain cardiovascular diseases. However, there is no direct evidence of the mechanisms that regulate vasa vasorum tone. Immunohistochemistry was used to study structure of arterial vasa vasorum isolated from porcine thoracic aorta. Tension and perfusion myography were used to directly investigate their reactivity and comparisons were made with other arteries of similar calibre.

Vasa consist of layers of smooth muscle oriented around a layer of endothelium and are innervated by nerves, predominantly sympathetic in origin. Vasa are sensitive to constrictors but the reactivity is different from the host vessel, the aorta, and other porcine small blood vessels. Whilst endothelin-1 (ET-1) produces potent contractions, vasa are relatively insensitive to noradrenaline (NA), thromboxane A2 (TXA2) mimetic or angiotensin II. ET-1, at low concentrations, potentiates contractions to NA but not TXA2 mimetic. Low concentrations of K+ potentiate responses to either constrictor. Vasa develop modest myogenic tone, which is considerably less than that in other resistance arteries. Vasa are sensitive to vasodilators bradykinin (BK), substance P (SP) and calcitonin gene-related peptide (CGRP). The endothelium is critical in mediating responses to BK and SP but not to CGRP.
Vasa vasorum consist of an entire vascular bed within conduit blood vessel walls that regulate tone in response to constrictors or dilators. Relative insensitivity to constrictors and pressure reflects the function of vasa vasorum and may allow vessel patency. The finding that the endothelium is obligatory in dilator responses and that sensitivity to constrictors can be altered has implications for conduit blood vessels in diseases involving endothelium dysfunction or depolarisation of smooth muscle.
ACKNOWLEDGEMENTS

This thesis is dedicated with thanks and love to my parents, whose encouragement and support I have enjoyed always. It has been their enthusiasm for achievement that has motivated me throughout my life, and for this I thank them very much. It is also dedicated with love to Mark.

I would like to thank Dr. Amrita Ahluwalia and Prof. Patrick Vallance for their continued support and guidance throughout the course of my Ph.D. I would also like to thank the Medical Research Council for their financial support.

Finally, thank you to my friends with whom I shared many memorable coffee breaks.

DECLARATION

All the work in this thesis is my own. No part of this thesis is, or has already been, submitted for a degree, diploma or other qualification at any other University.

Ramona Sumintra Scotland
PUBLICATIONS

This work in this thesis has lead to the following publications:


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<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AngI</td>
<td>angiotensin I</td>
</tr>
<tr>
<td>Ang II</td>
<td>angiotensin II</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
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<tr>
<td>BK</td>
<td>bradykinin</td>
</tr>
<tr>
<td>BQ123</td>
<td>Cyclo(-D-Trp-D-Asp-Pro-D-Val-Leu)</td>
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</tr>
<tr>
<td>Ca^{2+}</td>
<td>calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CGRP</td>
<td>calcitonin gene-related peptide</td>
</tr>
<tr>
<td>CPA</td>
<td>cyclopiazonic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>ECEs</td>
<td>endothelin converting enzymes</td>
</tr>
<tr>
<td>EDHF</td>
<td>endothelial-derived hyperpolarisation factor</td>
</tr>
<tr>
<td>EFS</td>
<td>electrical field stimulation</td>
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<tr>
<td>EGTA</td>
<td>ethyleneglycol-bis-(β-aminoethyl-ether)-N,N,N'N'-tetraacetic acid</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>eNOS KO</td>
<td>eNOS knockout</td>
</tr>
<tr>
<td>eNOS WT</td>
<td>eNOS wildtype</td>
</tr>
<tr>
<td>ET-1</td>
<td>endothelin-1</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>--------------</td>
<td>-------------</td>
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<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>haematoxylin and eosin</td>
</tr>
<tr>
<td>K^+</td>
<td>potassium</td>
</tr>
<tr>
<td>KPSS</td>
<td>PSS with equimolar (125 mM) substitution of K^+ for Na^+</td>
</tr>
<tr>
<td>L-NAME</td>
<td>NG-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>NA</td>
<td>noradrenaline</td>
</tr>
<tr>
<td>NANC</td>
<td>nonadrenergic noncholinergic</td>
</tr>
<tr>
<td>Ni^{2+}</td>
<td>nickel</td>
</tr>
<tr>
<td>NKA</td>
<td>neurokinin A</td>
</tr>
<tr>
<td>NKB</td>
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<td>nNOS</td>
<td>neuronal nitric oxide synthase</td>
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<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>NPY</td>
<td>neuropeptide Y</td>
</tr>
<tr>
<td>ODQ</td>
<td>1H-[1,2,4] oxadiazolo[4,3-a] quinoxalin-1-one</td>
</tr>
<tr>
<td>OxyHb</td>
<td>oxyhaemoglobin</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>phenylephrine</td>
</tr>
<tr>
<td>PGP 9.5</td>
<td>protein gene product 9.5</td>
</tr>
<tr>
<td>PSS</td>
<td>physiological salt solution</td>
</tr>
<tr>
<td>SKF 96365</td>
<td>1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1H-imidazole hydrochloride</td>
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<tr>
<td>αSMA</td>
<td>smooth muscle actin</td>
</tr>
<tr>
<td>S6c</td>
<td>sarafotoxin 6c</td>
</tr>
<tr>
<td>SNAP</td>
<td>S-nitroso-N-acetyl penicillamine</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Name</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>SP</td>
<td>substance P</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>TK</td>
<td>tachykinin</td>
</tr>
<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
</tr>
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<td>TxA$_2$</td>
<td>thromboxane A$_2$</td>
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<tr>
<td>U44069</td>
<td>9,11-Dideoxy-9α,11α-epoxy-methano prostaglandin F$_{2\alpha}$</td>
</tr>
<tr>
<td>U46619</td>
<td>11α,9α-epoxymethano-PGH$_2$</td>
</tr>
<tr>
<td>VIP</td>
<td>vasoactive intestinal peptide</td>
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CHAPTER 1

INTRODUCTION
1.1 PREFACE

The vasa vasorum, or "vessels of a vessel", were first described in 1757 by Haller. The vasa vasorum forms a network of microvessels in the adventitia and outer media of conduit blood vessels. The function of the vasa vasorum is thought to be to provide oxygen and nutrients to those parts of the vessel wall that are not adequately supplied by diffusion from the lumen. The importance of the vasa vasorum in maintaining normal physiology of conduit blood vessels has been highlighted by observations that disruption of blood flow through these microvessels produces medial necrosis (see for example Nakata and Shionoya, 1966). However, the mechanisms that regulate blood flow through the vasa vasorum are not clear. The vasa vasorum consists of small arteries that supply blood flow to the host vessel wall and small veins that carry blood away from the vessel wall. Resistance arteries are typically defined according to lumen diameter (diameter <500μm) (for review see Mulvany and Aalkjaer 1990) and thus according to this definition, the arteries of the vasa vasorum may similarly be defined as resistance arteries. This thesis describes investigations into the morphology of the small "resistance" arteries of the vasa vasorum and some of the possible mechanisms involved in regulating tone in these vessels. The reactivity of small arteries of the vasa vasorum is also compared to that of other resistance arteries since the function of these arteries are likely to be distinct from each other.
1.2 RESISTANCE ARTERIES

1.2.1 Morphology

Resistance arteries, like other arteries, consist of three layers (see fig 1):

1) The tunica adventitia forms the outer part of the vessel wall and consists mainly of connective tissue such as collagen and elastin. The adventitia also contains nerve axons that, in some instances, penetrate the media.

2) The majority of the small artery wall is made up of the tunica media, which contains layers of smooth muscle cells arranged circumferentially around the lumen of the artery. The number of smooth muscle layers in the media varies with diameter.

3) The intima consists of a single layer of squamous endothelial cells that are oriented in the direction of blood flow.

1.2.2 Regulation Of Blood Flow

Distribution of blood throughout the circulation is determined by the diameter of blood vessels, which is dependent on smooth muscle tone. Vascular tone of resistance arteries is influenced by a combination of local and circulating humoral substances, vasoactive agents released from nerve endings, and autoregulation. It is clear that there is considerable heterogeneity in the importance of these factors between vascular beds and species. Moreover these pathways do not act independently but interact with each other to provide complex regulation of smooth muscle tone, according to function and need. Some of the important mechanisms of regulation of tone in resistance arteries are briefly discussed below.
Figure 1. Diagram demonstrating three distinct layers of a resistance artery.
1.2.2.1 Hormonal Influences

1.2.2.1.1 Nitric Oxide

In 1980 Furchgott and Zawadzki showed that acetylcholine (ACh) induces relaxation of rabbit aorta via the release of a diffusible factor from the endothelium (Furchgott and Zawadzki, 1980). This factor was later identified as nitric oxide (NO) (Palmer et al. 1987; Ignarro et al. 1987). Subsequent to these initial findings, it has become clear that several different endothelium-derived factors, including NO, mediate and modulate vascular responses of blood vessels to various vasodilators, vasoconstrictors, neurotransmitters, and autoregulatory responses (see below). Nitric oxide is a gas that is synthesised by the conversion of amino acid L-arginine to L-citrulline, by the action of nitric oxide synthase (NOS). Three NOS isoforms have been identified (for review see Michel and Feron, 1997): endothelial NOS (eNOS), neuronal NOS (nNOS), and an inducible isoform (iNOS) that is considered to be absent under normal physiological conditions. NO, released from endothelial cells, can rapidly diffuse to adjacent smooth muscle to cause relaxation. This action of NO is mediated by stimulation of soluble enzyme guanylyl cyclase (sGC), which catalyses the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). The mechanisms of cGMP-mediated relaxation are not fully understood but include decrease in intracellular Ca\(^{2+}\) concentration via a complex combination of refilling of intracellular stores of Ca\(^{2+}\) (Cohen et al. 1999), and inhibition of Ca\(^{2+}\) influx through membrane ion channels, as well as activation of K\(^{+}\) channels (Onoue and Katusic 1997).
The eNOS isoform is a membrane-bound Ca\(^{2+}\)/calmodulin requiring enzyme that tonically releases NO. Basal NO release in arteries has an important role in establishing basal smooth muscle tone. This is demonstrated by the elevated blood pressure of mice with targeted disruption of the gene for eNOS (Huang et al. 1995) and an increase in resistance to forearm blood flow in the presence of NOS inhibition in man (Vallance et al. 1989). NO production may also be stimulated by several agonists such as bradykinin and substance P (see below). For example, NO mediates relaxation to ACh in human resistance arteries (Woolfson and Poston 1990). The activation of receptors on the endothelium by these agonists produces an increase in intracellular Ca\(^{2+}\) that subsequently activates eNOS and results in smooth muscle relaxation.

1.2.2.1.2 Prostacyclin

Prostacyclin, a metabolite of arachadonic acid produced by the action of cyclooxygenase, is also synthesised by endothelial cells (Moncada et al. 1976). The role of prostacyclin in endothelium-dependent relaxations is typically studied by inhibiting cyclooxygenase activity with drugs such as indomethacin. These studies demonstrate that prostacyclin is involved in receptor-stimulated relaxations such as those induced by ACh in perfused guinea-pig heart (Lee et al. 1990). Additionally, prostacyclin has a role in flow-mediated dilatation of certain small arteries (Koller et al. 1994). Similarly iloprost, a stable analogue of prostacyclin, can elicit relaxation as well as hyperpolarisation of smooth muscle of guinea-pig coronary and mammary arteries (Hammarstrom et al. 1999). However, the role of prostanoids in endothelium-dependent vasodilatation appears to be limited to specific vascular beds. The effects of
prostacyclin are considered to be mediated by activation of adenylyl cyclase, resulting
in the release of cyclic adenosine monophosphate (cAMP). However, recently it has
been suggested that responses to iloprost may occur independent of cAMP pathway
(Turcato and Clapp 1999).

1.2.2.1.3 EDHF

It is apparent that in certain preparations endothelium-dependent relaxation occurs
independent of NO or prostanoid release. These observations led to the suggestion of
the existence of a third unidentified endothelium-derived relaxing factor (Taylor and
Weston 1988). This factor has been termed endothelium-derived hyperpolarising
factor (EDHF) and can be released from the endothelium by different agonists to
produce relaxation via hyperpolarisation of smooth muscle cells (for reviews see
Mombouli and Vanhoutte, 1997; Edwards and Weston, 1998). The relative
contribution of NO, prostanoids and EDHF to endothelium-dependent relaxation and
hyperpolarisation varies between vascular bed (Triggle et al. 1999). Indeed several
studies have indicated that the role of EDHF increases with decrease in vessel size
(Shimokawa et al. 1996; Lagaud et al. 1999; Garland and Plane, 1996). Although the
identity of EDHF is not known, the effects of EDHF are inhibited by combined
inhibition of large conductance and small conductance K⁺ channels (Petersson et al.
1997; Doughty et al. 1999). Therefore EDHF is considered to mediate smooth muscle
relaxation by opening of K⁺ channel(s). The physiological role of EDHF in regulating
vascular tone is not known but it is clear that stimulated endothelial cells can release a
factor that is independent of NO and prostanoid pathways.
1.2.1.4 Endothelin

Endothelial cells can also release contracting factors. In 1988 Yanagisawa and coworkers isolated a potent contracting factor from porcine endothelial cells. This factor, termed endothelin (ET), is a 21 residue peptide (Yanagisawa et al. 1988) with a similar structure to other peptide neurotoxins such as sarafotoxin 6b, snake venom from Israeli asp (Kloog et al. 1988). ET is cleaved from a large precursor peptide, big-endothelin, by the action of widely expressed membrane-bound endothelin converting enzymes (ECEs) (for review see Russell and Davenport, 1999). Three ECE isoforms have been identified but the predominant isoform in humans appears to be ECE-1.

There are three related peptides in the endothelin family: endothelin-1 (ET-1), endothelin-2 (ET-2), and endothelin-3 (ET-3). Of these, ET-1 is the predominant isoform expressed in blood vessels and is localised within endothelial cells, indicating a local role of ET-1 in regulation of tone (for review see Haynes and Webb, 1999). Apart from endothelial cells, ET peptides are also produced by intestinal, nerve, glial, kidney, epithelial, neutrophil, and parathyroid cells. ET-1 release from endothelial cells may be produced by stimuli such as thrombin (Marsen et al. 1995), hypoxia (Rakugi et al. 1990), or pressure (Nguyen et al. 1999). Responses to ET-1 in arteries are mediated by at least two distinct G-protein coupled receptors: ET\textsubscript{A} and ET\textsubscript{B}. ET receptors are classified according to their affinity for ET peptides (for review see Bax and Saxena, 1994). The potency at ET\textsubscript{A} receptors is ET-1=ET-2>>ET-3 and at ET\textsubscript{B} is ET-1=ET-2=ET-3. ET\textsubscript{A} receptor subtype is typically located on vascular smooth muscle cells and mediates contraction. Whilst ET\textsubscript{B} receptor subtype is predominantly located on endothelial cells, there is evidence for ET\textsubscript{B} receptor expression on smooth muscle (Davenport et al. 1995) and perivascular nerves (Bacon et al. 1996).
Activation of ET\textsubscript{B} receptors on endothelial cells mediates smooth muscle relaxation via the release of NO. ET-induced contraction has been demonstrated in several vascular beds from several species including human coronary resistance artery (Pierre and Davenport 1998), human internal mammary artery (Maguire and Davenport 1995), rat mesenteric small artery (Mickley \textit{et al.} 1997), porcine coronary artery (Elmoselhi and Grover 1997), canine basilar artery (Awane-Igata \textit{et al.} 1997), and rabbit aorta (Komuro \textit{et al.} 1997). More importantly, ET-1 has been demonstrated to have a role in maintenance of basal tone in human arteries since infusion of an inhibitor of ECE or ET\textsubscript{A} receptor antagonist into the brachial artery produces up to 64\% dilatation (Haynes and Webb 1994). Furthermore, systemic administration of combined ET receptor antagonist in man produces a decrease in peripheral resistance and blood pressure (Haynes \textit{et al.} 1996). ET\textsubscript{B}-mediated vasodilatation may also have a significant role in regulating basal tone. Indeed dilatation in human forearm resistance vessels by blockade of ET\textsubscript{A} receptors is abolished by ET\textsubscript{B} receptor antagonist or inhibition of NOS, suggesting that these responses are mediated by ET\textsubscript{B}-stimulated NO release. Moreover, systemic blockade of ET\textsubscript{B} receptors in man causes peripheral vasoconstriction (Strachan \textit{et al.} 1999), suggesting that endogenous ET-1 also produces ET\textsubscript{B}-mediated vasodilatation \textit{in vivo}. 
Substance P is a member of the tachykinin (TK) family of peptides. It was first described in 1931 by von Euler and Gaddum, who noted that extracts of brain and gut tissue produced potent smooth muscle contraction and hypotension. However, it was not until 1971 that SP was sequenced (Chang et al. 1971). Two other peptides of the TK family were subsequently sequenced and named neurokinin A (NKA) and neurokinin B (NKB). The precursor peptides of TKs, prepro-TKs, are produced in neuronal cell bodies of capsaicin-sensitive sensory nerves, stored in vesicles and transported to nerve endings for final processing (for review see Lundberg 1996). SP-containing nerve terminals are abundant in the walls of several blood vessels. Immunohistochemical studies suggest that SP is also present in endothelial cells of several arteries from different species (Linnik and Moskowitz 1989; Cai et al. 1993; Ralevic et al. 1995). TKs exert a wide range of effects on smooth muscle, vascular endothelium, nerves, mast cells and the immune system (for review see Lundberg 1996). Pharmacological and molecular biology studies have demonstrated three receptors that mediate the biological effects of TKs, termed neurokinin 1 (NK1), neurokinin 2 (NK2), and neurokinin 3 (NK3). These receptors are classified according to their affinity for TKs. Although SP, NKA and NKB can all stimulate NK receptors, SP has the greatest affinity for NK1, NKA for NK2, and NKB for NK3. In blood vessels SP produces potent vasodilatation; an effect that is mediated by NK1 receptors. For example, exogenous application of peptide substance P (SP) produces relaxation of isolated resistance blood vessels in vitro (for example see D'Orleans-Juste et al. 1985). This response is endothelium-dependent (D'Orleans-Juste et al. 1985) and is typically mediated by NO release (see for example Whittle et al. 1989).
In addition to its vasodilator effects, SP produces smooth muscle contraction in certain blood vessels, such as rat mesenteric bed (D'Orleans-Juste 1991).

1.2.2.1.6 Bradykinin

Bradykinin (BK) is a nonapeptide synthesised from plasma and tissue kininogen by the action of kallikrein (for review see Boohla et al. 1992). BK is cleaved by kininase enzymes. Kininase I removes the C-terminal arginine from BK to yield des-Arg²-BK, which also exerts biological effects. Kininase II cleaves two amino acids from the C-terminus, and is the same as angiotensin converting enzyme (ACE) (see below). The effects of BK are due to stimulation of specific G-protein coupled cell surface receptors, termed B1 and B2 (for review see Regoli and Barabe 1980). These receptors are classified according to their affinity for des-Arg²-BK. This peptide is active only on a subset of BK receptors that are termed B1. B1 receptors appear to be normally absent from blood vessels but become evident in the presence of inflammatory and noxious stimuli. Most of the acute actions of BK however are mediated by the constitutively expressed B2 receptors. BK has been demonstrated to be a potent vasodilator, an effect that is typically mediated by the release of endothelium-derived relaxing factors (D'Orleans-Juste et al. 1985). For example, vasodilatation in human forearm is partly attenuated by NOS inhibition (O'Kane et al. 1994). Similarly, intravenous administration of BK in rats produces a decrease in blood pressure that is inhibited in the presence of a NOS inhibitor (Whittle et al. 1989). BK also produces potent endothelium-dependent relaxation of isolated blood vessels in vitro such as human omental resistance arteries (Ohlmann et al. 1997) and rat resistance mesenteric arteries (Lagaud et al. 1999), via the release of NO and
prostanoids. Indeed it has been demonstrated that BK produces a concentration-dependent release of NO from cultured endothelial cells (Palmer et al. 1987). It is also clear that in several vascular beds, including human coronary resistance arteries (Kemp and Cocks 1997), BK produces relaxation that may be mediated by EDHF (see above). Consistent with this hypothesis, it has recently been shown that BK can produce endothelium-dependent hyperpolarisation of cultured porcine coronary and guinea-pig carotid smooth muscle cells (Quignard et al. 1999).

1.2.2.1.7 Thromboxane

Like prostacyclin, thromboxane A₂ (TXA₂) is a metabolite of arachadonic acid produced by the action of cyclooxygenase. TXA₂ is the major cyclooxygenase product in platelets, and is released by these cells when stimulated (Fitzgerald et al. 1986). TXA₂ is a potent platelet aggregant and vasoconstrictor. The constrictor effects of TXA₂ are predominantly mediated by specific receptors on vascular smooth muscle, TP receptors (Coleman et al. 1984).

1.2.2.1.8 Angiotensin

In 1898 Tigerstedt and Bergman demonstrated that extracts of rat kidney injected into dogs produced increase in blood pressure. The agent responsible for this was named renin and was the first demonstration of the renin-angiotensin system. It was later established that the pressor response to renin was dependent on plasma and mediated by the release of two peptides. These peptides were the decapeptide angiotensin I (AngI) and octapeptide angiotensin II (AngII), which are released by the action of
renin on angiotensinogen, a substrate present in plasma. A membrane-bound enzyme was identified in the pulmonary circulation that was able to convert AngI to AngII, which was named angiotensin converting enzyme (ACE) (Ng and Vane 1968).

Further investigation of the renin-angiotensin system indicated that the primary site of AngI and AngII production is not the pulmonary circulation but that these peptides may also be produced locally in tissues. Indeed renin-like activity has been demonstrated in the walls of several blood vessels. In particular, immunohistochemical studies have demonstrated renin staining throughout the walls of human arteries (Okamura et al. 1992). The gene for renin substrate, angiotensinogen, has also been localised in blood vessels (Naftilan et al. 1991) and assays of bovine aortic endothelial cells indicate that these cells are capable of synthesising angiotensins (Kifor and Dzau 1987). Therefore, there is now considerable evidence for a local tissue renin-angiotensin system in the walls of blood vessels (for review see Webb and Cockcroft 1990), suggesting that these peptides may have a role in local regulation of tone (Samani 1991). Exogenous application of AngII in vitro produces potent concentration-dependent contraction that is predominantly mediated by angiotensin type 1 receptor stimulation. Several studies in man demonstrate that infusion of either AngI or AngII produces a dose-dependent decrease in forearm blood flow (see for example Webb et al. 1988; Benjamin et al. 1989). However, it is not clear whether angiotensins are involved in acute regulation of vascular tone in man particularly since inhibition of ACE (Webb et al. 1988) or angiotensin receptors has no effect on forearm vascular resistance (Newby et al. 1997).
1.2.2.2 Neuronal Influences

The walls of blood vessels are densely innervated by nerve fibres that exert a local control of blood flow (for review see Burnstock and Ralevic 1994). The autonomic nervous system is classically divided into two subpopulations: the sympathetic and parasympathetic nervous systems, which generally exert opposing effects. Although sympathetic nerves were regarded as containing noradrenaline (NA), it is now clear that these nerves also contain cotransmitters such as adenosine 5'-triphosphate (ATP) (Burnstock 1976) and neuropeptide Y (NPY) (Ekblad et al. 1984). The proportion of cotransmitters in sympathetic nerves varies between vascular beds. For example, ATP has been reported to be the sole transmitter in rabbit mesenteric resistance arteries whilst it has a minor role in rabbit ear artery (see Burnstock 1988). Therefore, cotransmission allows for regional variation and fine-tuning of neurogenic control of tone. The release of NA and ATP from nerve endings may mediate contraction of smooth muscle via activation of specific adrenergic and purinergic receptors respectively. Indeed contractile responses to electrical stimulation of sympathetic nerves in rat mesenteric small artery are inhibited by combined application of adrenergic and purinergic receptor antagonists (Sjoblom-Widfeldt 1990). ATP also produces relaxation of smooth muscle, for example in response to electrical field stimulation of rabbit mesenteric small artery (Kakuyama et al. 1998). In several human, rat, murine, rabbit, cat, and guinea-pig blood vessels, exogenous NPY produces little direct contractile effect (Ekblad et al. 1984). However, NPY potentiates contractions to exogenous NA, and ATP, as well as sympathetic nerve stimulation in these vessels (Ekblad et al. 1984). Conversely, blockade of NPY receptors attenuates sympathetic-induced vasoconstriction in pigs in vivo (Lundberg
and Modin 1995) and in perfused rat mesenteric bed in vitro (Han et al. 1998). Thus, NPY predominantly acts as a modulator of sympathetic nerve stimulation in blood vessels.

The neurotransmitter ACh is found in parasympathetic nerves and endothelial cells (Milner et al. 1989). ACh stimulates postjunctional receptors to mediate endothelium-dependent smooth muscle relaxation (Furchgott and Zawadzki 1980) contraction of visceral smooth muscle, and secretion from gland cells. Vasoactive intestinal peptide (VIP) is costored in these nerves and released with ACh upon nerve stimulation (Lundberg 1981). The predominant effect of VIP in several vascular beds is as a potent vasodilator, a response that is typically independent of the endothelium (Pernow 1989) and mediated by cAMP.

In addition to sympathetic and parasympathetic nerves, there are also a population of nerves that are nonadrenergic and noncholinergic (NANC). These include the primary afferent sensory nerves present throughout the circulation, which also have efferent functions on tissues via the release of neurotransmitters. The transmitters in NANC nerves include SP (see above) and calcitonin gene-related peptide (CGRP). CGRP is a 37 amino acid peptide produced by the alternative splicing of calcitonin gene. It is primarily found in primary afferent nerves and is often colocalised with SP (for review see Maggi 1995). It exerts its effects via the activation of specific G-protein coupled receptors (for review see Bell and McDermott 1996). CGRP1 is classified by its high affinity for CGRP fragment CGRP(8-37), which is an antagonist at these receptors. CGRP2 is classified by its low affinity for CGRP(8-37). Stimulation of
NANC nerves in rabbit mesenteric small artery in vitro produces a frequency-dependent relaxation that is partly mediated by CGRP release (Kakuyama et al. 1998), indicating a role for CGRP in local regulation of tone. Exogenous CGRP is a potent vasodilator of several vascular beds in different species. This effect is predominantly endothelium-independent (Greenberg et al. 1987) and involves hyperpolarisation by activation of ATP-sensitive K⁺ channels and elevation of cAMP levels. However, there are some reports of endothelium-dependent relaxations to CGRP in certain blood vessels, including rat aorta, which appear to be mediated by NO release (Gray and Marshall 1992).

NO itself is also a neurotransmitter (Garthwaite et al 1988). Unlike other neurotransmitters, it is not stored but is synthesised by a NOS enzyme (nNOS) on demand (Bredt et al. 1990). NO produces relaxation of smooth muscle (see above) and has been demonstrated to have an important role in mediating NANC vasodilator responses in several blood vessels including monkey (Toda and Okamura 1992) and canine mesenteric arteries (Toda and Okamura 1990). Nitrergic neurotransmission has also been demonstrated to have an important role in mediating NANC relaxation of rat anococygeus and bovine penile artery (Liu et al. 1991). Indeed NO is also colocalised with VIP in nerves supplying human penis where these two neurotransmitters mediate penile erections (Ehmke et al. 1995). Neuronal-derived NO release may also occur tonically and, like eNOS-derived NO, have a role in basal tone of smooth muscle (Gillespie et al. 1989).
1.2.2.3 Autoregulation

Another significant component of local regulation of vascular tone occurs independent of neuronal and humoral influences, termed autoregulation. Autoregulation can be defined as the innate response of blood vessels to the intraluminal forces of pressure (transmural force) and flow (shear stress) (for reviews see Schubert and Mulvany 1999; Koller and Kaley 1996) in order to regulate blood flow. Thus autoregulation describes responses to changes in the haemodynamic environment rather than humoral environment. The majority of studies on pressure and flow-induced responses involve measurement of responses to acute changes in flow or pressure. However, long-term alterations in pressure and flow may also result in structural and functional changes of the vessel wall (for review see Cowley 1992).

The acute response to transmural pressure is termed myogenic tone and describes the ability of certain blood vessels to respond to elevations in transmural pressure by constricting and to dilate in response to decreases in pressure (Bayliss 1902). Myogenic constriction allows for constant perfusion of a vascular bed over a range of intraluminal pressure and is also an important determinant of basal tone on which vasodilators and vasoconstrictors can act. The degree of myogenic tone that an artery develops, in general, increases with decreases in vessel size such that these responses predominate in small resistance arteries (Sun et al. 1992) whilst larger arteries show little, if any myogenic tone. Myogenic responses have been demonstrated in several species and vascular beds including human cerebral resistance artery (Wallis et al. 1996), human myometrial artery (Kublickienė et al. 1997), rat cerebral artery (Skarsgard et al. 1997), rabbit renal artery (Edwards 1983), rat mesenteric small artery
(Sun et al. 1992), rat gracilis muscle arteriole (Koller et al. 1994), and porcine coronary arteriole (Kuo et al. 1988). It has been suggested that the stimulus for myogenic tone is wall tension (VanBavel and Mulvany 1994) whereby pressure-induced increases in wall tension produce a decrease in lumen diameter and thus normalisation of wall tension according to the Laplace relationship (Wall tension = pressure x radius). The precise mechanisms involved in the myogenic response are not clear (for review see Davis and Hill 1999) but are considered to be due to a direct effect of pressure on smooth muscle, resulting in depolarisation (Knot and Nelson 1998) with a consequent increase in smooth muscle intracellular \(Ca^{2+}\).

Numerous studies have excluded a role for the endothelium in mediation of the myogenic response in a wide range of small blood vessels from several different species (for review see Meininger and Davis 1992). Recently, however there has been renewed interest in the role of the endothelium, not as a source of the mediator of myogenic tone but rather as a modulating influence over the myogenic response. Studies of mesenteric vessels from spontaneously hypertensive rats suggest that removal of the endothelium enhances myogenic constriction in response to pressure elevation (Garcia et al. 1997) possibly due to the removal of the vasodilator influence provided by endothelially-derived NO. Similarly, in a recent study of hamster cremaster arterioles in vivo NOS inhibitors potentiated the constriction of large but not small arterioles to increases in intraluminal pressure (de Wit et al. 1998). In addition to the inhibitory effect of NO on myogenic tone, the endothelium may also positively modulate pressure-induced constriction since endothelin receptor antagonists reduce myogenic tone in pressurised rabbit mesenteric small arteries (Nguyen et al. 1999).
Flowing blood exerts a frictional force on blood vessels in the same axis as blood flow (shear stress). The level of shear stress throughout the circulation is relatively constant despite differences in vessel calibre and rates of flow. This is due to responses of blood vessels to changes in flow in order to normalise shear stress since shear stress is inversely related to diameter. Thus changes in flow can initiate dilatation or constriction. The most common observation to changes in flow is dilatation, both in vitro and in vivo. However, flow-induced constriction has been demonstrated in vitro at low and high levels of tone but has not been demonstrated in vivo (for review see Koller and Kaley 1996). Therefore, flow-induced constriction may serve to modulate dilator responses and thus prevent excessive flow-induced dilatation. In contrast to myogenic tone, flow-induced responses have been demonstrated in conduit blood vessels, such as canine femoral and coronary artery (Rubanyi et al. 1986), as well as resistance vessels such as human resistance myometrial arteries (Kublickiene et al. 1997) and rat gracilis muscle arterioles (Koller et al. 1994).

The endothelium is ideally situated between flowing blood and smooth muscle to regulate and mediate responses to changes in flow. This hypothesis is supported by the observations that removal of the endothelium abolishes flow-induced dilatation (Koller et al. 1994). In 1986 Rubanyi et al measured flow-induced prostanoid release in canine coronary artery and demonstrated an increase in prostacyclin release that was inhibited by indomethacin. However, the effluent was able to relax segments of endothelium-denuded artery in the presence of cyclooxygenase inhibition indicating that another endothelium-derived factor is released in response to changes in flow.
These authors identified this factor as NO. Similarly, endothelium-derived NO mediates flow-induced dilatation in other vascular beds. For example, in human resistance myometrial arteries (Kublickienė et al. 1997) inhibition of NOS abolished flow-induced dilatation and instead these vessels developed constriction in response to flow. In some preparations endothelium-derived prostanoid release, in addition to NO, has been implicated in mediating flow-induced dilatation. Flow-mediated responses are partly attenuated by inhibition of either NOS or prostanoids but completely abolished by combined application of both inhibitors in isolated rat (Koller et al. 1994) and murine (Sun et al. 1999) gracilis muscle arterioles in vitro. Increases in flow are associated with increases in eNOS expression in porcine coronary arterioles (Woodman et al. 1999), and cultured rat mesenteric endothelial cells (Redmond et al. 1998). Changes in flow also alter the capacity of G-protein signalling in cultured rat mesenteric smooth muscle cells via an endothelium-dependent, indomethacin-sensitive mechanism (Redmond et al. 1998). Therefore, it is clear that the endothelium has a critical role in mediating responses to flow.

It is clear that myogenic tone is opposed by flow-mediated dilatation and that responses to flow are similarly dependent on the level of myogenic constriction (Kuo et al. 1991). Therefore, the actual net tone due to autoregulatory processes in vivo depends on the sum of pressure and flow-mediated responses.
1.2.3 Contraction of smooth muscle

The regulation of vascular tone due to the above factors is mediated by complex regulation of changes in intracellular calcium concentrations ([Ca$^{2+}$]), (for reviews see Somlyo and Somlyo 1994; Hughes 1998; Shaw and McGrath 1996). Contraction of smooth muscle is mediated by an increase in [Ca$^{2+}$], and conversely relaxation of smooth muscle is mediated by a decrease in [Ca$^{2+}$]. Changes in [Ca$^{2+}$], may be achieved by influx of extracellular Ca$^{2+}$ through voltage-sensitive Ca$^{2+}$ channels (VOC), which are sensitive to changes in membrane potential and increase in open probability when the membrane is depolarised. The predominant VOC in vascular smooth muscle is the L-type channel, which is characterised by its high voltage activation and sensitivity to dihydropyridines, such as nifedipine. Another important mechanism of Ca$^{2+}$ influx is through receptor-operated Ca$^{2+}$ channels (ROC), which are less selective for Ca$^{2+}$ over other ions like Na$^+$ and K$^+$ than VOC and are directly linked to the receptor.

Increases in [Ca$^{2+}$], may also be achieved by the release of Ca$^{2+}$ from intracellular sources, although it appears that this is less important in resistance arteries than in capacitance vessels. Mobilisation of Ca$^{2+}$ from intracellular stores occurs independent of changes in membrane potential and is mediated by changes in concentrations of inositol-1,4,5-triphosphate (IP3), a metabolite of membrane phospholipid phosphatidylinositol 4,5,-biphosphate (PIP2) by the action of phospholipase C enzymes. IP3 binds to specific receptors on intracellular stores to stimulate efflux of Ca$^{2+}$. Uptake of Ca$^{2+}$ into intracellular stores is via a Ca$^{2+}$ ATPase that is reversibly inhibited by cyclopiazonic acid (CPA) and irreversibly thapsigargin. Indeed these drugs are often used to deplete Ca$^{2+}$ stores to indicate the importance of intracellular Ca$^{2+}$ release in mediating contractile responses. Although IP3 is probably the most important mediator of intracellular Ca$^{2+}$ release, Ca$^{2+}$ stores in smooth muscle also contain ryanodine-sensitive ion channels, the role of which remains unclear.

Elevation of [Ca$^{2+}$], induces contraction of smooth muscle through the formation of a complex with the ubiquitously expressed intracellular protein calmodulin (CaM). The Ca$^{2+}$/CaM complex can activate several enzymes including the myosin light chain kinase (MLCK), which is required for contraction of vascular smooth muscle. The contractile apparatus of smooth muscle cells consist of thin actin filaments and thick myosin filaments.
There are two anatomically distinct patterns of vasa; first order vasa run longitudinally to the lumen of the host vessel; while second order vasa are arranged circumferentially around the host vessel (Kwon et al. 1998a). Arterial vasa are readily distinguishable from venous vasa since they have a straight course whereas the course of venous vasa is more tortuous (Lowenberg and Shumacker, 1940). It is likely that this relates to the difference in wall thickness between the small arteries and veins. Also, arterial vasa are less numerous with fewer branches and have a smaller lumen than the small veins. The vasa vasorum in the aortic media has been observed to consist of a layer of smooth muscle around an endothelial layer. The smooth muscle of the vasa vasorum is oriented in relation to the vasa rather than the aortic media (Heistad and Marcus, 1979).

There is evidence that the vasa vasorum may be neurally innervated. Nerve fibres have been observed near the vasa vasorum of the aortic media (Heistad and Marcus, 1979). In addition, it has been shown that the vasa vasorum of the human saphenous vein are closely associated with unmyelinated sympathetic nerve fibres (Herbst et al. 1992). NPY, VIP and dopamine β-hydroxylase-immunoreactive nerves have also been demonstrated near the vasa vasorum of deep dorsal penile vein (Crowe et al. 1991). NPY immunoreactivity has also been demonstrated near vasa vasorum of human uterine artery (Stjernquist et al. 1991). Although the neural innervation associated with the vasa vasorum appears to be mainly sympathetic, other nerve types may also be present in some blood vessels. For example, CGRP and SP-containing nerves have been demonstrated around the vasa vasorum of human saphenous vein (Herbst et al. 1992).
1992) and rat carotid arteries (Milner et al. 1997). It should be emphasised that these observations are of whole sections of the host vessel wall and that direct innervation of vasa vasorum has not yet been demonstrated.

The presence of the vasa vasorum in blood vessels is likely to be related to nutritional needs. Comparison of different species revealed that vasa vasorum are present in the media of the aorta of those animals in which the aortic wall thickness exceeds 0.5mm (Geiringer, 1951). Wolinsky and Glagov (1967) redefined this critical depth in terms of lamellar units instead of absolute wall thickness where a lamellar unit is a fibromuscular unit consisting of an adjacent compartment of circumferentially oriented collagen, elastin, and smooth muscle. The thoracic aorta of adult small animals, such as rat, has less than 29 lamellae and is avascular in these species. Conversely, the inner 29 lamellae of the thoracic aorta in large animals are avascular and the outer lamellae are supplied by the vasa vasorum. Moreover, the number rather than the thickness of lamellar units may be a critical factor since avascular portions of the aorta of newborn humans is approximately half the thickness (0.16mm) of the adult feline aorta, although it contains 29 lamellae. Consistent with Wolinsky and Glagov’s definition, the media of human abdominal aorta has 28 lamellae and is avascular. Interestingly, the avascular regions of the human abdominal aorta also show the greatest propensity for atherosclerosis (Wolinsky and Glagov, 1967), implying that the blood supply provided by the vasa vasorum may be protective in this respect.

Therefore, one could speculate that if the vasa vasorum are important in providing protection of the host vessel, decreases in blood flow through these microvessels may
contribute to atherosclerosis. Coronary arteries are an exception to the above definitions since the critical wall thickness is less in coronary arteries (0.35mm) than in the aorta (Geiringer 1951). This anomaly suggests that the above definition may not hold true for all vessels and that as well as wall thickness, an important determinant for the presence of vasa vasorum may be luminal oxygen tension. In support of this is the finding that large veins, which have thin walls but low luminal oxygen tension, are also supplied by a dense network of vasa (Brook 1977). The observation that the supply of vasa vasorum is more extensive to veins than arteries implies that these microvessels are sensitive to oxygen tension. Indeed, measurement of vasa vasorum blood flow to the canine aorta suggests that vasa vasorum dilate in response to acute systemic hypoxia (Heistad et al. 1986). This may represent an important mechanism whereby the supply of oxygen to a blood vessel wall is increased when diffusion of oxygen from the lumen is limited. However, blood flow to the walls of large veins such as the inferior vena cava is greater than that to veins with thinner media such as the superior vena cava (Heistad et al. 1986) indicating that wall thickness is also an important determinant of the presence of vasa venarum. Similar to veins, the pulmonary artery, which also has low luminal oxygen tension, has a more extensive supply of vasa vasorum in the adventitia and outer media than systemic arteries (Sobin et al. 1962). Indeed measurement of blood flow to canine pulmonary artery is equal to that to the aorta despite a difference in wall thickness and lamellar units (29 compared to 49) (Heistad et al. 1986). Therefore, both oxygen tension and wall thickness are important determinants of the presence of vasa, which may have important implications for the host vessel in diseases which result in increases in wall thickness or hypoxia (see later). The distribution of the vasa is not fixed. It is clear
that in certain cardiovascular diseases (see later), including atherosclerosis, the number and density of vasa changes and it seems that this relates closely to changes in blood supply to the conduit vessel wall.

1.3.2 Regulation Of Blood Flow

The presence of smooth muscle implies that the vessels of the vasa vasorum actively regulate their own tone rather than serving as a passive channel for blood flow. The first studies supporting this hypothesis investigated vasa vasorum reactivity to vasoactive agents in dogs in vivo (Heistad et al. 1978). This study exploited the microsphere technique in which microspheres labeled with gamma-emitting isotopes were injected into the left atria, distributed throughout the circulation and, since they are too large to pass through capillaries, extracted following a single passage through the vasa vasorum. The results of this study suggested that the diameter of the vasa vasorum of canine thoracic aorta increased in response to intravenous infusion of adenosine (Heistad et al. 1978). In contrast another study, using the same technique, showed no change in blood flow through the vasa vasorum of carotid arteries of monkeys during infusion of either the vasoconstrictor phenylephrine (PE) or 5-hydroxytryptamine (5-HT) (Williams et al. 1988a). Ohhira and Ohhashi (1992) removed sections of the vasa vasorum attached to the canine thoracic aorta and examined the reactivity of the vasa vasorum in vitro by measurement of perfusion pressure. In this study the vasa vasorum appeared sensitive to a range of constrictor agents: 5-HT>> noradrenaline (NA)=adrenaline>>dopamine. Indeed, in vitro receptor autoradiography has demonstrated dense binding of [3H]-5-HT in the vasa vasorum of human saphenous vein (Dahm et al. 1996).
The vasa vasorum is also sensitive to several vasodilators. *In vivo* studies, again using microspheres, showed that ACh, histamine, isoprenaline, ATP, adenosine diphosphate, adenosine or sodium nitroprusside all depress perfusion pressure through the vasa vasorum (Ohhira and Ohhashi, 1992).

Whilst it is has been demonstrated that flow through the vasa vasorum may be regulated by vasoactive substances, the contribution of neurotransmitters on regulation of tone in vasa vasorum is not known. Heistad *et al.* (1979) suggested that blood flow through the vasa vasorum may be sensitive to neural stimulation since stimulation of the stellate ganglion of dogs decreased blood flow to the outer media of the thoracic aorta and stimulation of carotid sinus baroreceptors increased blood flow. Therefore, in addition to certain humoral factors, tone of vasa vasorum may also be regulated by neuronal influences. However, there are no studies that have demonstrated direct neural responses in isolated vasa vasorum.

Whilst these studies appear to support the hypothesis that the vasa vasorum regulates its own tone, in both systems vasa vasorum reactivity was not studied in isolation. The possibility that the responses seen were secondary to effects on the host vessel and not direct effects of the agonists on the vascular smooth muscle of the vasa vasorum cannot be excluded.
1.3.3 Role Of Vasa Vasorum In Cardiovascular Diseases

The presence of the vasa vasorum in blood vessel walls is considered to be required for the maintenance of conduit vessel physiology. Evidence to support this hypothesis has been provided by experimental disruption of blood flow through the vasa vasorum of healthy blood vessels. Furthermore there is considerable evidence that the vasa vasorum may be involved in certain cardiovascular diseases, including atherosclerosis.

1.3.3.1 Atherosclerosis

Atherosclerosis is a progressive inflammatory disease of large arteries (for reviews see Ross 1999; Schachter 1998). It is a leading cause of death in the industrialised world (Ross 1993). The response to injury hypothesis proposes that endothelial dysfunction is the first step in development of atherosclerosis (Ross and Glomset, 1973). Endothelial dysfunction may be induced by several factors including elevated levels of low-density lipoprotein, smoking, diabetes, hypertension, genetic factors and certain infectious agents. Disruption of normal endothelium function is associated with increased expression of some cell adhesion molecules leading to increased adhesion of leukocytes and platelets to the endothelium in addition to increasing permeability so that monocytes and T lymphocytes adhere and penetrate the endothelial layer to form the initial lesion. Endothelial dysfunction also alters the anticoagulant properties of the endothelium to procoagulant, and induces vasoactive agents, cytokines and growth factors. Continued inflammation leads to proliferation and migration of smooth muscle cells to form an intermediate lesion. Further, monocyte-derived macrophages form foam cells within the lesion and also multiply leading to the release of hydrolytic enzymes, cytokines, chemokines, and growth factors. Repeated cycles of accumulation of inflammatory cells, migration and proliferation of smooth muscle, and formation of fibrous tissue lead to formation of a fibrous cap over a complex lesion containing lipids such as esterified cholesterol. These lesions protrude into the lumen of the vessel and alter blood flow, which may lead to ischaemia and infarction of the heart, brain and extremities. Additionally, lesions containing large amounts of lipids may be unstable and rupture, resulting in thrombosis.
It has been suggested that the vasa vasorum in the adventitia of large arteries may have a crucial role in the pathogenesis of atherosclerosis. In 1966 Nakata and Shionoya demonstrated that occlusion of blood flow through the vasa vasorum of canine abdominal aorta, with a thrombin and gelatin mix, results in an initial intimal thickening and smooth muscle proliferation with subsequent lipid deposition and hypertrophy of the neointima. More recently, Martin et al. (1991) described a model of atherosclerosis in rabbits in which placement of an inert silastic collar around the outside of the carotid arteries results in the formation of an atheroma-like intimal lesion beneath an anatomically and apparent functionally intact endothelium. These authors argue that occlusion of the adventitial vasa vasorum by the collar leads to hypoxia of the vessel wall and that this initiates smooth muscle cell proliferation and migration. However, the mechanisms of collar-induced lesions are controversial. Intimal thickening was also observed in the rabbit thyroid artery, a small branch of the carotid artery, despite the absence of vasa vasorum in these arteries (De Mayer et al. 1997). Nevertheless Barker et al. (1994) demonstrated that removal of the adventitia containing the vasa vasorum of carotid arteries, from rabbits, induces an intimal lesion containing smooth muscle cells and macrophages. Furthermore ligation of the side branches of femoral arteries, and hence disruption of vasa vasorum blood flow, in the Yucatan miniature pig produces significant intimal hyperplasia (Barker et al. 1993). Similarly, removal of the periaortic fat containing the vasa vasorum of canine ascending aorta results in extensive medial necrosis and an acute decrease in aortic distensibility (Stefanadis et al. 1993). Collectively these studies suggest that impairment of nutrient blood flow through the vasa vasorum may contribute to vessel
wall hypoxia and that they might allow predisposition to atherosclerosis or other degenerative conditions of the host vessel.

The number of vasa vasorum supplying large arteries remains constant throughout adult life. However, vasa can proliferate in response to acute arterial injury. In particular, studies on the vasa vasorum of coronary arteries from hypercholesterolemic pigs suggest that the three-dimensional pattern of second order vasa increases and becomes disorganised (Kwon et al. 1998b). As mentioned above, the vasa vasorum are sensitive to oxygen tension and therefore the proliferation of vasa vasorum in arteries occluded by atheromatous plaques may be due to decreased oxygen tension in the vessel wall. The severity of atherosclerosis is directly related to the density of adventitial vasa vasorum (Williams et al. 1988) but the role of newly formed microvessels in the chronic processes of atherogenesis is unknown. There are currently two postulates (Pels et al. 1997); firstly, microvessels that grow into the media and intima may nourish and stabilise the growing plaque by delivering growth factors and hormones. Support for this hypothesis is provided by immunohistochemical and confocal microscopy studies on microvessels in human coronary arteries (Zhang et al. 1993). These studies demonstrated the presence of microvessels in thickened intimas and atherosclerotic plaques that was directly related to the size of the plaque and inversely related to lumen diameter. This rich neovascularisation could be traced from adventitial vasa vasorum through the media and occurred predominantly at the base of the plaque at the border with the normal intima. Albumin and fibrinogen leakage was associated with these microvessels and immunoglobulin containing cells were observed surrounding microvessels indicating
that intimal vascularisation contributes to nourishment of atheromatous plaques and inflammation in conduit blood vessel walls. Unlike the resident vasa, these newly developed vasa are typically fragile endothelial channels without smooth muscle layers (Williams et al. 1988b) and therefore do not have the capacity to regulate tone. Furthermore, these vessels may rupture and therefore have a role in intraplaque haemorrhage and plaque rupture. In contrast, a study by Williams et al. (1988a) in monkeys demonstrates vasa in the intima-media with a single layer of smooth muscle, suggesting that these vessels may have some capacity to regulate tone. This study also suggests that the smooth muscle of vasa vasorum of atherosclerotic arteries is more sensitive to vasoconstrictors (Williams et al. 1988a). A decrease in blood flow to the vessel wall would contribute to further vessel wall hypoxia and presumably thereby aid the progression of atheroma formation.

In contrast, it has been suggested that these microvessels may limit the progression of the lesion by maintaining nutrient blood flow to the thickened vessel wall. Barker et al (1994) used loosely placed PVC tubing to promote the formation of a "neoadventitia" around rabbit carotid arteries with intimal lesions and demonstrated that lesions regress under these conditions. Thus maintenance of blood flow through the vasa vasorum might limit neointimal formation.

Therefore there is a paradox in the benefit of proliferation of vasa vasorum in atherosclerosis. Whilst these vessels provide a significant increase in nutrient blood flow to the thickened artery wall, several studies suggest that these vessels are "leaky" and prone to rupture. These vessels may thereby contribute to intraplaque haemorrhage,
plaque rupture and formation of thrombi. In addition if, in some cases, these vessels do contain smooth muscle, it appears to be more sensitive to constrictors and therefore blood flow through these particular vessels may be compromised.

1.3.3.2 Restenosis

Transluminal angioplasty is now a common method of restoring blood flow in vessels that are occluded with atheromatous plaques. However, within 6 months, vessel size often returns to preangioplasty dimensions (restenosis) (O'Brien et al. 1994). Recently, a three-dimensional study of the anatomy of normal and balloon-injured porcine coronary arteries has demonstrated that there is a decrease in the ratio of first order to second order vasa 28 days after balloon injury (Kwon et al. 1998a). Basic fibroblast growth factor, which is both mitogenic and angiogenic may mediate this proliferation of vasa vasorum in response to local injury (Edelman et al. 1992). Although the density of vasa is directly related to the severity of stenosis (Kwon et al. 1998a), it is not yet clear whether these changes in the vasa vasorum are a consequence or a cause of changes in the vessel wall.

Balloon angioplasty is associated with stretching and splitting of the intima/media as well as the adventitia of the vessel (Scott et al. 1996). Additionally, several studies clearly show endothelial damage of the large vessel following this procedure. Of interest is the possibility that these procedures may also damage the endothelium of the vasa vasorum resulting in impaired endothelium-dependent control of blood flow to the vessel wall and subsequent vessel wall hypoxia. Hypoxia itself is a stimulus for the induction of several growth factors and cytokines and in this way may contribute
to the mechanisms of luminal narrowing. Several authors have investigated the effect of angioplasty on the morphology and blood flow through the vasa vasorum but the findings are inconclusive. In 1982, Train et al observed a fine vascular network around the femoral arteries of 3 patients immediately following angioplasty that may have represented hypertrophied vasa. Yet Cragg et al (1983) did not observe any acute (up to 7 days postangioplasty) morphological changes in the vasa vasorum of dilated canine carotid arteries. In contrast, studies on the long-term effects of angioplasty in dogs showed considerable stretching and rupture of the vasa vasorum that was followed by extensive proliferation of vasa (Zollikofer et al. 1987). However, this increased vascularisation following angioplasty completely regresses by 18 months (Pisco et al. 1994). The reasons for the regression of these microvessels are not known but may be a component of inadequate remodeling (Pels et al. 1999). The functional effects of the morphological changes in the vasa vasorum are not clear; measurements of vasa vasorum blood flow, using the microsphere technique in dogs, showed that blood flow may be increased (Cragg et al. 1983) or decreased (Eisenhauer et al. 1990) immediately after angioplasty.

1.3.3.3 Hypertension

Power-Doppler imaging of blood flow through the vasa vasorum of normal human carotid arteries demonstrate that perfusion of vasa vasorum occurs after the main flow velocity in the lumen of the carotid artery (Belcaro et al. 1996). This suggests that, similar to the coronary circulation, the vasa vasorum fill during diastole. It may therefore be expected that an increase in arterial pressure in the host vessel leads to a reduction in perfusion of the vasa vasorum. In order to test this hypothesis Sacks
(1975) used a model of a section of the aortic wall, comprising a simulated vasa embedded in a block of soft material, and measured the patency of the vasa (flow per unit pressure drop). In this model, elevation of radial stress on the vasa resulted in decreased patency of vasa vasorum. The author suggests that elevation of blood pressure and thus compression of the aortic wall reduces blood flow through the vasa vasorum. Furthermore, measurements of blood flow in canine thoracic aortic wall, using the microsphere technique, indicate a substantial reduction in the vasodilator capacity of vasa vasorum in animals with chronic hypertension (Marcus et al. 1985). Therefore it is possible that associated increases in wall tension in hypertension, distort the vasa vasorum thus leading to underperfusion of blood vessel walls, and thereby compound the situation by producing vessel wall hypoxia.

1.3.3.4 Deep Vein Thrombosis

The extensive supply of microvessels to the walls of large veins suggests that the vasa venarum is likely to be the most important source of nutrition in these blood vessels. Indeed O'Neill (1947) demonstrated that experimental disruption of the vasa venarum of canine jugular vein results in an increase in permeability of endothelial cells. Subsequent accumulation of fluid beneath the endothelial layer caused the endothelial cells to lift off. It is apparent that damage to endothelial cells is the precursor for thrombus formation. Therefore, disruption of blood flow through microvessels supplying large veins may have a role in endothelial damage and subsequent thrombosis.
1.4 AIMS AND OBJECTIVES

Disruption of blood flow in the vasa vasorum of healthy blood vessels results in vascular lesions. Moreover certain vascular diseases are associated with changes in the vasa vasorum that are directly related to the severity of the disease. Therefore, the regulation of blood flow through the vasa vasorum may have important implications for the physiology and pathophysiology of conduit blood vessels. Although it has been suggested that the vasa vasorum can actively regulate its tone, there is no direct evidence to support this idea. Moreover, there are no investigations into the mechanisms involved in constrictor and dilator responses in these vessels. The aim of this thesis is to directly examine the morphology and reactivity of the vasa vasorum of normal conduit arteries by studying these microvessels in isolation in vitro. The arterial vessels of the vasa vasorum will be focused on since these vessels provide nutrient blood flow to the vessel wall. The arterial vasa vasorum is unique in that the organ it supplies is a blood vessel and thus the reactivity of these arteries is likely to be distinct from other arteries of a similar calibre. In order to test this hypothesis, the reactivity and morphology of the arterial vasa vasorum will be compared to that of other systemic resistance arteries. For these studies the mesenteric bed will be used. Finally, the reactivity of the vasa vasorum will also be compared to the host conduit vessel to test the hypothesis that the regulation of tone in these two arteries is also distinct.
CHAPTER 2

METHODS
2.1 TISSUE PREPARATION

For all studies physiological salt solution (PSS) of the following composition (mM) was used: NaCl 119, KCl 4.7, CaCl$_2$.2H$_2$O 2.5, MgSO$_4$.7H$_2$O 1.2, NaHCO$_3$ 25, KH$_2$PO$_4$ 1.2 and glucose 5.5.

2.1.1 Porcine Thoracic Aortic Vasa Vasorum

Porcine thoracic aorta from mixed breed, adult pigs were collected from an abattoir and placed immediately in PSS and transported to the laboratory by courier within 2 hours of death. Second order vessels were traced from large adventitial first order vessels under a dissection microscope (Nikon SMZ-2B). Vessels at the adventitial-medial border were dissected out of the large vessel wall and cleaned of surrounding tissue, using micro-dissection.

2.1.2 Porcine Aortic Strips

For large vessel studies the porcine thoracic aorta was cleaned of extraneous tissue and helical strips of approximately 3mm thickness and 1cm length were cut.

2.1.3 Porcine Mesenteric Small Arteries

Porcine mesenteries were collected from an abattoir and placed immediately in PSS. Small arteries penetrating the wall of the jejunum were dissected and cleaned of surrounding tissue, using micro-dissection.
2.1.4 *Rat Mesenteric Small Arteries*

Male Sprague-Dawley rats (180-220g) were killed by cervical dislocation. The mesentery was dissected out and immediately placed in cold PSS. Third order mesenteric arteries were cleaned of surrounding fat using micro-dissection.

2.1.5 *Murine Mesenteric Small Arteries*

Male mice (25-30g) were killed by cervical dislocation. The mesentery was dissected out and immediately placed in cold PSS. Third order mesenteric arteries were cleaned of surrounding fat using micro-dissection.

2.2 **HISTOLOGICAL STUDIES**

Longitudinal sections of porcine thoracic aorta (2cm³), isolated vasa vasorum or mesenteric small arteries were placed in formal saline (10% formaldehyde) overnight. Vessels were then embedded in paraffin wax and longitudinal or cross-sections taken (4μm) and mounted on slides. To demonstrate general structures, sections were stained with haematoxylin and eosin (H&E).

2.3 **IMMUNOHISTOCHEMICAL STUDIES**

To demonstrate smooth muscle cells, some sections were incubated with a mouse monoclonal antibody to smooth muscle actin (αSMA, 1:1000). The labelling was visualised using the standard streptavidin-biotin technique. To demonstrate neural innervation some vessels were placed in Zamboni's fixative (2% paraformaldehyde) (Stefanini *et al.* 1967), immediately after dissection, for 16 hours at 4°C. Vessels were rinsed in phosphate-buffered saline (PBS) containing 15% sucrose and 0.01% sodium azide and placed on slides for whole mount
indirect immunofluorescence, as previously described (Gulbenkian et al. 1987). Briefly, following immersion for one hour in 0.1M phosphate buffered saline (PBS) containing 0.02% (w/v) Triton X-100, arteries were stained with 0.05% (w/v) Pontamine Sky Blue in PBS containing 0.5% dimethyl sulphoxide (DMSO) for 30 min. to counterstain background fluorescence. Vessels were then washed with PBS and incubated overnight at room temperature in a humid atmosphere with one primary antibody of the following dilution: calcitonin gene-related peptide (CGRP) 1:200, neuropeptide Y (NPY) 1:1500, protein gene product 9.5 (PGP 9.5) 1:1000, S-100 1:200, or tyrosine hydroxylase (TH) 1:200. These concentrations were chosen since they were found to be the lowest dilution that produced good staining. Preparations were then rinsed three times with PBS and incubated with fluorescein isothiocyanate (FITC)-labelled swine anti-rabbit immunoglobulin G (1:40) for one hour at room temperature. Staining was visualised using a fluorescence microscope and photographs were taken at various magnifications.

2.4 STUDIES IN THE TENSION MYOGRAPH

Arteries were mounted between two stainless steel wires (40μm in diameter) in an automated tension myograph (Danish Myotechnology, Aarhus, Denmark). Isometric tension was continuously measured and recorded on a pen chart recorder (BS-272, Gould Electronics Ltd.) (see fig 2). Vessels were bathed in PSS gassed with 5% CO$_2$ in O$_2$ at 37°C. Arteries were stretched in a stepwise manner to determine the relationship between passive tension and internal circumference according to Laplace’s equation (Tension = pressure x radius). From this relationship the internal diameter was determined. Vessels were then stretched to
90% of the diameter achieved when the vessel was under an effective transmural pressure of 100mmHg. These parameters were chosen since the maximum active tension response in small arteries of this size is achieved at this resting tension and because a transmural pressure of 100mmHg approximates to physiological conditions for resistance arteries of 100-350μm diameter in vivo (for review see Mulvany and Aalkjaer 1990). To confirm that these settings were also optimal for force production in vasa vasorum, the response to PSS with equimolar (125 mM) substitution of K⁺ for Na⁺ (KPSS) was tested at varying basal tension by increasing the distance between the wires. The maximum contractile response to KPSS in vasa vasorum was found to occur at a basal tension of approximately 1.0 mN and was equivalent to the maximum response to KPSS under the settings described above (n=4). The mean basal tension of vasa vasorum was 1.1 ± 0.03 mN (n=293).

Following the normalisation procedure, vessels were repeatedly contracted with KPSS until contractions were constant. Those vessels that did not produce active tension responses at least equivalent to that produced in response to an effective transmural pressure of 100mmHg were rejected.
Figure 2. Schematic of a tension myograph.
2.5 STUDIES IN THE PERFUSION MYOGRAPH

The perfusion myograph (Living Systems, Burlington, VT, USA) was used to study the reactivity of isolated resistance arteries under pressure. The vessel was mounted onto two opposing PSS-filled cannulae of equal flow-resistance, with luminal diameter similar to that of the vessel (see fig 3A). The proximal end of the vessel was mounted onto the proximal cannula, which was connected to a flow pump in order to mimic the direction of blood flow in vivo, and secured using fine nylon thread (see fig 3B). The vessel was briefly perfused at 10μl/min to remove any clotted blood from the lumen. Then the distal end of the vessel was mounted and secured onto the distal cannula, which was connected to a pressure servo control to maintain a set intraluminal pressure. Pressure leaks were detected by increasing intraluminal pressure to 40 mmHg to verify that intraluminal pressure was maintained.

The 10ml vessel chamber was continuously superfused with preheated PSS at 37°C, pH 7.4 and gassed with 21% O₂, 5% CO₂ in N₂ at a rate of 10ml/min so that the volume of the chamber was exchanged every minute. Test drugs were added to the superfusing PSS. The vessel chamber was placed on the stage of an inverted microscope (Nikon TMS) and the vessel visualised using a video camera (VM-902, Hitachi Denshi Ltd.). The internal diameter was determined using a video dimension analyser (V94, Living Systems Inc.), sampled at 50 Hz, and recorded on a pen chart recorder (BS-272, Gould Electronics Ltd.). In-line pressure
Figure 3. (A) Photograph depicting a rat mesenteric small artery (passive diameter = 135μm) mounted onto two glass cannulae and fixed with fine nylon thread (x4).

(B) Schematic of a perfusion myograph.
transducers on the proximal and distal ends of the set up continuously monitored mean intraluminal pressure. Vessels were equilibrated for 45 min at an intraluminal flow rate of 10μl/min and pressure of 10mmHg prior to constructing pressure-diameter curves. At the end of each experiment the passive diameter of the vessel at 80mmHg was determined by replacing the superfusing solution with calcium-free PSS containing 2mM EGTA (ethyleneglycol-bis-(β-aminoethyl-ether)-N,N,N’N’-tetraacetic acid).

2.6 STUDIES IN THE ORGAN BATH

Using silk thread, porcine aortic strips were suspended between 0.5mm diameter stainless steel wires in 25ml jacketed glass organ baths (see fig 4). The upper hook was attached to a micrometer and suspended from a force displacement transducer (Grass FT-03, Grass Instrument Company, USA) to measure changes in isometric tension, which were recorded on a multi-pen recorder (Rikadenki).

Tissues were equilibrated at 2g resting tension for one hour in PSS bubbled with 5% CO₂ in O₂ with regular washing at 15 min intervals. Tissues were repeatedly contracted with KPSS until contractions were constant.
Figure 4. Schematic of organ bath.
2.7 DATA ANALYSIS

Contractile responses were expressed as a percentage of the maximum increase in tone to KPSS. Relaxation responses were expressed as a percentage reversal of ET-1-induced pre-contraction. Individual response curves were fitted to a sigmoidal logistic curve (Microcal Origin 4.1). Using these curves, the negative logarithm of the mean concentration required to achieve 50% of the maximum effect (EC$_{50}$) was calculated. Wall tension (N/m) was calculated using Laplace’s relationship: wall tension = transmural pressure x vessel radius, where 1mmHg = $1.33 \times 10^{-4}$ N/m$^2$. Myogenic tone was calculated from the following equation: myogenic tone (%) = (Do-D)/Do x 100%, where D is actual vessel diameter (µm) and Do is passive diameter (µm). Comparisons between responses in the absence or presence of inhibitors or antagonists were performed using a two-tailed Student’s unpaired t-test for responses in separate vessels and a paired Student’s t-test for responses in the same vessel. One way ANOVA followed by Tukey’s post-hoc test was performed for multiple comparisons. Significant difference was considered at $P<0.05$.

2.8 PREPARATION OF DRUGS

Noradrenaline, L-NAME, nickel, atropine and guanethidine were made in saline. Indomethacin was made in 1% sodium carbonate in saline. Nifedipine was made in DMSO. Stock solutions of endothelin-1, bradykinin, angiotensin II, neuropeptide Y, CGRP, substance P, SKF 96365 and TTX were made in sterile water. Stock solutions of sarafotoxin 6c were made in 0.1% acetic acid. Stock solutions of U44069, BQ123, ODQ, SNAP and cyclopiazonic acid were made in DMSO. Stock solutions of BQ788 were made in 50% methanol. Stock solutions of U46619 were made in ethanol. All stock solutions were stored frozen except SKF 96365, which was stored at room temperature and TTX, which was stored at
4°C. Nifedipine, nickel, atropine, guanethidine, L-NAME, indomethacin, SNAP and noradrenaline were made on day of use. OxyHb was prepared by reduction of methaemoglobin with dithionite (Di Iorio 1981). All dilutions were made in saline. The maximum concentration of DMSO, methanol or ethanol in the bath at any time was 0.005%.

All antibodies were diluted in PBS containing 0.05% bovine serum albumin (BSA) and 0.01% sodium azide.

2.9 SOURCE OF DRUGS

Bachem, UK
Cyclo(-D-Trp-D-Asp-Pro-D-Val-Leu) (BQ123)
N-cis-2,6-dimethylpiperidinocarbonyl-β-tBu-Ala-D-Trp(1-methoxycarbonyl)-D-Nle-OH (BQ788)
Calcitonin gene-related peptide CGRP
Endothelin-1 (human,porcine,dog,rat)
Neuropeptide Y (porcine)

Biomol, UK
11α,9α-epoxymethano-PGH₂ U46619

Calbiochem, UK.
Cyclopiazonic acid
SKF 96365 hydrochloride.
Dr. R. Corder, London, UK.

Rabbit anti-NPY (porcine) was a kind gift from Dr. R. Corder, London, UK.

Dako, UK.

Fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit immunoglobulins

Mouse monoclonal anti-smooth muscle actin

Rabbit anti-cow anti-S-100

Eugene Tech (Affiniti, UK).

Rabbit polyclonal antibody to tyrosine hydroxylase (TH).

Hammersmith Hospital, London, UK.

Rabbit polyclonal antibodies to calcitonin gene-related peptide (CGRP) and substance P (SP).

Medicinal Chemistry Department, UCL

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

S-nitroso-N-acteyl penicillamine (SNAP)

Novabiochem, UK

Sarafotoxin 6c

Sigma Chemical Co., UK.

acetylcholine chloride

angiotensin II
atropine sulphate
bradykinin acetate
ethyleneglycol-bis-\((\beta\text{-aminoethyl-ether})\)-N,N,N’N’-tetraacetic acid (EGTA)
guanethidine
\(\text{N}\omega\text{-L-nitro L-arginine methyl ester hydrochloride (L-NAME)}\)
nickel sulphate
nifedipine
noradrenaline bitartrate
Pontamine Sky Blue
substance P acetate
tetrodotoxin
\(9,11\text{-Dideoxy-9\alpha,11\alpha-epoxy-methano prostaglandin F}_{2\alpha} \text{ (U44069)}\)
CHAPTER 3

MORPHOLOGY
All photographs included in this chapter are typical of experiments carried out in vessels from at least 4 different animals.

3.1 PORCINE ARTERIAL VASA VASORUM

3.1.1 Histological Studies

Figure 5 is a typical photograph of haematoxylin and eosin staining of porcine thoracic aorta showing an arterial vasa vasorum at the adventitial-medial border. The basic dark purple dye, haematoxylin, stains cell nuclei and the acidic lighter pink dye, eosin stains cytosolic elements. This staining demonstrates that the vasa vasorum consists of several layers of smooth muscle. These smooth muscle cells are oriented concentrically around the lumen of the vessel and not in relation to the aorta. Nuclei of a single layer of endothelial cells, which protrude into the lumen of the vasa vasorum, lie perpendicular to these smooth muscle cells.

3.1.2 Immunohistochemical Studies

Incubation of isolated vasa vasorum with antibody to smooth muscle actin (αSMA) supported histological findings. Figure 6 shows a longitudinal section of an isolated porcine vasa vasorum. The dark brown staining demonstrates the presence of several layers of smooth muscle cells.

Incubation of isolated porcine vasa with antibody to either general neural marker PGP 9.5 (see fig 7) demonstrated a network of nerve fibres that surround and penetrate the vessel. Similar patterns of labelling are seen with antibody to NPY (see fig 8), and to a lesser extent with anti-TH (see fig 9), indicating that these nerve fibres are primarily of sympathetic origin. Sparse labelling with anti-CGRP was occasionally seen in some vessels (see fig 10).
Figure 5. Haematoxylin and eosin staining of porcine thoracic aorta showing an arterial vasa vasorum at the adventitial-medial border (x400).
IMMUNOHISTOCHEMICAL DEMONSTRATION OF SMOOTH
MUSCLE OF VASA VASORUM

Figure 6. Smooth muscle actin immunoreactivity of isolated porcine thoracic aortic vasa vasorum (x400).
Figure 7. Whole mount indirect immunofluorescence (yellow) of isolated porcine thoracic aortic vasa vasorum (red) incubated with antibody to protein gene product 9.5 (PGP 9.5, x125).
Figure 8. Whole mount indirect immunofluorescence (yellow) of isolated porcine thoracic aortic vasa vasorum (red) incubated with antibody neuropeptide Y (NPY, x160).
Figure 9. Whole mount indirect immunofluorescence (yellow) of isolated porcine thoracic aortic vasa vasorum (red) incubated with antibody to tyrosine hydroxylase (TH, x125).
Figure 10. Whole mount indirect immunofluorescence (yellow) of isolated porcine thoracic aortic vasa vasorum (red) incubated with antibody to calcitonin gene-related peptide (CGRP).
3.2 PORCINE MESENTERIC SMALL ARTERIES

3.2.1 Immunohistochemical Studies

Figure 11 is a typical photograph of smooth muscle actin immunoreactivity of a porcine mesenteric small artery and demonstrates several layers of smooth muscle associated with these vessels. The pattern of neural innervation of porcine mesenteric small arteries, as demonstrated by S-100, was similar to that seen in porcine vasa (see fig 12). These nerves were also predominantly NPY- (see fig 13) or TH-containing (see fig 14) however CGRP immunoreactivity was also evident (see fig 15).
IMMUNOHISTOCHEMICAL DEMONSTRATION OF SMOOTH
MUSCLE OF PORCINE MESENTERIC SMALL ARTERY

Figure 11. Smooth muscle actin immunoreactivity of porcine mesenteric small artery (x400).
Figure 12. Whole mount indirect immunofluorescence (yellow) of porcine mesenteric small artery (red) incubated with antibody to S-100 (x160).
Figure 13. Whole mount indirect immunofluorescence (yellow) of porcine mesenteric small artery (red) incubated with antibody to neuropeptide Y (NPY, x125).
Figure 14. Whole mount indirect immunofluorescence (yellow) of porcine mesenteric small artery (red) incubated with antibody to tyrosine hydroxylase (TH, x125).
IMMUNOHISTOCHEMICAL DEMONSTRATION OF NEURAL
INNERVATION OF PORCINE MESENTERIC SMALL ARTERY (4)

Figure 15. Whole mount indirect immunofluorescence (yellow) of porcine mesenteric small artery (red) incubated with antibody to calcitonin gene-related peptide (CGRP, x160).
3.3 Summary

Standard histological studies to demonstrate the basic structure of porcine vasa vasorum showed that these vessels consist of layers of smooth muscle oriented radially around a single layer of endothelial cells. Immunohistochemical studies confirmed the presence of several layers of smooth muscle cells. Furthermore, indirect immunofluorescence with general neural markers demonstrated a network of nerves surrounding and penetrating arterial vasa vasorum. These nerves were predominantly of sympathetic origin since they mainly contained NPY and TH. However, there was also evidence of CGRP-containing nerves.

The morphology of porcine mesenteric small arteries was similar to that of porcine vasa vasorum. These small arteries also consisted of layers of smooth layers around a single layer of endothelium. The pattern of neural innervation evident in mesenteric arteries was of a similar density to that in vasa and was also predominantly sympathetic in origin.
CHAPTER 4

CONSTRUCTOR RESPONSES
4.1 PORCINE ARTERIAL VASA VASORUM

4.1.1 Characterisation Of Contractile Reactivity

4.1.1.1 Experimental Protocol

In order to characterise the contractile reactivity of the vasa vasorum, concentration response curves were constructed to noradrenaline (NA, 1-10000 nM), angiotensin II (Ang II, 0.01-300 nM), endothelin-1 (ET-1, 0.01-300nM) and the thromboxane A₂ mimetic (TxA₂-mimetic) U-44069 (9α,11α-epoxymethano-PGH₂, 1-1000nM) in porcine arterial vasa. To compare the reactivity of porcine vasa with another porcine blood vessel of similar diameter, concentration-response curves were constructed to the same contractile agents in porcine mesenteric arteries.

4.1.1.2 Results

The mean diameter of vasa vasorum in this study was 157.1 ± 5.9 μm (n=23). ET-1 (0.01-300nM) produced potent concentration-dependent contractions of isolated porcine arterial vasa vasorum (see fig 16A) with an EC₅₀ value of 4.7 ± 1.4 nM (n=6, see fig 17). NA (1-10000nM) also caused concentration-dependent contraction of vasa vasorum (see fig 16B) but was a weak constrictor of these vessels with an EC₅₀ > 3000nM and a maximum response of 15.7± 7.0% (n=5, see fig 17). Unlike in the other porcine blood vessels (see sections 4.2.2 and 4.3.2), neither TxA₂-mimetic (n=5, see fig 17) nor Ang II (n=7, see fig 17) had any contractile effect (see Table 1).
Figure 16. Typical traces depicting responses of porcine thoracic aortic vasa vasorum to (A) endothelin-1 (ET-1) and (B) noradrenaline (NA).
Figure 17. Contractile concentration-response curves to endothelin-1 (ET-1), thromboxane A2-mimetic U44069, noradrenaline (NA) and angiotensin II (AngII) in porcine thoracic aortic vasa vasorum (n=5-7). Values are shown as arithmetic mean ± s.e.m.
### COMPARISON OF CONTRACTILE REACTIVITY IN PORCINE BLOOD VESSELS

<table>
<thead>
<tr>
<th>Constrictor</th>
<th>ET-1</th>
<th>U44069</th>
<th>NA</th>
<th>Ang II</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Porcine Vasa Vasorum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC(_{50})</td>
<td>4.7 ± 1.4 nM</td>
<td>&gt;30,000 nM</td>
<td>&gt;30,000 nM</td>
<td>&gt;30,000 nM</td>
</tr>
<tr>
<td>Max (%KPSS)</td>
<td>143.0 ± 11.0</td>
<td>0</td>
<td>15.7 ± 7.0</td>
<td>0</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td><strong>Porcine Mesenteric Artery</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC(_{50})</td>
<td>13.9 ± 5.2 nM</td>
<td>41.6 ± 16.8 nM</td>
<td>3.4 ± 1.3 μM</td>
<td>4.2 ± 1.6 nM</td>
</tr>
<tr>
<td>Max (%KPSS)</td>
<td>110.7 ± 11.3</td>
<td>59.6 ± 5.1</td>
<td>68.7 ± 5.7</td>
<td>6.1 ± 1.7</td>
</tr>
<tr>
<td>n</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td><strong>Porcine Aorta</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC(_{50})</td>
<td>5.8 ± 1.3 nM</td>
<td>30.0 ± 6.2 nM</td>
<td>7.5 ± 1.0 μM</td>
<td>9.9 ± 1.9 nM</td>
</tr>
<tr>
<td>Max (%KPSS)</td>
<td>91.6 ± 11.4</td>
<td>140.0 ± 34.4</td>
<td>220.0 ± 34.4</td>
<td>59.4 ± 7.3</td>
</tr>
<tr>
<td>n</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 1. Comparison of the potency of selected contractile agonists in porcine vasa vasorum, mesenteric small artery and aortic strips. Values are shown as arithmetic mean ± s.e.m.
4.1.2 Characterisation Of ET Receptors In Porcine Vasa Vasorum

4.1.2.1 Experimental Protocol

In a separate series of experiments in porcine vasa, cumulative concentration-response curves were constructed to ET-1 (0.01-300nM) or the ET$_B$-selective receptor agonist, sarafotoxin 6c (S6c) (Williams et al. 1991). To determine whether the response to ET-1 was mediated by ET$_A$ or ET$_B$ receptors some vessels were pretreated with either the selective ET$_A$ receptor antagonist BQ123 (10μM) (Ihara et al. 1992a,b), the ET$_B$ receptor antagonist BQ788 (1μM) (Ishikawa et al. 1994) or a combination of both antagonists for 30 min. Concentration-response curves were also constructed to S6c in the presence of BQ788.

4.1.2.2 Results

The diameter of vessels used in this study was 138.1 ± 3.7 μm (n=62). ET-1 produced slow developing and sustained concentration-dependent contractions of isolated arterial vasa vasorum (EC$_{50}$ = 15.3 ± 0.8 nM and maximum response = 138.3 ± 2.3%, n=32). Concentration-response curves to ET-1 were shifted to the right and became biphasic in the presence of selective ET$_A$ antagonist, BQ123 (10μM, n=5) (see fig 18), consisting of a high potency (EC$_{50}$=9.8 ± 1.4 nM, maximum response = 54.3 ± 4.8%) response and a low potency (EC$_{50}$ > 800 nM, maximum response > 120%) response.

BQ788, the selective ET$_B$ antagonist (1μM, n=7), produced a significant (P<0.05) rightward shift (approximately 5-fold) of the ET-1 concentration-response curve (see fig 19). EC$_{50}$ values were 19.5 ± 5.2 nM and 100.5 ± 34 nM in the absence and presence of the antagonist respectively. Pretreatment with a combination of both receptor antagonists (n=4) produced a significant (P<0.05) rightward shift
(approximately 20-fold) (see fig 18) giving EC$_{50}$ values of 19.7 ± 7.8 nM and >400 nM in the absence and presence of the antagonists respectively.

Like ET-1, S6c, the selective ET$_B$ agonist was a potent contractile agonist of porcine vasa with an EC$_{50}$ of 3.6 ± 0.2 nM and a maximum response of 87.4 ± 1.6% (n=7). Contractions to S6c were significantly (P<0.01) shifted to the right (approximately 80-fold) by BQ788 (EC$_{50}$ = 294.3 ± 10.1 nM, n=6) (see fig 19).

**4.1.3 Characterisation Of Ca$^{2+}$ Mobilisation In Responses To ET-1 In Porcine Vasa Vasorum**

**4.1.3.1 Experimental Protocol**

To investigate the mechanisms involved in ET-1-induced contractions of vasa, some vessels were pretreated with various inhibitors of both intracellular and extracellular Ca$^{2+}$ flux and the response to ET-1 assessed. Contractile response curves to ET-1 were constructed in the absence or presence of (i) Ca$^{2+}$-free PSS containing 2 mM EGTA, (ii) nifedipine (10 µM, 30 min), the L-type Ca$^{2+}$-channel blocker, (iii) SKF 96365 (30 µM, 30 min), the purported inhibitor of receptor-operated Ca$^{2+}$ channel (Merritt et al. 1990) or (iv) nickel (1mM, 60 min) an inhibitor of non-selective cation entry (Shetty and DelGrande 1994). To investigate the role of Ca$^{2+}$ release from intracellular stores, some vasa were pretreated with cyclopiazonic acid (CPA, 10 µM, 30 min), an inhibitor of sarcoplasmic reticulum CaATPase (Seidler et al. 1989). Control experiments in the absence of the antagonist or inhibitors were carried out at the same time on paired rings from the same vessel.
4.1.3.2 Results

The diameter of vessels used in this study was 145.2 ± 4.5 μm (n=68). In Ca²⁺-free PSS the contractile response to ET-1 was abolished (n=4). Pretreatment with nifedipine (10 μM, n=5) suppressed ET-1-induced contractions such that the maximum response was approximately 44% lower than in untreated tissues but did not affect the potency of ET-1 i.e. EC₅₀ = 11.7 ± 0.2 nM (see fig 20). SKF 96365 (30 μM, n=5) treatment also suppressed ET-1-induced contractions with a 61% suppression of the maximum response (see fig 20). In contrast, whilst Ni²⁺ (1mM, n=4) attenuated ET-1-mediated contractions (approximate 55% suppression of the maximum response) (see fig 21) a decrease in potency of ET-1 was also evident (EC₅₀ values were 21.2 ± 0.4 and 44.2 ± 2.3 nM in the absence and presence of Ni²⁺ respectively). Combination of nifedipine with SKF 96365 (n=5) had a greater effect than either inhibitor alone and attenuated the maximum response to ET-1 by approximately 75% (see fig 20).

Similarly to ET-1, S6c-induced contractions were also attenuated by nifedipine treatment by approximately 55% (n=7) (see fig 22). The contractile response to ET-1 that remained following BQ123 treatment was significantly (P<0.05) suppressed by addition of nifedipine (n=7, see fig 23A) whilst that remaining following BQ788 was not (n=5, see fig 23B). EC₅₀ values in the presence of BQ788 were 15.5 ± 2.8 and 11.7 ± 2.2 nM (n=5) with maximum contractions of 135.9 ± 16% and 110.2 ± 20.3% in the absence and presence of nifedipine respectively (not significantly different). The contractions to ET-1 were not altered by depletion of intracellular Ca²⁺ stores using CPA (10 μM, n=5, see fig 24), an inhibitor of sarcoplasmic reticulum CaATPase (EC₅₀ values were 20.4 ± 2.6 nM and 15.3 ± 1.1 nM in the absence and presence of CPA respectively).

Preliminary (n=2) experiments using CaATPase inhibitor thapsigargin (1μM) showed that thapsigargin alone produced transient contractions of 22.0% and 31.6%, indicating that vasa vasorum do possess intracellular Ca²⁺ stores. Thapsigargin was not used in experiments since it is also an inhibitor of L-type Ca²⁺ channels.
Figure 18. Contractile concentration-response curves to endothelin-1 (ET-1) in porcine thoracic aortic vasa vasorum in the absence and presence of ET$_A$ receptor antagonist BQ123 (10µM, n=5), or ET$_B$ receptor antagonist BQ788 (1µM, n=7), or a combination of both antagonists (n=4). Values are shown as arithmetic mean ± s.e.m.
CONTRACTILE RESPONSES TO ET$_B$ AGONIST IN VASA VASORUM

Figure 19. Contractile concentration-response curves to sarafotoxin 6c (S6c) in porcine thoracic aortic vasa vasorum in the absence and presence of ET$_B$ receptor antagonist BQ788 (1µM, n=6). Values are shown as arithmetic mean ± s.e.m.
Figure 20. Contractile concentration-response curves to endothelin-1 (ET-1) in porcine thoracic aortic vasa vasorum in the absence and presence of inhibitors of L-type (nifedipine, 10μM, n=5) or receptor-operated calcium channels (SKF 96365, 30μM, n=5), or a combination of both inhibitors (n=5). Significant difference from control values is shown as *P<0.05 and **P<0.01 using one-way ANOVA followed by Tukey’s multiple comparison.
ROLE OF NON-SELECTIVE CATION INFLUX IN CONTRACTILE RESPONSES TO ET-1 IN VASA VASORUM

Figure 21. Contractile concentration-response curves to endothelin-1 (ET-1) in porcine thoracic aortic vasa vasorum in the absence or presence of Ni\(^{2+}\), an inhibitor of non-selective cation channels (1mM, n=4). Values are shown as arithmetic mean ± s.e.m. Significant difference from control values is shown as * P<0.05 using unpaired two-tailed Student’s t-test.
ROLE OF CA\(^{2+}\) INFLUX IN CONTRACTILE RESPONSES TO ET\(_B\) AGONIST IN VASA VASORUM

Figure 22. Contractile concentration-response curves to sarafotoxin 6c (S6c) in porcine thoracic aortic vasa vasorum in the absence or presence of nifedipine, an inhibitor of L-type calcium channels (10\(\mu\)M, \(n=7\)). Values are shown as arithmetic mean ± s.e.m. Significant difference from control values is shown as * P<0.05 using unpaired two-tailed Student’s t-test.
ROLE OF CA\textsuperscript{2+} INFLUX IN CONTRACTILE RESPONSES TO ACTIVATION OF ET\textsubscript{A} AND ET\textsubscript{B} RECEPTORS IN VASA VASORUM

A

Figure 23. Contractile concentration-response curves to endothelin-1 (ET-1) in porcine thoracic aortic vasa vasorum in the presence of (A) ET\textsubscript{A} (n=7) or (B) ET\textsubscript{B} (n=7) receptor blockade in the absence or presence of nifedipine, an inhibitor of L-type calcium channels (10\textmu M). Values are shown as arithmetic mean ± s.e.m. Significant difference is shown as * P<0.05 using unpaired two-tailed Student’s t-test.
ROLE OF INTRACELLULAR CA\(^{2+}\) RELEASE IN CONTRACTILE RESPONSES TO ET-1 IN VASA VASORUM

![Graph showing contractile concentration-response curves to endothelin-1 (ET-1) in porcine thoracic aortic vasa vasorum in the absence or presence of cyclopiazonic acid, an inhibitor of CaATPase (10\(\mu\)M, n=5). Values are shown as arithmetic mean ± s.e.m.]

Figure 24. Contractile concentration-response curves to endothelin-1 (ET-1) in porcine thoracic aortic vasa vasorum in the absence or presence of cyclopiazonic acid, an inhibitor of CaATPase (10\(\mu\)M, n=5). Values are shown as arithmetic mean ± s.e.m.
4.1.4 Potentiation Of Contractile Reactivity In Porcine Vasa Vasorum

4.1.4.1 Experimental Protocol

To investigate whether raising tone with threshold concentrations of ET-1 alters the vasoconstrictor reactivity profile of vasa vasorum the following experiments were carried out:

In any single tissue control concentration-response curves were constructed to NA (1-100 000 nM) or thromboxane A2 - mimetic (U44069, 0.1-1 000 nM) in the absence and then in the presence of ET-1 (1-3 nM) or K+ (10-20 mM). In each case the concentration of ET-1 or K+ used precontracted the vessel to approximately 10% of the initial KPSS response. In a separate study, control concentration-response curves were constructed to NA (1-100 000 nM) in the absence and then in the presence of NPY (100nM). The responses to NA and U44069 following precontraction were compared to responses to those agents in non-contracted time-matched controls.

4.1.4.2 Results

The diameter of vessels used in this study was 156.3 ± 5.6 μm (n=61). Threshold concentrations of ET-1 (1-3 nM) induced contractions of 7.5 ± 0.9% (n=11) and significantly (P<0.05) potentiated the responses to NA (n=7, see fig 25A). Maximum responses to NA of 8.7 ± 0.7% and 32.1 ± 0.8% were achieved in the absence and presence of ET-1 respectively whilst the potency of NA was not significantly altered (EC50 values were 1.1 ± 0.3 μM and 0.7 ± 0.1 μM in the absence and presence of ET-1 respectively). The potentiated responses to NA were blocked following pretreatment with an L-type Ca2+ channel inhibitor,
nifedipine (n=4, see fig 25B) whilst depletion of intracellular Ca\(^{2+}\) stores with CPA had no effect on potentiated responses to NA (n=4, see fig 26). EC\(_{50}\) values were 0.41 ± 0.1 \(\mu\text{M}\) and 0.46 ± 0.1 \(\mu\text{M}\) in the absence and presence of CPA respectively. In contrast to NA, responses to U44069 were not altered by elevation of tone with ET-1 (n=4, see fig 27).

In contrast to ET-1, raising basal tone by 8.2 ± 0.6\% (n=11) using K\(^{+}\) (10-20 mM) significantly (P<0.05) enhanced the responses to both NA (n=6, see fig 28) and U44069 (n=5, see fig 29). Maximum contractions to NA were 13.1 ± 1.5\% and 50.7 ± 1.8\% in the absence and presence of K\(^{+}\) respectively whilst the potency was not significantly altered (EC\(_{50}\) values were 3.0 ± 1.0 \(\mu\text{M}\) in the absence and 1.4 ± 0.2 \(\mu\text{M}\) in the presence of K\(^{+}\)). In the absence of K\(^{+}\) U44069 had no contractile effect but in the presence of K\(^{+}\) the TXA\(_2\)-mimetic produced potent concentration-dependent contraction with an EC\(_{50}\) of 54.4 ± 7.6 nM and a maximum effect of 83.6 ± 3.8\%. The potentiated response to U44069 was unaffected by pretreatment with CPA (n=7, see fig 30). (EC\(_{50}\) = 72.7 ± 7.4 nM and 78.7 ± 4.0 nM in the absence and presence of CPA respectively, not significantly different).

NPY (100nM) produced weak, transient contraction (1.3 ± 0.5\%, n=5) of porcine vasa but did not potentiate contractile responses to NA (see fig 31). Maximum contractions to NA were 8.5 ± 6.5\% and 2.9 ± 2.7\% (n=5) in the absence and presence of NPY respectively.
Figure 25. Contractile concentration-response curves to noradrenaline (NA) in porcine thoracic aortic vasa vasorum in the absence and (A) presence of endothelin-1 (ET-1, 1-3nM) alone (n=7) or (B) in the presence of combination of endothelin-1 (ET-1, 1-3nM) with L-type calcium channel inhibitor nifedipine (10µM, n=4). Values are shown as arithmetic mean ± s.e.m. Significant difference is shown as * P<0.05 and ** P<0.01 using paired two-tailed Student’s t-test.
ROLE OF INTRACELLULAR CA\(^{2+}\) RELEASE IN POTENTIATION OF CONTRACTILE RESPONSES TO NA BY ET-1 IN VASA VASORUM

Figure 26. Contractile concentration-response curves to noradrenaline in porcine thoracic aortic vasa vasorum in absence and presence of a combination of endothelin-1 (1-3nM) with CaATPase inhibitor cyclopiazonic acid (10\(\mu\)M, n=4). Values are shown as arithmetic mean ± s.e.m. Significant difference is shown as * P<0.05 using paired two-tailed Student’s t-test.
Figure 27. Contractile concentration-response curves to TXA$_2$ mimetic U44069 in porcine thoracic aortic vasa vasorum in the absence and presence of endothelin-1 (ET-1, 1-3nM, n=4). Values are shown as arithmetic mean ± s.e.m.
Figure 28. Contractile concentration-response curves to noradrenaline in porcine thoracic aortic vasa vasorum in the absence and presence of K⁺ (10-20mM, n=5). Values are shown as arithmetic mean ± s.e.m. Significant difference is shown as * P< 0.05 using paired two-tailed Student’s t-test.
POTENTIATION OF CONTRACTILE RESPONSES TO TXA$_2$ MIMETIC BY K$^+$ IN VASA VASORUM

Figure 29. Contractile concentration-response curves to TXA$_2$ mimetic U44069 in porcine thoracic aortic vasa vasorum in the absence and presence of K$^+$ (10-20 mM, n=5). Values are shown as arithmetic mean ± s.e.m. Significant difference is shown as * P<0.05 and ** P<0.01 using paired two-tailed Student’s t-test.
Figure 30. Contractile concentration-response curves to TXA$_2$ mimetic U44069 in porcine thoracic aortic vasa vasorum in the absence and presence of a combination of K$^+$ (10-20mM) with CaATPase inhibitor cyclopiazonic acid (10µM, n=6). Values are shown as arithmetic mean ± s.e.m. Significant difference is shown as * P<0.05 and ** P<0.01 using paired two-tailed Student’s t-test.
EFFECT OF NPY ON CONTRACTILE RESPONSES TO NA IN VASA VASORUM

Figure 31. Contractile concentration-response curves to noradrenaline in porcine thoracic aortic vasa vasorum in the absence and presence of neuropeptide Y (100nM, n=5). Values are shown as arithmetic mean ± s.e.m.
4.2 PORCINE MESENTERIC SMALL ARTERY

4.2.1 Experimental Protocol

To compare the reactivity of porcine vasa with another porcine blood vessel of similar diameter, porcine small mesenteric arteries were mounted in a tension myograph and concentration-response curves were constructed to ET-1 (0.01-300nM), U-44069 (0.1-1000nM), NA (1-30000 nM) or Ang II (0.1-300 nM). One curve was constructed in each tissue.

4.2.2 Results

The mean diameter of vessels in this study was 189.0 ± 13.9 μm (n=20). Similar to porcine vasa vasorum, ET-1 produced potent concentration-dependent contractions of porcine mesenteric small arteries with an EC_{50} of 13.9 ± 5.2 nM (n=4) and maximal contractions of 110.7 ± 11.3% (see Table 1). The thromboxane A2 mimetic U44069 (n=5) also produced potent concentration-dependent contractions of porcine mesenteric arteries with an EC_{50} of 41.6 ± 16.8 nM and maximum response of 59.6 ± 5.1% (see Table 1). Contraction elicited by NA (n=5) were concentration-dependent with an EC_{50} of 3.4 ± 1.3 μM and maximal effect of 68.7 ± 5.7%. In contrast, Ang II (n=6) was a weak agonist and produced transient contractions with an EC_{50} of 4.2 ± 1.6 nM and maximum contraction of 6.1 ± 1.7 % (see Table 1).

4.3 PORCINE THORACIC AORTA

4.3.1 Experimental Protocol

To compare the reactivity of porcine vasa with the host vessel from which they were dissected, strips of thoracic aorta were mounted in an organ bath (see section 2.6), and cumulative concentration-response curves were constructed to ET-1
(0.01-300nM), U-44069 (1-3000nM), NA (1-30000 nM) or Ang II (0.01-300 nM).
One curve was constructed in each tissue.

4.3.2 Results

ET-1 (n=5) produced slow developing contraction of porcine aortic strips with an EC$_{50}$ of 5.8 ± 1.3 nM and maximum contraction of 91.6 ± 11.4% (see Table 1). TxA$_2$-mimetic U44069 (n=5) produced potent concentration-dependent contractions of with an EC$_{50}$ of 30.0 ± 6.2 nM and a maximal effect of 140.0 ± 34.4 % (see Table 1). Similarly, NA (n=5) produced contractions of porcine aortic strips but was a less potent agonist with an EC$_{50}$ of 7.5 ± 1.0 μM but a maximal contraction of 220.0 ± 34.4 % (see Table 1). Ang II (n=4) also produced concentration-dependent contractions with an EC$_{50}$ of 9.9 ± 1.9 nM and maximum contraction of 59.4 ± 7.3 % (see Table 1).
4.4 SUMMARY

Arterial vasa vasorum, isolated from the adventitial-medial border of porcine thoracic aorta, mounted in a tension myograph, produced potent contractions to ET-1 and only weak contractions to NA. However, these vessels were insensitive to contractile agonists U44069 and AngII. This profile of contractile reactivity was markedly different to that observed in other porcine blood vessels. Porcine mesenteric small arteries, of a similar diameter to the vasa vasorum, contracted to ET-1, U44069 and NA. Furthermore, strips of porcine aorta, mounted in an organ bath, also produced potent concentration-dependent contractions to ET-1, NA, U44069 and AngII.

The contractile response to ET-1 in the vasa vasorum was mediated by both $\text{ET}_A$ and $\text{ET}_B$ receptor subtypes and involved the influx of extracellular $\text{Ca}^{2+}$ through L-type and non-L-type $\text{Ca}^{2+}$ channels. Moreover, threshold concentrations of ET-1 (1-3nM) potentiated contractile responses to NA, an effect that was abolished by inhibition of L-type $\text{Ca}^{2+}$ channels. There was some specificity of this effect of ET-1 on NA responses since ET-1 had no effect on responses to U44069. In contrast, similar increases in tone with $\text{K}^+$ (10-20mM) potentiated contractions to NA and uncovered contractile responses to U44069.
CHAPTER 5

VASODILATOR RESPONSES
5.1 CHARACTERISATION OF RELAXANT REACTIVITY OF VASA VASORUM

5.1.1 Experimental Protocol

Vasa vasorum were precontracted with a submaximal concentration of ET-1 (10-30nM) to give approximately 70% of the tone induced by KPSS. Once the contraction reached a plateau, cumulative relaxation concentration-response curves were constructed to bradykinin (BK, 0.01-30 nM), substance P (SP, 0.01-30 nM) or calcitonin gene-related peptide (CGRP, 0.01-300 nM). Only one peptide was tested in any one preparation. The possibility of adrenergic agonists causing relaxations was determined by constructing concentration-response curves to non-selective adrenoceptor agonist NA (1-10000 nM), and the selective β-adrenoceptor agonist isoprenaline (0.1-1000 nM) in ET-1 precontracted vasa vasorum.

5.1.2 Results

The mean diameter of vessels in this study was 179.3 ± 6.0 (n=85). BK caused concentration-dependent relaxation of ET-1 precontracted porcine arterial vasa vasorum (see fig 32A) with an EC\textsubscript{50} of 3.8 ± 0.5 nM and a maximal effect of 71.5 ± 3.3% reversal of the precontraction (n=19, see fig 33). Similarly, CGRP produced potent concentration-dependent relaxation of ET-1 precontracted vasa with an EC\textsubscript{50} of 6.7 ± 1.5 nM (n=12) and maximum relaxation of 84.1 ± 2.4% (see fig 33). Whilst SP also produced potent concentration-dependent relaxation the response underwent rapid desensitisation at concentrations above 10nM (see fig 32B) giving an EC\textsubscript{50} of 3.5 ± 0.7 nM (n=18) and maximal effect of 39 ± 5.1% (see fig 33). Neither the non-selective adrenoceptor agonist NA nor selective β-adrenoceptor agonist isoprenaline had any relaxant effects in precontracted tissues (n=4 in each case). Application of ACh (1-3000 nM) had no relaxant effect (n=2).
Figure 32. Typical traces depicting responses of porcine thoracic aortic vasa vasorum to (A) bradykinin (BK) and (B) substance P (SP).
Figure 33. Relaxation concentration-response curves to bradykinin (BK), calcitonin gene-related peptide (CGRP), and substance P (SP) of endothelin-1 (10-30nM) precontracted porcine thoracic aortic vasa vasorum (n=12-19). Values are shown as arithmetic mean ± s.e.m.
5.2 ROLE OF ENDOTHELium-DERIVED FACTORS

5.2.1 Experimental Protocol

To investigate the involvement of endogenous NO or prostanoids in the responses seen, concentration-response curves were constructed in the presence or absence of the following inhibitors:

(i) To determine whether the NO-guanylyl cyclase pathway (see fig 34) was involved vessels were pretreated for 30 min with NG-nitro-L-arginine methyl ester (L-NAME, 300 μM) or 1H-[1,2,4] oxadiazolo[4,3-a] quinoxalin-1-one (ODQ, 1 μM) to inhibit NO synthase and soluble guanylyl cyclase respectively (Garthwaite et al. 1995). The effect of oxyhaemoglobin (OxyHb), the NO scavenger was also investigated. Vessels were pretreated with OxyHb (10 μM) for 30 min prior to ET-1 application and then again at the time of ET-1 application to give a total concentration of 20μM.

(ii) To determine whether generation of prostanoids was involved, vessels were pretreated with the cyclooxygenase inhibitor indomethacin (5μM) for 30 min.

(iii) To determine the possible involvement of an endothelial-derived hyperpolarisation factor(s) (EDHF), some vessels were precontracted with 40-50 mM K⁺ (to give 70% KPSS increase in tone) to inhibit K⁺ channels.

Following incubation with inhibitors, preparations were precontracted with ET-1 to give a level of tone approximately 70% of the control contractile response to K⁺ (i.e.
Figure 34. Schematic of the NO/L-arginine pathway and its inhibitors.
equivalent to the tone used for control responses). Relaxation responses in drug-treated vasa were compared to those produced in control untreated vessels dissected from the same segment of blood vessel.

5.2.1.1 Removal of endothelium

For certain studies, following normalisation, the endothelium was removed by passing a hair 10 times through the lumen of the vessel. It was not possible to determine successful removal of the endothelium since the responses to ET-1 undergo tachyphylaxis and hence vasa vasorum could only be precontracted once. However, previous studies in small vessels have demonstrated that this procedure results in selective removal of the endothelium (Kakuyama et al. 1998). This procedure had no effect on contractions to KPSS and the smooth muscle was still sensitive to non-endothelium dilator NO donor, S-nitroso-N-acetyl penicillamine (SNAP,10μM).

5.2.2 Results

The responses to BK (n=3, see fig 35) and SP (n=3, see fig 36) were abolished by removal of the endothelium whilst the response to CGRP was unaffected (see fig 37). The EC$_{50}$ for CGRP was 61.2 ± 4.7 nM in intact tissues and 30.6 ± 6.3 nM (n=4) in endothelium-denuded vessels (not significantly different). Endothelium-dependent relaxations to SP were attenuated in the presence of the non-selective NOS inhibitor L-NAME (300μM, n=5, see fig 38). In contrast, L-NAME had no effect on relaxations to BK (n=5, see fig 39). Responses to BK were also unaffected by inhibition of the L-arginine/NO pathway at the level of guanylyl cyclase since
pretreatment with ODQ (n=8, see fig 40) had no inhibitory effect. Similarly removal of free NO, using NO scavenger OxyHb (n=5, see fig 40), had no significant effect on these responses. Indomethacin, the non-selective cyclooxygenase inhibitor (5μM), had no significant effect on responses to either BK (see fig 41) or SP (see fig 42). The EC$_{50}$ for BK was 6.1 ± 1.27 nM (n=9) and 6.4 ± 1.4 nM (n=9) respectively in the absence and presence of indomethacin (not significantly different). The EC$_{50}$ for SP was 1.7 ± 2.5 nM (n=5) and 1.2 ± 3.6 nM (n=5) respectively in the absence and presence of indomethacin (not significantly different). Combination of indomethacin with L-NAME (n=4, see fig 43) produced no inhibition of relaxations to BK.

Responses to BK were significantly (n=5, P<0.05) lower in vasa precontracted with 40mM K$^+$ (equimolar substitution of Na$^+$) than in vessels precontracted with ET-1 (see fig 44). Maximal relaxations to BK were 24.5 ± 4.7% in tissues precontracted with K$^+$ whilst relaxations in ET-1 precontracted tissues were 69.0 ± 9.5 %. The potency of BK in K$^+$ precontracted vessels was not significantly different (P<0.05) from that in ET-1 precontracted vessels with EC$_{50}$ values of 7.9 ± 2.9 nM and 4.7 ± 2.4 nM in K$^+$ precontracted and ET-1 precontracted tissues respectively.
Figure 35. Relaxation concentration-response curves to bradykinin of endothelin-1 (10-30nM) precontracted porcine thoracic aortic vasa vasorum in the absence or presence of the endothelium (n=3). Values are shown as arithmetic mean ± s.e.m. Significant difference is shown as * for \( P < 0.05 \) and ** for \( P < 0.01 \) using unpaired two-tailed Student’s t-test.
Figure 36. Relaxation concentration-response curves to substance P of endothelin-1 (10-30nM) precontracted porcine thoracic aortic vasa vasorum in the absence or presence of the endothelium (n=3). Values are shown as arithmetic mean ± s.e.m. Significant difference is shown as * for P< 0.05 and ** for P<0.01 using unpaired two-tailed Student’s t-test.
EFFECT OF REMOVAL OF ENDOTHELIUM ON RELAXANT RESPONSES TO CALCITONIN GENE-RELATED PEPTIDE IN VASA VASORUM

Figure 37. Relaxation concentration-response curves to calcitonin gene-related peptide (CGRP) of endothelin-1 (10-30nM) precontracted porcine thoracic aortic vasa vasorum in the absence or presence of the endothelium (n=4). Values are shown as arithmetic mean ± s.e.m.
Figure 38. Relaxation concentration-response curves to substance P (SP) of endothelin-1 (10-30nM) precontracted porcine thoracic aortic vasa vasorum in the absence or presence of the nitric oxide synthase inhibitor L-NAME (300μM, n=5). Values are shown as arithmetic mean ± s.e.m. Significant difference is shown as * for P< 0.05 using unpaired two-tailed Student’s t-test.
EFFECT OF INHIBITION OF NITRIC OXIDE SYNTHASE ON RELAXANT RESPONSES TO BRADYKININ IN VASA VASORUM

Figure 39. Relaxation concentration-response curves to bradykinin (BK) of endothelin-1 (10-30nM) precontracted porcine thoracic aortic vasa vasorum in the absence or presence of the nitric oxide synthase inhibitor L-NAME (300μM, n=5). Values are shown as arithmetic mean ± s.e.m.
Figure 40. Relaxation concentration-response curves to bradykinin (BK) of endothelin-1 (10-30nM) precontracted porcine thoracic aortic vasa vasorum in the absence or presence of the inhibitor of soluble guanylyl cyclase, ODQ (1μM, n=8), or the scavenger of free nitric oxide, oxyHb, (20μM, n=5). Values are shown as arithmetic mean ± s.e.m.
EFFECT OF INHIBITION OF CYCLOOXYGENASE ON RELAXANT RESPONSES TO BRADYKININ IN VASA VASORUM

Figure 41. Relaxation concentration-response curves to bradykinin (BK) of endothelin-1 (10-30nM) precontracted porcine thoracic aortic vasa vasorum in the absence or presence of the cyclooxygenase inhibitor indomethacin (5µM, n=9). Values are shown as arithmetic mean ± s.e.m.
EFFECT OF INHIBITION OF CYCLOOXYGENASE ON RELAXANT RESPONSES TO SUBSTANCE P IN VASA VASORUM

Figure 42. Relaxation concentration-response curves to substance P (SP) of endothelin-1 (10-30nM) precontracted porcine thoracic aortic vasa vasorum in the absence or presence of the cyclooxygenase inhibitor indomethacin (5μM, n=5). Values are shown as arithmetic mean ± s.e.m.
EFFECT OF COMBINED INHIBITION OF NITRIC OXIDE SYNTHASE AND CYCLOOXYGENASE ON RELAXANT RESPONSES TO BRADYKININ IN VASA VASORUM

Figure 43. Relaxation concentration-response curves to bradykinin (BK) of endothelin-1 (10-30nM) precontracted porcine thoracic aortic vasa vasorum in the absence or presence of combined inhibition of nitric oxide synthase (L-NAME, 300μM) and cyclooxygenase (indomethacin, 5μM, n=5). Values are shown as arithmetic mean ± s.e.m.
Figure 44. Relaxation concentration-response curves to bradykinin (BK) of endothelin-1 (10-30nM) or K⁺ (40-50mM, n=5) precontracted porcine thoracic aortic vasa vasorum. Values are shown as arithmetic mean ± s.e.m. Significant difference is shown as *P<0.05 using unpaired two-tailed Student’s t-test.
5.3. SUMMARY

Isolated porcine arterial vasa vasorum precontracted with ET-1 relaxed in a concentration-dependent manner to BK, SP and CGRP. The responses to BK and SP were endothelium-dependent whilst those to CGRP were not. Furthermore endothelium-dependent relaxant responses to SP were predominantly mediated by the release of NO. In contrast, BK-induced responses were independent of activation of the L-arginine/NO pathway and prostanoid activity but were significantly inhibited by raising extracellular [K$^+$] (40-50mM).
CHAPTER 6

NEURAL RESPONSES
6.1. PORCINE ARTERIAL VASA VASORUM

6.1.1 Contractile Responses To EFS

6.1.1.1 Experimental Protocol

To stimulate nerves in porcine vasa vasorum, vessels were mounted in tension myographs with platinum electrodes connected to a stimulator (Grass S88, Grass Instrument Company, USA) to provide transmural EFS. Following normalisation, frequency-response curves were constructed (1-32Hz) at a supra-maximal voltage of 28V, 0.3ms duration for 5s. The involvement of sympathetic nerve activity in responses seen was determined by adding 5µM guanethidine, the adrenergic neurone blocker, and repeatedly stimulating at a submaximal frequency (16Hz). To confirm that responses seen were neuronally-derived tetrodotoxin (TTX, 1µM), the Na\(^+\) channel blocker was added at the end of the curve and vessels were repeatedly stimulated at 16Hz.

6.1.1.2 Results

Mean diameter of porcine thoracic aortic vasa vasorum in this study was 144.1 ± 11.6 µm (n=6). Transmural EFS for 5s (28V, 0.3ms duration) of nerves in porcine vasa vasorum produced frequency-dependent contractions in the presence of threshold concentrations of ET-1 (1-3nM, see fig 45A) that were not inhibited by TTX (1µM, n=6).
6.1.2 Relaxant Responses To EFS

6.1.2.1 Experimental Protocol

To study relaxant responses to transmural EFS in porcine vasa vasorum, vessels were precontracted with ET-1 (3-30 nM). The vessels were not equilibrated with KPSS since K+ depolarises nerves and therefore ET-1 concentrations were chosen to achieve a level of contraction at least as great as the tension achieved at an effective transmural pressure of 100mmHg. This contraction is approximately 50% of the contraction usually produced by KPSS. To inhibit contractile responses to EFS, some vessels were pretreated with guanethidine (5µM) and atropine (1µM) for 30 min to inhibit sympathetic and cholinergic neurotransmission respectively and then precontracted with a submaximal concentration of ET-1 prior to EFS. Transmural EFS (1-32Hz) was applied at 28V, 0.3ms duration for either 5s, 20s or 30s.

6.1.2.2 Results

Mean diameter of porcine thoracic aortic vasa vasorum in this study was 169.8 ± 10.2 µm (n=18). Transmural EFS (28V, 0.3ms duration) for 5s (n=3), or 20s (n=4) or 30s (n=3) produced decrease in tone of vasa vasorum precontracted with ET-1. However, this response was neither frequency-dependent nor inhibited by treatment with TTX (1µM, n=10). Similarly, guanethidine (5µM) and atropine (1µM) treatment had no effect on these TTX-insensitive responses to EFS (n=8, see fig 45B).
Figure 45. Typical trace depicting EFS stimulation of porcine thoracic aortic vasa vasorum (A) in the presence of endothelin-1 (ET-1, 3nM)(diam = 126μm) and (B) precontracted with endothelin-1 (ET-1, 30 nM) in the presence of guanethidine (5μM) and atropine (1μM)(diam = 116μm).
6.2 PORCINE MESENTERIC SMALL ARTERIES

6.2.1 Contractile Responses To EFS

6.2.1.1 Experimental Protocol

In order to compare contractile responses to EFS in porcine vasa vasorum to porcine blood vessels of a similar diameter, porcine mesenteric small arteries were mounted in a tension myograph and EFS was carried out as described above (section 6.1.1.1).

6.2.1.2 Results

Mean diameter of porcine mesenteric arteries was 199.4 ± 23.3 μm (n=5). In contrast to vasa, porcine mesenteric arteries produced frequency-dependent contractions to EFS (1-32 Hz, n=5, see fig 46A). Maximal contractions at 32Hz were 8.5 ± 2.6 mN (n=5, see fig 46B). Contractions at 16Hz were significantly (P<0.05) attenuated but not abolished following treatment with guanethidine (5μM, n=5, see fig 47). Contractions at 16Hz were 4.4 ± 0.9 mN and 1.5 ± 0.2 mN (n=5) in the absence and presence of guanethidine respectively. Inhibition of nerve transmission with TTX (1μM, n=5) had no additional inhibitory effect following application of guanethidine.

6.2.2 Relaxant Responses To EFS

6.2.2.1 Experimental Protocol

In order to study relaxant responses to EFS in porcine mesenteric small arteries, vessels were precontracted with ET-1 (3-30nM) and EFS was carried out as described above (section 6.1.2.1).
6.2.2.2 Results

Mean diameter of porcine mesenteric arteries was $185.7 \pm 6.1 \mu m$ (n=4). Similar to porcine vasa vasorum, porcine mesenteric small arteries precontracted with ET-1 did not produce frequency-dependent relaxations in response to EFS (28V, 0.3ms duration)(see fig 48).
Figure 46. Frequency-dependent contractile responses to 5s stimulation EFS (28V, 0.3ms duration) in porcine small mesenteric arteries. (A) Typical trace (diam = 249μm) and (B) frequency-response curve. Values are shown as arithmetic mean ± s.e.m.
EFFECT OF INHIBITION OF SYMPATHETIC NERVE TRANSMISSION
ON CONTRACTILE RESPONSES TO ELECTRICAL FIELD
STIMULATION IN PORCINE MESENTERIC SMALL ARTERY

Figure 47. Contractile responses to 5s stimulation EFS (28V, 0.3ms duration) at 16Hz in porcine mesenteric small arteries in the absence and presence of guanethidine (5µM). Values are shown as arithmetic mean ± s.e.m. Significant difference is shown as * P<0.05 using paired two-tailed Student's test.
Figure 48. Typical trace depicting EFS stimulation of porcine mesenteric small artery (diam = 198μm) precontracted with endothelin-1 (ET-1, 30 nM) in the presence of a combination of guanethidine (5μM) with atropine (1μM).
6.3 SUMMARY

EFS (28V, 0.3ms duration, 5s stimulation) did produce TTX-sensitive responses of isolated porcine arterial vasa vasorum in the absence or presence of threshold concentrations of ET-1 (1-3 nM). In contrast, EFS produced frequency-dependent contractions of porcine mesenteric small arteries that were significantly attenuated by inhibition of sympathetic nerve transmission. EFS did not produce frequency-dependent relaxation in ET-1 precontracted vasa vasorum or mesenteric small arteries.
CHAPTER 7

MYOGENIC TONE
7.1 PORCINE ARTERIAL VASA VASORUM

7.1.1 Pressure-Induced Responses

7.1.1.1 Experimental Protocol

Porcine arterial vasa vasorum were mounted in a perfusion myograph (see section 2.5). Following equilibration the vessels were pressurised to 100mmHg in the absence of flow. Vessels were constricted with 300nM U44069 and the endothelium was then tested with endothelium-dependent vasodilator BK (300nM). Pressure-diameter curves were constructed, in the absence of flow, by increasing intraluminal pressure in 10mmHg steps from 10mmHg to 100mmHg. Each pressure was maintained until the diameter remained constant (approximately 4 min.). A micrometer attached to the proximal cannula was used to increase the length of the vessel with each increase in pressure.

To investigate the role of endogenous NO in pressure-induced responses, curves were also constructed in the absence and then in the presence of L-NAME (300µM, 30 min.)

7.1.1.2 Results

The passive diameter of porcine vasa vasorum mounted in a perfusion myograph at 100mmHg in the absence of Ca\(^{2+}\) was 165.0 ± 9.5 µm (n=5) and wall thickness was 23.0 ± 1.2 µm (n=5). Mean diameter of vasa at 10mmHg was 84.0 ± 4.8 µm. Increasing intraluminal pressure to 20mmHg produced a large increase in internal diameter (38.0 ± 12 µm). Further stepwise 10mmHg increases in pressure from 20 to 50mmHg produced stepwise increases in diameter. However, subsequent increases in
intraluminal pressures from 60 to 100mmHg produced no further increase in diameter (see fig 49). Although there was no apparent decrease in diameter to increases in intraluminal pressure, the active diameter at 100mmHg was less than the passive diameter indicating a slight level of myogenic tone (7.1 ± 4.2 %, n=5).

Pretreatment of vessels with L-NAME (300μM, 30 min.) had no effect on the pressure-diameter relationship between 10 and 60mmHg. However, the diameter of vasa was lower than control values at pressures above 70mmHg in the presence of NOS inhibition, although this was only significant (P<0.05) at 100mmHg (see fig 50). Myogenic tone in the presence of L-NAME was 22.0 ± 4.6% (n=5). Similarly, L-NAME significantly (P<0.05) attenuated wall tension at 100mmHg (see fig 51, n=5).
RESPONSE OF VASA VASORUM TO CHANGES IN INTRALUMINAL PRESSURE

Figure 49. Typical trace depicting intraluminal pressure-diameter relationship in porcine thoracic aortic vasa vasorum.
Figure 50. Intraluminal pressure-diameter relationship in porcine thoracic aortic vasa vasorum in the absence and presence of NOS inhibitor L-NAME (300µM, n=5). Values are shown as arithmetic mean ± s.e.m. Significance is shown as * P<0.05 using paired two-tailed Student’s t-test.
Figure 51. Intraluminal pressure-wall tension relationship in porcine thoracic aortic vasa vasorum in the absence and presence of nitric oxide synthase inhibitor L-NAME (300µM, n=5). Values are shown as arithmetic mean ± s.e.m. Significance is shown as * P<0.05 using paired two-tailed Student’s t-test.
7.1.2 Reactivity Studies in the Perfusion Myograph

7.1.2.1 Experimental Protocol

To determine the reactivity of isolated vasa vasorum under isobaric conditions, cumulative concentration-response curves were constructed to thromboxane A2-mimetic U44069 (0.1-300nM).

To investigate the importance of endogenous NO in constrictor responses to U44069, concentration-response curves were also constructed in the absence and then in the presence of L-NAME (300μM, 30 min).

7.1.2.2 Results

In contrast to isometric studies in the tension myograph, porcine vasa vasorum responded to thromboxane A2 mimetic U44069 under isobaric conditions in the perfusion myograph (see fig 52A). Constrictions to U44069 were completely reversed by 300nM BK (see fig 52B). At a transmural pressure of 100mmHg, in the absence of flow, U44069 produced a concentration-dependent decrease in diameter (see fig 53). Basal diameter at this pressure was less in the presence of NOS inhibition and consequently the diameter at each concentration of U44069 was also significantly (P<0.05) less than control values. However, despite a reduction in diameter, the potency of U44069 was not significantly affected by NOS inhibition (see fig 53). EC50 values were 43.9 ± 14 nM and 14.2 ± 4.9 nM (n=4) in the absence and presence of L-NAME respectively.
Figure 52. Photographs of a porcine thoracic aortic vasa vasorum mounted in a perfusion myograph in the presence of (A) 300 nM U44069 (diam = 95μm) and (B) 300 nM BK (diam = 150μm)
Figure 53. Concentration-response curves to thromboxane A$_2$ mimetic U44069 in porcine thoracic aortic vasa vasorum pressurised at 100mmHg in the absence and presence of nitric oxide synthase inhibitor L-NAME (300μM, n=4). Values are shown as arithmetic mean ± s.e.m. Significant difference is shown as * P<0.05 and ** P<0.01 using paired two-tailed Student’s t-test.
7.2 RAT MESENTERIC SMALL ARTERIES

7.2.1 Pressure-Induced Responses

7.2.1.1 Experimental Protocol

Third order rat mesenteric small arteries were mounted in a perfusion myograph (see section 2.5). Following equilibration the vessels were pressurised to 80mmHg in the absence of flow. Vessels that did not develop spontaneous tone (i.e. sustained decrease in diameter) were rejected. The endothelium was then tested with endothelium-dependent vasodilator acetylcholine (ACh, 10μM). Pressure-diameter curves were constructed, in the absence of flow, by increasing intraluminal pressure in 10mmHg steps from 10mmHg to 80mmHg. Each pressure was maintained until the diameter remained constant (approximately 4 min.).

To investigate the role of endogenous NO in pressure-induced responses, curves were also constructed in the absence and then in the presence of L-NAME (300μM, 30 min.).

7.2.1.2 Results

Fig 54 shows a typical pressure-response curve constructed in a rat mesenteric artery. Stepwise increases in intraluminal pressure from 10mmHg to 50mmHg produced incremental increases in diameter of rat mesenteric small arteries. The response reached a plateau at 60mmHg. Subsequent increases in intraluminal pressure above 70mmHg resulted in development of myogenic tone, as shown by a decrease in diameter. Mean myogenic tone at 80mmHg was 29.2 ± 3.0% (n=6)
Pretreatment with NOS inhibitor L-NAME (300μM, 30 min) lowered the pressure at which the onset of myogenic tone occurred to 40mmHg and resulted in a significant (P<0.01) depression of the pressure-diameter curve (n=5, see fig 55) such that the magnitude of myogenic tone was greater than control values. Myogenic tone at 80mmHg was 37.2 ± 3.0%.
Figure 54. Typical trace depicting intraluminal pressure-diameter relationship in rat mesenteric small artery.
EFFECT OF INHIBITION OF NITRIC OXIDE SYNTHASE ON PRESSURE-INDUCED RESPONSES IN RAT SMALL MESENTERIC ARTERY

Figure 55. Intraluminal pressure-diameter relationship in rat mesenteric small artery in the absence and presence of NOS inhibitor L-NAME (300μM, n=6). Values are shown as arithmetic mean ± s.e.m. Significant difference is shown as ** P<0.01 using a paired two-tailed Student’s t-test.
7.3 MURINE MESENTERIC SMALL ARTERIES

7.3.1 Pressure-Induced Responses

7.3.1.1 Experimental Protocol

Third order mesenteric small arteries from eNOS wildtype (eNOS WT) or eNOS knockout (eNOS KO) mice were mounted in a perfusion myograph (see section 2.5). Pressure-diameter curves were constructed as described above (section 7.2.1). In some experiments a second curve was constructed in the presence Ca^{2+} free PSS containing 2mM EGTA to determine pressure-passive diameter relationship. To investigate the role of endogenous NO in pressure-induced responses, curves were also constructed in the absence and then in the presence of L-NAME (300µM, 30 min.).

7.3.1.1.1 Removal of endothelium

In certain studies, following construction of a control pressure-diameter curve, the endothelium was removed by slowly injecting 2ml of air through the lumen of the vessel, pressurised at 80 mmHg via the proximal cannula. The vessel was then flushed with PSS at 100µl/min and the air was removed from the system. Smooth muscle tone was increased by applying 10nM U46619 (11α,9α-epoxymethano-PGH₂) and the integrity of the endothelium was verified by applying endothelium-dependent dilator ACh before and after injection of air. Vessels were considered endothelium-denuded when ACh responses were less than 10% of control change in diameter. In vessels that were not successfully denuded a further 1ml of air was injected through the lumen. The sensitivity of the smooth muscle to NO was tested at the end of the experiment by applying non-endothelium dependent dilator SNAP (10µM).
7.3.1.2 Results

Mean passive diameter at 80 mmHg in eNOS WT was 198.3 ± 9.7 μm (n=18). The pressure-diameter relationship in murine mesenteric small arteries was similar to that in rat mesenteric blood vessels (see fig 56). The onset of myogenic tone occurred at pressures above 40 mmHg (see fig 57). Myogenic tone at 80 mmHg was 21.6 ± 1.1% (n=6). Mean passive diameter of eNOS KO arteries was 208.0 ± 9.0 μm (n=10). There was no significant (P<0.05) difference between passive diameter or wall thickness of blood vessels from eNOS WT and eNOS KO animals (see Table 2). The active pressure-diameter relationship in arteries from eNOS KO mice was similar to that in eNOS WT mice. The magnitude of myogenic tone (22.8 ± 2.7%) was not significantly (P<0.05) different in eNOS KO. Indeed calculated pressure-wall tension relationship was not significantly (P<0.05) different between the two groups (see fig 58).

In eNOS WT, selective removal of the endothelium (n=6) significantly (P<0.05) increased myogenic tone at pressures of 50, 60, and 70 mmHg (see fig 58). Endothelium denuded vessels dilated by 80.5 ± 7.4% of maximum (n=6) to application of SNAP. Similarly, inhibition of NOS with L-NAME (300 μM, n=6) increased myogenic tone at all pressures above 40 mmHg (see fig 59). In contrast to the wildtype animals, myogenic tone in arteries from eNOS KO was not altered by inhibition of NOS (n=5, fig 60).
RESPONSE OF MURINE ENOS WILDTYPE MESENTERIC SMALL ARTERY TO CHANGES IN INTRALUMINAL PRESSURE

Figure 56. Typical trace depicting intraluminal pressure-diameter relationship in murine mesenteric small artery.
Figure 57. Intraluminal pressure-diameter relationship in mesenteric small arteries from eNOS WT mice in the presence of normal PSS or Ca$^{2+}$-free PSS containing 2mM EGTA (n=6). Values are shown as arithmetic mean ± s.e.m. Significance is shown as * P<0.05 and ** P<0.01 using paired two-tailed Student’s t-test.
DIAMETER AND WALL THICKNESS OF MESENTERIC SMALL ARTERIES FROM ENOS WILDTYPE AND KNOCKOUT MICE

<table>
<thead>
<tr>
<th></th>
<th>eNOS WT</th>
<th>eNOS KO</th>
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<tr>
<td>n</td>
<td>18</td>
<td>10</td>
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<tr>
<td>Passive diameter (μm)</td>
<td>198.3 ± 9.7</td>
<td>208.0 ± 9.0</td>
</tr>
<tr>
<td>Wall thickness (μm)</td>
<td>14.2 ± 0.6</td>
<td>15.5 ± 1.2</td>
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Table 2. Comparison of mean passive diameter at 80mmHg and wall thickness of mesenteric small arteries from eNOS WT and eNOS KO.
PRESSURE-WALL TENSION RELATIONSHIP IN MESENTERIC SMALL ARTERY OF ENOS WILDLTYPE AND ENOS KNOCKOUT MICE

![Graph showing pressure-wall tension relationship](image)

Figure 58. Intraluminal pressure-wall tension relationship in mesenteric small arteries from eNOS WT mice (n=6) and eNOS KO mice (n=5).
EFFECT OF REMOVAL OF ENDOTHELIUM ON PRESSURE-INDUCED RESPONSES IN ENOS WILDTYPE MURINE SMALL MESENTERIC ARTERY

Figure 59. Intraluminal pressure-diameter relationship in mesenteric small arteries from eNOS WT mice in the absence and presence of the endothelium (n=6). Values are shown as arithmetic mean ± s.e.m. Significance is shown as * P<0.05 using paired two-tailed Student’s t-test.
EFFECT OF INHIBITION OF NITRIC OXIDE SYNTHASE ON PRESSURE-INDUCED RESPONSES IN ENOS WILDLIGHT MURINE SMALL MESENTERIC ARTERY

Figure 60. Intraluminal pressure-diameter relationship in mesenteric small arteries from eNOS WT mice in the absence and presence of NOS inhibitor L-NAME (300μM, n=6). Values are shown as arithmetic mean ± s.e.m. Significance is shown as * P<0.05 and ** P<0.01 using paired two-tailed Student’s t-test.
Figure 61. Intraluminal pressure-diameter relationship in mesenteric small arteries from eNOS KO mice in the absence and presence of NOS inhibitor L-NAME (300μM, n=5). Values are shown as arithmetic mean ± s.e.m.
7.4 SUMMARY

The internal diameter of isolated porcine arterial vasa vasorum increased in response to increases in intraluminal pressure from 10 to 100mHg. These vessels developed slight myogenic tone. However, in the presence of NOS inhibition the diameter at pressures above 70 mmHg was decreased such that myogenic tone was significantly increased. Furthermore in contrast to reactivity studies in the tension myograph, vasa vasorum studied under isobaric conditions in the perfusion myograph produced concentration-dependent decrease in diameter to TXA₂-mimetic. Pretreatment with NOS inhibitor increased the magnitude of the response to U44069 without a significant change in the potency.

In contrast to vasa vasorum, rat mesenteric small arteries developed myogenic tone above 50mmHg. However, similar to the vasa vasorum, the pressure-diameter curve was suppressed in the presence of NOS inhibition such that myogenic tone was significantly (P<0.01) increased at intraluminal pressures of 40, 50, and 60mmHg. Similar to rat blood vessels, murine mesenteric small arteries were capable of generating considerable myogenic tone at pressures above 60mmHg. Selective removal of the endothelium resulted in a significant (P<0.05) depression of the pressure-diameter curve. Moreover, inhibition of NOS resulted in a similar increase in myogenic tone. Mesenteric small arteries from mice with targeted disruption of eNOS gene demonstrated a similar response to pressure as the wild type arteries but NOS inhibition no longer modified the pressure-diameter response curve.
CHAPTER 8

GENERAL DISCUSSION
8.1 METHODS

The vasa vasorum form a network of small arteries and veins in the walls of conduit blood vessels. They are considered to provide oxygen and nutrients to those parts of the vessel wall that are not adequately supplied by diffusion from luminal blood. The hypothesis that the vasa vasorum is important in maintaining physiology of conduit blood vessels is supported by the observation that experimental disruption of these microvessels results in vascular lesions. Furthermore, certain diseases of conduit arteries are associated with changes in the vasa vasorum. In some cases the density of vasa vasorum is directly related to the severity of the disease. Indirect measurement of perfusion pressure and blood flow through vasa vasorum suggest that the vasa vasorum may actively regulate blood flow to the host vessel wall. The presence of the vasa vasorum in blood vessel walls is dependent on wall thickness and partial oxygen tension such that the supply of vasa vasorum is greater in veins than in arteries and greater in larger arteries than smaller ones. Therefore, this thesis was undertaken to study the vasa vasorum of the largest conduit artery, the aorta, in order to gain insight into some of the mechanisms involved in nutrition of this artery. The species used in this thesis was the pig since porcine aorta is of a similar size to human aorta and thus the extent and diameter of vasa vasorum is also likely to be similar. Furthermore, the thoracic part of the aorta was used since the density of vasa vasorum in human aorta is greatest in this region, whilst in contrast the abdominal aorta has surprisingly few vessels in the media (Heistad and Marcus 1979a). From this it may be speculated that vasa vasorum play a more important role in providing nutritional support in the thoracic region of the human aorta than in the abdominal
aorta. Although the vasa vasorum is an entire vascular bed, consisting of arteries and veins, only the arterial vessels were studied since these vessels provide the site of control of nutrient blood flow to the host vessel wall. Second order vasa vasorum were traced from large adventitial first order vessels and dissected out from the adventitial-medial border of porcine thoracic aorta. The vessels were dissected free from surrounding aortic tissue and the responses of isolated arterial vasa vasorum were studied.

In order to study directly the reactivity of isolated vasa vasorum, the tension myography technique was used. The tension myograph allows measurement of the reactivity of small vessels of a minimum diameter of 80µm. It is clear from previous work that the diameter of the vasa vasorum penetrating the media is often as small as 50µm. Whether it is these medial vessels or the vessels studied that are the most important with regards to regulation of perfusion of the large vessel wall is not known. However, the internal diameter of the vessels used was approximately 150µm indicating that these small arteries are likely to contribute significantly to the overall resistance of the vasa vasorum (Mulvany and Aalkjaer 1990; Johnson and Hanson 1962) and it seems likely therefore that modulation of the tone of adventitial-medial vasa vasorum would have an impact on blood flow in the walls of large blood vessels.

8.2 MORPHOLOGY

The histological studies demonstrate that vasa vasorum consist of layers of smooth muscle oriented radially around the lumen of the vessel. A single layer of
endothelial cells protruding into the lumen of the vessel was also evident. The immunohistochemical studies with antibody to αSMA confirm the presence of several layers of smooth muscle associated with these vessels. Immunohistochemical studies of porcine small mesenteric arteries, of a similar diameter, demonstrated that these arteries have a similar structure to the arterial vessels of the vasa vasorum. Thus the small arteries of the porcine thoracic aortic vasa vasorum, like other resistance arteries, are equipped with the necessary elements to actively regulate tone and indicates that they may not be passive channels for blood flow.

Indirect immunofluorescence of whole arterial vessels of porcine vasa vasorum, using general neural markers, demonstrated a network of nerves lying longitudinally along the length of the artery with smaller branches lying radially across the vessel. It is clear that these nerves are primarily of sympathetic origin since the densest immunoreactivity was displayed using antibodies against NPY and TH. These antibodies are commonly used to identify markers of sympathetic nerve fibres and NPY and TH are often colocalised (Gulbenkian et al. 1993). However, the labeling of NPY in vasa vasorum was more extensive than that to TH. Indeed NPY staining is often denser than TH. For example, around the vasa vasorum of human uterine artery TH-immunoreactivity corresponds to 50% of NPY-immunoreactive nerve fibres although TH was always colocalised with NPY (Stjernquist et al. 1991). This suggests that NPY is also present in nerve fibres other than sympathetic fibres. Indeed there is evidence that NPY may also coexist with other neuropeptides such as SP (Weihe et al. 1990). Sparse and less intense CGRP-immunoreactivity was also evident in porcine vasa vasorum. CGRP and SP
are markers of sensory C and Aδ fibres, indicating that these nerve types are also present in vasa vasorum.

The pattern of neural innervation of porcine mesenteric small arteries was similar to that observed in vasa vasorum. The nerves identified in porcine mesenteric small arteries by general neural markers were also predominantly NPY and TH-containing. Like the vasa vasorum, sparse CGRP-immunoreactivity was also demonstrated in these arteries. Therefore, the innervation of porcine vasa vasorum is typical of porcine resistance arteries. Together the immunohistochemical studies suggest that the morphology of isolated porcine thoracic aortic vasa vasorum is similar to that of porcine mesenteric small arteries.

8.3 RESPONSE TO CONSTRUCTORS

The immunohistochemical studies demonstrate the presence of several layers of smooth muscle around the vasa vasorum and it is clear that the vasa vasorum have sufficient radial smooth muscle to generate considerable tension. In accordance with the presence of smooth muscle, the vasa vasorum contracted in response to ET-1. The response to ET-1 was concentration-dependent and had a potency similar to that in porcine mesenteric small arteries and indeed very similar to that seen in the parent aorta from which the vasa vasorum were dissected (Table 1). Furthermore, the potency of ET-1 in these studies was as low as that previously reported in canine mesenteric small arteries (Awane-Igata et al. 1997) and human coronary small arteries (Pierre and Davenport 1998). Thus the response to ET-1 in porcine vasa vasorum was typical of that in other porcine blood vessels and also other resistance arteries in general. However, unlike the contractile responses in
porcine mesenteric small artery and aorta, the vasa vasorum did not contract to TXA2-mimetic. AngII produced concentration-dependent contractions of porcine aorta but did not elicit similar contractions in either vasa vasorum or mesenteric small arteries. Previous studies in rabbit (Dunn et al. 1994) and porcine (Feletou and Tesseire 1990) resistance arteries have also demonstrated a lack of contractile effect of AngII under isometric conditions which has been suggested to be due to receptor desensitisation prior to generation of sufficient stimulus to cause contraction (Dunn et al. 1994). In contrast, NA produced only a low level of contraction in vasa vasorum at high concentrations (EC50 >3000 nM) whilst NA was a potent contractile agonist in both porcine mesenteric small arteries and aorta. Therefore, the profile of contractile reactivity of vasa vasorum to TXA2-mimetic and NA is not characteristic of porcine arteries. Thus despite a similar architecture, the contractile reactivity of porcine vasa vasorum is distinct from other porcine resistance-size arteries, suggesting that under certain conditions decreased sensitivity to constrictor agents may be characteristic of vasa vasorum.

The physiological significance of the differences in contractile responses between the vasa vasorum and other blood vessels and whether these differences exist in vivo are not known. However, it seems reasonable to speculate that it might be important for the vasa vasorum to be resistant to certain mediators that control tone in the large host artery itself. For example, these results indicate that NA released during increased sympathetic activity might constrict the major vessel, but would not affect the tone of vasa vasorum. This would ensure a patent system for the nutrition and oxygenation of the host vessel wall even during sustained sympathetic activation and vasoconstriction. Activated platelets release
What do we know about climate change?
ET-1 in Prima
burst
considerable amounts of thromboxane (Fitzgerald et al. 1986) and thus the failure of the vasa vasorum to respond to thromboxane $A_2$ might similarly protect blood flow to the host vessel wall from the constrictor effects of activated platelets.

The reactivity of vasa vasorum to ET-1 and the considerable $[^{125}\text{I}]$ET-1 binding to vasa vasorum of both human saphenous vein and porcine femoral arteries (Dashwood et al. 1993) suggests that ET-1 may have an important role in regulating nutrient blood flow through the vasa vasorum. Therefore, contractile responses to ET-1 in vasa vasorum were characterised with respect to the receptor subtype and mechanisms of Ca$^{2+}$ mobilisation involved. Contraction of vascular smooth muscle to ET-1 is mediated by at least two distinct receptor subtypes: ET$_A$ and ET$_B$. The distribution of receptor subtype and the contribution that they make to ET-1-induced contraction varies between vascular beds (for review see Bax and Saxena 1994). Blockade of ET$_B$ receptors using the selective ET$_B$ antagonist BQ788 significantly shifted the ET-1 response curve to the right indicating that ET$_B$ receptors mediate at least part of the ET-1 induced contraction of vasa. Confirmation of the presence of ET$_B$ receptors was provided by the demonstration that S6c, the selective ET$_B$ agonist, produced potent concentration-dependent contraction that was significantly shifted to the right by BQ788. Blockade of ET$_A$ receptors, using the selective ET$_A$ antagonist, BQ123, also shifted the concentration-response curve to ET-1 to the right demonstrating that ET$_A$ receptors are involved in ET-1 induced contractions. The response to ET-1 became biphasic in the presence of BQ123, such that responses to high concentrations of ET-1 were shifted to the right whilst the response to low concentrations were BQ123-resistant. Similar sensitivity to BQ123 has also been
reported in other arteries such as rat mesenteric arteries (Mickley et al. 1997) and porcine coronary artery (Awane-Igata et al. 1997). The persisting ET-1-induced contraction in the presence of ET\textsubscript{A} antagonism, which was of a comparable magnitude to that to S6c, may be ET\textsubscript{B}-mediated since BQ788 abolished the BQ123-resistant component of the ET-1 response. Blockade of both ET\textsubscript{A} and ET\textsubscript{B} receptors resulted in a parallel monophasic shift of the concentration-response curve. Curiously, although ET\textsubscript{B} antagonism shifted the entire ET-1 concentration-response curve it did not have an additional effect in the presence of ET\textsubscript{A} antagonism. The explanation of these observations is unclear but is consistent with a permissive role of ET\textsubscript{B} in mediation of ET-1 contraction at the higher concentrations. Nevertheless, these results indicate that in the vasa vasorum of the porcine aorta ET-1-induced contraction is produced by activation of both ET\textsubscript{A} and ET\textsubscript{B} receptors.

The mechanisms of ET-1-stimulated Ca\textsuperscript{2+} influx have been studied extensively (for review see Decker and Brock 1998) but the results are contradictory. The possible mechanisms include Ca\textsuperscript{2+} influx through L-type and non-L-type Ca\textsuperscript{2+} channels in addition to release of Ca\textsuperscript{2+} from intracellular stores. The contractile response of the vasa vasorum to ET-1 is clearly dependent on Ca\textsuperscript{2+} entry since bathing the vessels in a Ca\textsuperscript{2+}-free solution abolished the response, whereas depletion of intracellular Ca\textsuperscript{2+} stores with CPA had no effect. The results indicate that L-type and non-L-type Ca\textsuperscript{2+} channels are important in mediating the Ca\textsuperscript{2+} entry in response to ET-1 in these vessels.
In untreated vessels ET-1 produced slowly developing, sustained contractions that were attenuated in the presence of nifedipine, an inhibitor of L-type Ca\(^{2+}\) channels. Other studies have suggested that ET-1 causes a biphasic increase in intracellular Ca\(^{2+}\) in vascular smooth muscle (Somlyo and Somlyo 1994), suggesting the involvement of at least two different mechanisms of calcium mobilisation. To test whether ET-1 stimulates an increase in Ca\(^{2+}\) through non-L-type channels the effects of agents that block Ca\(^{2+}\) entry through receptor-operated channels were explored. SKF 96365, a purported inhibitor of receptor-operated Ca\(^{2+}\) channels (Merritt et al. 1990), inhibited contractions to ET-1 by about 50%. Ni\(^{2+}\), a non-selective inhibitor of cation entry (Shetty and DelGrande 1994), produced a similar inhibition of ET-1-induced contractions. In addition to its effect on receptor-operated channels, SKF 96365 is also an inhibitor of L-type Ca\(^{2+}\) channels (Merritt et al. 1990). However combination of nifedipine with SKF 96365 produced a greater effect than either inhibitor alone resulting in almost complete blockade of ET-1 responses, supporting a role for nifedipine-insensitive channels in mediating the responses to ET-1.

Since both BQ123 and BQ788 modified ET-1-induced contractions the possibility that the activation of two distinct Ca\(^{2+}\) mobilisation pathways reflects the activation of two distinct receptors were investigated. Contractions to the selective ET\(_B\) agonist S6c were partially inhibited following pretreatment with nifedipine whilst ET-1-induced contractions seen in the presence of ET\(_B\) antagonism were resistant to nifedipine. These findings would be consistent with the possibility that in porcine vasa vasorum ET\(_B\) receptors are coupled to voltage-sensitive L-type channels whilst the ET\(_A\)-mediated component of ET-1 contractions is likely to be
predominantly due to influx of Ca\(^{2+}\) through non-L-type channels. However, in the presence of BQ123 nifedipine suppressed both phases of the resulting contractions and thus the mechanisms of Ca\(^{2+}\) mobilisation following ET\(_{A}\) or ET\(_{B}\) activation in these vessels are not clear.

ET-1 not only produced potent contraction but also sensitised arterial vasa vasorum to the constrictor effects of NA such that in the presence of ET-1 the maximum contraction to NA increased 4-fold. Similar ET-1-induced enhancement of contractile responses to \(\alpha\)-adrenergic agonists or adrenergic nerve stimulation has been reported in rat (Tabuchi et al. 1991; Tabuchi et al. 1989) and canine (Zhang et al. 1996) isolated mesenteric arteries. ET-1 also potentiates contractions to NA and 5HT in human internal mammary arteries (Yang et al. 1990). Nifedipine abolished ET-1-induced potentiation of NA responses in vasa vasorum implicating influx of extracellular Ca\(^{2+}\) through L-type channels in mediation of this enhanced response to NA. Similar effects of inhibition of L-type channels have been seen in human internal mammary arteries (Yang et al. 1990) and perfused rat mesenteric arteries (Tabuchi et al. 1989). In contrast to its effects on NA-induced contractions, ET-1 did not potentiate responses to U44069 indicating some specificity in the potentiating action of ET-1. However, depolarisation of smooth muscle with K\(^+\), which would also be expected to open voltage-gated Ca\(^{2+}\) channels, significantly potentiated the responses to both NA and TXA\(_2\)-mimetic. The reasons for the differences in potentiation of responses by ET-1 or K\(^+\) are not clear. Both stimuli increased basal tension to the same degree (10%) and both would be expected to open voltage-gated Ca\(^{2+}\) channels. These differences may reflect different mechanisms of Ca\(^{2+}\) mobilisation by NA and U44069. Due to the
lack of selective pharmacological tools it was not possible to test this hypothesis since Ni\(^{2+}\) and SKF 96365 inhibit K\(^+\) induced precontraction. However, one could speculate that contractions to U44069 are mediated by influx of Ca\(^{2+}\) through non-L-type Ca\(^{2+}\) channels.

The finding that low levels of ET-1 alters the responses to NA may be of considerable physiological and pathophysiological importance. The isolated vasa vasorum is normally resistant to NA-induced contraction and, as already indicated, this might be important to allow maintenance of vessel wall perfusion when the host conduit vessel is contracting to sympathetic stimulation. However, the present findings suggest that an increase in ET-1 levels would expose reactivity to NA and that depolarisation would enhance responses to both NA and TXA\(_2\).

Consistent with these observations, in a study using monkeys fed an atherogenic diet, a model associated with elevated ET-1 levels (Lerman et al. 1993), the diameter of the vasa vasorum of coronary arteries decreased in response to PE or 5-HT whilst these constrictors had no effect in healthy animals (Williams et al. 1988). Circulating plasma levels of ET-1 are elevated in several diseases including atherosclerosis (Lerman et al. 1995), hypertension, congestive heart failure, asthma and diabetes (for review see Brooks et al. 1998). Furthermore, radioimmunoassay studies have demonstrated a significant increase in endogenous ET in human aorta with atheromatous plaques (Bacon et al. 1996). Indeed chronic ET\(_A\) receptor blockade normalises NO-mediated endothelial dysfunction and reduces atheroma formation in a mouse model of human atherosclerosis (Barton et
ET-1 synthesis (Bodi et al. 1995) and release in both resistance (Rakugi et al. 1990) and conduit (Pape et al. 1997) vessels can also be stimulated by hypoxia or thrombin (Marsen et al., 1995). The results of these experiments suggest that various pathophysiological states might significantly alter the reactivity of the vasa vasorum which in turn may lead to underperfusion of the host vessel wall. In this respect endothelin receptor antagonists might have additional useful effects to preserve or restore patency of the vasa vasorum.

Despite the dense innervation of porcine vasa vasorum with NPY-containing nerves, exogenous NPY produced no contractile effect in these arteries. Whilst NPY is able directly to induce moderate constriction in perfused rat mesenteric bed, an effect that is mediated by postjunctoinal Y1 receptors (Han et al. 1998), it is thought that the function of NPY is as a modulator of contraction rather than a mediator. Several studies have failed to demonstrate a direct contractile effect of NPY in different small artery preparations including human epicardial coronary arteries (Gulbenkian et al. 1993), human temporal arteries (Jansen Oleson et al. 1995), human uterine arteries (Stjernquist et al. 1991) and rabbit femoral arteries (Ekblad et al. 1984). NPY increases the potency of NA-induced contractions in human epicardial coronary (Gulbenkian et al. 1993), temporal (Jansen Oleson et al. 1995), and uterine arteries (Stjernquist et al. 1991). Furthermore NPY potentiates contractions elicited by electrical field stimulation in rabbit femoral arteries (Ekblad et al. 1984). In contrast to these studies, high concentrations of NPY (100nM) that potentiate responses to NA (Stjernquist et al. 1991) and electrical field stimulation (Ekblad et al. 1984) in other preparations had no effect on responses to NA in isolated porcine vasa vasorum. Therefore there was no
apparent postjunctional effect of NPY in vasa vasorum. It is possible that NPY has a prejunctional role to modulate transmitter release in vasa vasorum. Indeed several studies have demonstrated that NPY can inhibit NA release and contractions to EFS without affecting contractile effects of NA (for review see Linton-Dahlof and Dahlof, 1993). It was not possible to test the hypothesis that NPY modulates NA release in vasa vasorum since no specific prejunctional NPY receptors are commercially available.

8.4 RESPONSE TO DILATORS

The vasa vasorum is also sensitive to vasodilators. Vessels precontracted with ET-1 produced concentration-dependent relaxations to SP with a potency similar to that reported in other resistance arteries (for example see Lagaud et al. 1999). Indeed autoradiography studies have suggested that SP receptors are present in adventitial vasa vasorum of blood vessels of equine synovial membranes (Bowker et al. 1993). BK also produced concentration-dependent relaxation of precontracted vasa vasorum. The potency of BK in vasa vasorum was similar to that previously reported in other resistance arteries such as human coronary small arteries (Kemp and Cocks 1997). The relaxant responses to BK and SP were abolished by removal of the endothelium. Endothelial denudation resulted in selective removal of the endothelium without damage to the underlying smooth muscle; since contractile responses of endothelium-intact vessels, to either ET-1 or KPSS, were not significantly different from responses in endothelium-denuded vessels. Several studies have demonstrated a similar obligatory role of the endothelium in mediating smooth muscle relaxation to BK and SP (see for example D’Orleans-Just et al. 1985). Endothelium-dependent responses may be
mediated by relaxing factors NO, and prostacyclin, and EDHF. Experiments were carried out to identify which mediator was involved in the endothelium-dependent responses to both SP and BK. The results demonstrate that responses to SP in vasa vasorum are predominantly mediated by endothelium-derived NO since the relaxations were attenuated by inhibition of NOS but were unaffected by inhibition of prostanoid production. These findings mirror previous studies that demonstrate a similar sensitivity of SP-induced vasodilatation in rats in vivo to an inhibitor of NOS (Whittle et al. 1989). In contrast to their effects on the response to SP, neither L-NAME nor indomethacin, either alone or in combination, significantly altered the responses to BK in vasa vasorum. Thus the response to BK was endothelium-dependent but appeared to be independent of NO or prostanoids. It is unlikely that the lack of effect of L-NAME was due to insufficient block of NOS since the concentrations used have previously been shown to provide almost total inhibition of enzyme activity (Rees et al. 1990) and indeed were effective in blocking the response to SP. The suggestion that the endothelium-dependent response to BK was independent of NOS activity was supported by the lack of effect of ODQ (inhibitor of guanylyl cyclase) or OxyHb (binding of free NO). Thus in the vasa vasorum BK evokes an endothelium-dependent response which is not fully accounted for by generation of NO and is independent of prostanoids.

In addition to NO and prostanoids, the endothelium is capable of releasing a third as yet unidentified relaxing factor, endothelium-derived hyperpolarising factor (EDHF) (Taylor and Weston 1988). The response attributed to EDHF is the portion of relaxation and/or hyperpolarisation that is resistant to inhibition of NO
and prostanoids. The effects of EDHF appear to be mediated by activation of $K^+$ channel(s) (Petersson et al. 1997). It is apparent that EDHF has a more important role in endothelium-dependent responses in resistance-size blood vessels than conduit blood vessels (Urakami-Harasawa et al. 1997; Shimokawa et al. 1996). Therefore it is possible that endothelium-dependent relaxations to BK in vasa vasorum are mediated by the release of EDHF. Consistent with this possibility, raising the concentration of extracellular $K^+$ concentration (50mM) diminished the response to BK. The conductance of $K^+$ channels is dependent upon the extracellular $K^+$ concentration such that raising the concentration produces depolarisation and inhibition of $K^+$ channels (for review see Nelson and Quayle 1995) and this property is often exploited to separate EDHF responses from NO and prostanoid-mediated responses (Adeagbo and Triggle 1993). Thus in vasa vasorum BK may produce relaxant responses by stimulating the release of EDHF.

Similar to its effects in other isolated blood vessels in vitro (for review see Maggi 1995), CGRP produced potent relaxations of isolated vasa vasorum. The responses to CGRP in these arteries were not affected following removal of the endothelium, as demonstrated in most other vessels (for example see Greenberg et al. 1987) although CGRP in certain blood vessels, such as rat aorta, produces endothelium-dependent relaxations that are probably mediated by the release of NO (Gray and Marshall 1992).

The finding that vasa vasorum respond to vasoactive agents and that the endothelium modulates responses may have implications for the pathophysiology of disease affecting the host vessel. The endothelium of resistance vessels is
important to co-ordinate the distribution of blood flow to tissues (Griffith et al. 1987). If endothelial function of vasa vasorum is altered by disease or following physical manoeuvres, such as balloon angioplasty of the large host vessel, this would affect blood flow in the vessel wall. Several studies suggest that the vasa vasorum may be affected by angioplasty of the host vessel (Zollikofer et al. 1987; Cragg et al. 1983; Pisco et al. 1994). The number of microvessels in the aortic wall increases following angioplasty in a canine disease model (Pisco et al. 1994) and it would be interesting to determine whether the reactivity of the vessels is also affected. Furthermore in a balloon-injury model in the rat carotid artery it has been shown that there is an initial decrease in neuronal CGRP and SP-immunoreactivity around the vasa vasorum of the injured vessel and a compensatory increase in the control contralateral vessel (Milner et al. 1997). The functional consequences of these changes in vasa vasorum are not known but, from the findings of this thesis, it is clear that CGRP and SP may alter the tone of the vasa vasorum. Therefore a decrease in the supply of these peptides would presumably decrease vasa vasorum blood flow to the host vessel wall.

8.5 NEURAL RESPONSES

It is well documented that neurogenic control of vascular smooth muscle tone includes the release of NA from sympathetic nerve endings at the adventitial-medial border of several arteries, including resistance arteries such as rat mesenteric small arteries (Sjoblom-Widfeldt 1990). NA is often colocalised with ATP and NPY in these nerves which act as cotransmitters upon nerve stimulation (Sjoblom-Widfeldt 1990). It is clear from immunohistochemical studies that arterial vasa vasorum of porcine thoracic aorta are also innervated by sympathetic
nerves. These nerves demonstrated immunoreactivity to TH, the enzyme required for NA synthesis, and NPY. Consistent with sympathetic regulation of tone of the vasa vasorum, stimulation of the stellate ganglion of dogs decreases blood flow to the outer media of the thoracic aorta (Heistad et al. 1979b). However, there is no direct evidence for neural control of blood flow through the vasa vasorum. Transmural EFS of isolated porcine vasa vasorum produced frequency-related contractions. TTX is an inhibitor of sodium conductance and thereby abolishes action potential propagation (Nakamura et al. 1965) and is commonly used to irreversibly inhibit nerve transmission. Responses to EFS in vasa vasorum were not sensitive to treatment with TTX indicating that these contractions were not neurogenic but likely to be due to direct stimulation of smooth muscle. This result is surprising since electrical activation of smooth muscle usually requires pulse duration as high as 30ms (Sjoblom-Widfeldt 1990). However, there is evidence for a subpopulation of nerves that are not sensitive to TTX (Baker and Rubinson 1975). In contrast to vasa vasorum porcine mesenteric small arteries, that appeared to have a similar density of sympathetic nerve fibres, did produce frequency-dependent contractions under identical experimental conditions. Moreover, these responses were inhibited by guanethidine, the inhibitor of sympathetic nerve transmission (Burnstock et al. 1971). Thus the lack of contractile neurogenic responses in vasa vasorum is likely to be characteristic of the vasa vasorum.

The pharmacological studies of vasa vasorum demonstrate that these vessels are not sensitive to NA under basal conditions but that low levels of ET-1 uncover contractile responses to NA in vasa vasorum. It is therefore reasonable to assume
that sympathetic nerve stimulation does not affect tone in vasa vasorum under basal conditions but does in the presence of threshold concentrations of ET-1 (1-3 nM). However, no neuronal responses in vasa vasorum were observed even in the presence of ET-1. Previous studies in rat tail artery have demonstrated that these concentrations of ET-1 may in fact inhibit rather than potentiate sympathetic contractions via activation of prejunctional \( \text{ET}_B \) receptors (Mutafova-Jambolieva and Westfall 1998) and this may account for the lack of effect of EFS in the presence of ET-1 in vasa vasorum.

The lack of contractile responses to EFS in isolated porcine vasa vasorum is not surprising given its relative insensitivity to normally potent arterial constrictors. These arteries appear to be more sensitive to dilators than constrictors and thus it may be expected that EFS produces mainly relaxation and not contraction in vasa vasorum. In particular exogenous CGRP was a potent vasorelaxant of vasa vasorum precontracted with ET-1 and immunohistochemical studies demonstrated sparse CGRP-immunoreactivity of vasa vasorum. In order to study EFS-induced relaxations, vessels were pretreated with inhibitors of sympathetic nerve transmission and cholinergic receptors, guanethidine and atropine respectively. Under these conditions EFS failed to produce frequency-related relaxations sensitive to TTX. Similarly, no neuronal frequency-dependent responses were observed in porcine mesenteric small arteries. These results indicate that stimulation of CGRP-containing nerves in these blood vessels was not sufficient to produce relaxation. However, it is not possible to exclude the possibility that the lack of EFS-induced relaxation in either of these preparations is due to collection of the tissue from the abattoir and the time delay involved in
transportation. For example, neural responses may be more sensitive to temperature than receptor-mediated responses. Indeed cold storage is a method used to selectively destroy nerves in blood vessels in certain studies (Jensen 1995). Therefore, the lack of relaxation to EFS may not be a true reflection of the role of dilator responses to nerve stimulation in these blood vessels in vivo.

8.6 MYOGENIC TONE

The myogenic response is a major determinant of smooth muscle tone and hence vascular resistance in several vascular beds including the mesenteric microcirculation (Sun et al. 1992). This capacity to respond to pressure occurs in small arteries of most species, including humans (Wallis et al. 1996). However, the pressure- diameter relationship in isolated vasa vasorum demonstrated that these arteries possess little myogenic tone over a pressure range of 10-100mmHg. The intraluminal pressure of porcine thoracic aortic vasa vasorum in vivo is not known. The mean aortic pressure in conscious pigs is approximately 90mmHg (Adachi and Nishino 1998) and therefore it seems reasonable to assume that the mean pressure in porcine thoracic aortic vasa vasorum is similar. However, it has been suggested that, like the coronary circulation, the vasa vasorum fill during diastole (Belcaro et al. 1996) and thus the pressure in vasa vasorum may be considerably lower than 90mmHg. Nonetheless, there was little change in diameter over the pressure range 50-100mmHg. This suggests that the vasa vasorum exist at near maximal diameter over the pressure range studied. The reasons for the low level of myogenic tone in vasa vasorum are not clear but may reflect the unique function of these small arteries. It is possible that the modest myogenic tone in vasa vasorum ensures a constant supply of nutrient blood flow.
to the host vessel wall. Agonist sensitivity is greater under isobaric conditions compared to isometric (Dunn et al. 1994), suggesting an interaction between myogenic and agonist-stimulated constriction. Therefore, the low level of myogenic constriction in vasa vasorum may also decrease the sensitivity of these vessels to certain vasoconstrictors. The results of this thesis have demonstrated that isolated porcine vasa vasorum are not sensitive to TXA$_2$ mimetic when studied under isometric conditions in the tension myograph. However, despite a low level of myogenic tone, TXA$_2$ mimetic produced a concentration-dependent constriction of vasa vasorum under isobaric conditions. The level of myogenic tone at these pressures (~10%) is equal to the level of K$^+$ induced contraction required to uncover reactivity to TXA$_2$ mimetic in the tension myograph. This suggests that pressurised vasa vasorum may be depolarised to a similar extent as that induced by low concentrations of K$^+$ and that under these conditions vasa vasorum respond to TXA$_2$ mimetic. Thus if vasa vasorum are exposed to similar intraluminal pressure in vivo, these results suggest that these vessels would be sensitive to the contractile effects of TXA$_2$.

In contrast to vasa vasorum, third order mesenteric small arteries from rats and eNOS WT mice exhibited typical pressure-dependent decrease in diameter within a physiologically relevant pressure range. This response is considered to involve influx of extracellular Ca$^{2+}$ (Knot and Nelson 1998) and consistent with this hypothesis, myogenic tone in eNOS WT arteries was abolished in the absence of extracellular Ca$^{2+}$. The pressure-passive diameter curve (fig 57) demonstrates that these blood vessels develop myogenic tone at pressures above 40 mmHg. It is possible that the differences in myogenic responses between the vasa vasorum and
other resistance arteries is due to a greater basal release of vasodilators. In particular, basal NO release is an important determinant of basal tone (Vallance et al. 1989). In support of this hypothesis, inhibition of NO release significantly potentiated myogenic responses such that in the presence of NOS inhibition, myogenic tone in vasa vasorum at 100mmHg was comparable to that seen in other resistance arteries. However, inhibition of NO release in eNOS WT mesenteric small arteries also significantly decreased intraluminal diameter only in the pressure range at which myogenic tone was observed (i.e. above 40 mmHg). The lack of effect of L-NAME on the diameter of these arteries at intraluminal pressures less than 50mmHg indicates that the extent of basal NO release has minimal impact on vascular tone of these vessels at low pressures. It is likely that this modulation of myogenic tone by basal NO is a common mechanism in small arteries since myogenic tone in rat mesenteric small arteries was also significantly increased in the presence of NOS inhibition.

In order to investigate whether the NO influencing myogenic tone in eNOS WT was derived from the endothelium, pressure-diameter curves were constructed before and after removal of the endothelium. The endothelium was removed by bolus injection of air through the vessel. The vessel was considered to be denuded of endothelium if dilation to the endothelium-dependent dilator ACh was lost. This procedure did not damage the underlying smooth muscle since the contractile response to TXA₂ mimetic was unaffected and vessels had the capacity to dilate to the endothelium independent dilator SNAP. Removal of the endothelium from eNOS WT arteries significantly increased the magnitude of myogenic tone at intraluminal pressures of 50, 60 and 70 mmHg. These findings are in agreement
with a recent study in mesenteric small arteries of spontaneously hypertensive rats in which myogenic tone was significantly greater in the absence of the endothelium (Garcia and Bund 1998). In contrast, several other studies have demonstrated that removal of the endothelium has no effect on the pressure-diameter relationship in a variety of different vascular beds and species (for review see Meininger and Davis 1992) including human cerebral resistance arteries (Wallis et al. 1996) and rat mesenteric small arteries (Sun et al. 1992). The difference between these studies and the present study may relate to the method of endothelium removal since many of the above studies used chemical means to remove the endothelium instead of physical removal using air. The increase in the magnitude of the myogenic response in eNOS WT mesenteric arteries in the absence of the endothelium was similar to that observed in the presence of NOS inhibition. Together these findings indicate that the endothelium functionally moderates myogenic tone and that the endothelium-derived factor responsible for this effect is NO.

Since the NOS inhibitors show little selectivity for the different NOS isoforms, mice with targeted disruption of eNOS gene were used to determine whether the NO influencing myogenic constriction was due to the activity of eNOS. This isoform was specifically concentrated on since it is the main isoform found in the endothelium, although there have been some studies demonstrating nNOS in these cells (Loesch and Burnstock 1996). Unlike small arteries from eNOS WT, in mesenteric small arteries from eNOS KO the pressure-diameter relationship was not significantly altered by NOS inhibition. This result demonstrates that the NOS isoform responsible for the production of NO inhibition in moderating myogenic
responses is eNOS. Surprisingly, the pressure-diameter curve in eNOS KO was not significantly different from that in the wildtype animals. Indeed the calculated wall tension (the considered stimulus for myogenic constriction) was not altered by the absence of the eNOS gene, suggesting that other factors compensate for the lack of NO. These results imply developmental adaptation. Indeed other studies on eNOS KO have demonstrated similar adaptation. The absence of eNOS is compensated for by nNOS in pial arteries (Meng et al. 1998) and by cyclooxygenase in mesenteric arteries (Chataigneau et al. 1999) in responses to ACh and in flow-mediated responses in gracilis muscle arterioles of eNOS KO mice (Sun et al. 1999).

Therefore, whilst the vasa vasorum does possess myogenic tone at 100mmHg this is markedly lower than that observed in other resistance arteries of the mouse and rat. However, similar to the other resistance arteries studied, the magnitude of myogenic constriction in vasa vasorum was significantly enhanced by NOS inhibition. NO is an important mediator of vascular autoregulation and it is well documented that, in isolated resistance arteries, changes in flow (shear stress) result in eNOS-derived NO release from the endothelium to produce dilatation and thereby normalize shear stress (Koller et al. 1994; Woodman et al. 1999). The results of this thesis demonstrate that NO is also involved in transmural pressure-mediated responses. It is clear that the source of NO in murine arteries is eNOS. Immunohistochemical studies have demonstrated the presence of eNOS in vasa vasorum of human aorta (Wilcox et al. 1997) and it seems reasonable to speculate that eNOS-derived NO also modulates myogenic tone in porcine vasa vasorum and rat arteries. Moreover, these results imply that impairment of normal
endothelium and thereby eNOS function would result in an increase in myogenic tone and thus may decrease nutrient blood flow through the vasa vasorum.
8.7 SUMMARY AND CONCLUSIONS

Vasa vasorum supply oxygen and nutrients to the walls of conduit blood vessels. The control of blood flow to the blood vessel wall is determined by the diameter of the small arteries of the vasa vasorum. It is considered that blood flow through vasa vasorum has an important role in maintaining physiology of the host vessel. Previous studies in vivo and in vitro have indicated that vasa vasorum respond to vasoactive agents. However, the direct effects of these substances and the mechanisms involved have not been investigated. This thesis describes studies carried out on arterial vasa vasorum isolated from porcine thoracic aorta. The morphology and reactivity of these vessels was studied and compared to other blood vessels of a similar diameter, and also to the host vessel itself. The major findings of these studies are:

1) Arterial vasa vasorum have a similar structure and innervation to other resistance arteries. The morphology of arterial vasa vasorum of porcine thoracic aorta is similar to that of porcine mesenteric small arteries. Vasa vasorum consist of layers of smooth muscle oriented radially around a single layer of endothelium. These arteries are innervated by a network of nerves that predominantly contain TH and NPY. In addition, some CGRP-containing nerves are also evident.

2) Arterial vasa vasorum are sensitive to vasodilators but relatively insensitive to vasoconstrictors. Vasa vasorum contract to ET-1, an effect that is mediated by both ET$_A$ and ET$_B$ receptor subtypes and involves the influx of Ca$^{2+}$ through L-type and non-L-type Ca$^{2+}$ channels. The profile of contractile reactivity in
vasa vasorum is markedly different from that in porcine mesenteric small arteries in that the vasa vasorum are not sensitive to NA or TXA$_2$ mimetic. However, the sensitivity of vasa vasorum to certain constrictors is altered in the presence of low concentrations of ET-1 or K$^+$. In contrast, the potency and mechanism of relaxant responses in vasa vasorum was typical of that previously reported in other arteries of a similar diameter.

3) *Arterial vasa vasorum possess less myogenic tone than other resistance arteries.* Vasa vasorum are capable of producing modest myogenic constriction, which is considerably less than that observed in murine and rat mesenteric small arteries. Similar to the other resistance arteries studied, myogenic constriction in vasa vasorum is modulated by basal release of nitric oxide.
8.8 FUTURE STUDIES

1. *Effect of pressure.* The results from this thesis demonstrate that porcine aortic vasa vasorum are not sensitive to certain vasoconstrictors when studied in the tension myograph but are when studied in the perfusion myograph. This difference in sensitivity has been demonstrated in several small arteries from different vascular beds and species. Vessels mounted in a tension myograph are isometric preparations, where the diameter is fixed and application of vasoconstrictor agents results in an increase in wall tension. Vessels mounted in a perfusion myograph are isobaric preparations, where the pressure is constant and the diameter is variable. In isobaric situations, vessel diameter decreases in response to a vasoconstrictor and subsequently wall tension remains low. The studies of vasa in perfusion myograph demonstrate that these vessels are capable of developing modest myogenic tone at high pressures (above 90mmHg), which are not achievable in the tension myograph. The basal tension (approximately 1 mN) at which vasa were set in the tension myograph (i.e. a level of tension that gives maximal force production to KPSS), is equivalent to an effective pressure of approximately 20mmHg. Therefore, it would be of interest to study the reactivity of vasa to vasoconstrictors and vasodilators at higher pressures to test whether this alters the sensitivity of vasa vasorum to vasoactive agents and also whether these vessels are capable of developing myogenic tone and thus possess a vasodilator reserve.

2. *Vasa vasorum from other vascular beds and species.* The vasa vasorum of small arteries of large animals and of large arteries of small animals are
technically too small to study using the techniques in this thesis. However, it would be valuable to study the reactivity of vasa vasorum isolated from large arteries of other large animals, such as dogs, to determine whether the characteristics of the vasa vasorum of porcine thoracic aorta are similar to those of vasa vasorum in other species. Since the distribution of vasa vasorum is not uniform throughout the circulation, it would also be important to study the reactivity of vasa vasorum of other porcine arteries and veins to determine whether the reactivity of porcine thoracic aortic vasa are characteristic of porcine vasa vasorum in general.

3. Hypoxia. The extensive distribution of vasa vasorum in veins and arteries with low luminal oxygen tension suggest that these vessels are sensitive to oxygen tension. In vivo studies have already suggested that the diameter of vasa vasorum increases in response to hypoxia. It would therefore be interesting to study the effects of low oxygen tension on reactivity in vasa vasorum.

4. Models of atherosclerosis. There is considerable evidence that the morphology of vasa vasorum is altered in atherosclerosis. Indeed the proliferation of vasa vasorum in atherosclerosis is directly associated with the severity to the disease. These studies indicate that the vasa vasorum may have a role in supplying nutrient blood flow and/or inflammatory cells to the thickened artery. It has also been suggested that atherosclerosis may develop secondary to impaired vasa vasorum blood flow. Therefore, in order to further test this hypothesis, the reactivity of vasa vasorum of blood vessels from animals fed a high cholesterol diet, a model for atherosclerosis, could be studied to
determine whether these vessels are more sensitive to constrictors and/or less sensitive to dilators in this model.

5. Balloon angioplasty. It is unclear what the effects of balloon angioplasty are on vasa vasorum blood flow and morphology. It is possible that these manoeuvres distort vasa vasorum and also possibly damage the endothelium of these vessels. Such changes in vasa vasorum morphology may therefore alter nutrient blood flow to the host artery wall. To test this hypothesis the morphology and reactivity of isolated vasa vasorum from ballooned arteries could be studied in vitro.

6. Effect of metabolites. Vasa vasorum penetrate conduit vessel walls and may therefore be sensitive to changes in metabolites in the vessel wall. Previous studies using the microsphere technique suggest that vasa vasorum may be sensitive to adenosine. To determine whether this effect of adenosine and other metabolites eg lowered pH and modestly raised potassium directly affect the vasa vasorum, studies could be carried out in vitro.
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