THE EFFECT OF CHEMICAL DENERVATION ON THE ACTION OF ACETYLCHOLINE AND SUBSTANCE P IN THE ISOLATED PERFUSED MESENTERIC ARTERIAL BED OF THE RAT

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THE EFFECT OF CHEMICAL DENERVATION ON THE ACTION OF ACETYLCHOLINE AND SUBSTANCE P IN THE ISOLATED PERFUSED MESENTERIC ARTERIAL BED OF THE RAT

by

© Margaret E. Miller, B.Sc.

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ABSTRACT

Vascular endothelial cells respond to certain vasoactive agents by releasing factors which act on medial smooth muscle to cause relaxation or contraction of blood vessels. One of the substances responsible for endothelium-dependent relaxation to acetylcholine has recently been identified as nitric oxide. We have tested the hypothesis that the ability of vascular endothelium to cause relaxation in response to stimulation by vasoactive agents is related in some way to the pattern of perivascular innervation. The actions of acetylcholine and substance P were tested in the presence of methoxamine induced tone in the isolated perfused mesenteric arterial bed of the rat. Tissues were tested from untreated normal 12 week old Sprague-Dawley rats and from rats which had been treated from birth with capsaicin to prevent the development of peptidergic perivascular innervation or 6-hydroxydopamine to prevent development of catecholaminergic innervation. Concentration dependent endothelium-dependent relaxations were observed in response to acetylcholine. The concentration response curve to acetylcholine was shifted 1.2 log units to the right in the capsaicin-treated group but no change was observed with 6-hydroxydopamine treatment. Substance P caused a dose dependent potentiation of the methoxamine induced tone which was not dependent on the presence of an intact endothelium. Relaxations to substance P were not observed at any dose. Sympathectomy with neonatal 6-OHDA treatment resulted in an increase

in the substance P pressor response, but no changes were observed with capsaicin treatment. Thus, it appears that altering the peptidergic perivascular innervation results in a decreased sensitivity of the mesenteric arterial bed to acetylcholine and changes in the catecholaminergic fibre plexus result in an enhancement of the substance P modulation of adrenergic vasoconstriction.

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TABLE OF CONTENTS

INTRODUCTION

1.1. The Control of Vascular Tone

The control of the cardiovascular system involves a variety of components which influence smooth muscle tone. The distribution of blood to tissues in the body is determined by the flow of blood from the heart and the regulation of blood vessel diameter. The complex system responsible for this function includes the innervation of the vasculature, circulating factors in the blood, and the endothelium.

1.1.1. Vascular Innervation

One of the most powerful mechanisms for the overall control of the cardiovascular system is the innervation provided by the autonomic nervous system. The nerve fibres which supply the blood vessels are called perivascular nerves since they form a mesh-like terminal plexus at the adventitial-medial border of the blood vessel wall.

For a long time the innervation of the vasculature was thought to consist of sympathetic nerves containing catecholamines. However, with the development of immunological techniques capable of identifying and defining neurotransmitter substances, these fibre networks have been shown to be more complex (Kenigsberg and Cuello, 1987). Three different types of perivascular nerves, adrenergic,

cholinergic, and peptidergic have been distinguished. In addition, some nerve fibres have been shown to contain more than one of these types of neurotransmitter co-localized at the nerve terminals (Hökfelt *et al.*, 1987).

(a) Adrenergic Innervation of the Vasculature

The most important component of autonomic motor control of the vasculature is the sympathetic nervous system, in which noradrenaline is the peripheral neurotransmitter. The adrenergic nerve supply to most vessels in the body originates in the pre- or para-vertebral ganglia of the sympathetic nervous system. However, the innervation of some blood vessels in the brain may originate in central catecholaminergic neurons (Edvinsson *et al.*, 1973).

There is a great variation in the pattern and density of the sympathetic innervation of vascular smooth muscle (Burnstock, 1975). It is likely that such variation is related to the physiological role of the blood vessel in the tissue it supplies. In general, large elastic arteries show a sparse pattern of innervation and, as the size of the artery decreases, the density of innervation increases. Capillaries, venules and small veins are considered to have very little adrenergic nerve supply. Veins usually have a much less dense innervation than arteries.

(b) Cholinergic Innervation of the Vasculature

Innervation of blood vessels by postganglionic cholinergic nerve fibres is generally thought to provide an inhibitory control of vascular tone. However, the nature of this vasodilatory action is understood less than the actions of adrenergic nerves. These cholinergic nerves have a less widespread distribution in the cardiovascular system than do the sympathetic fibres. However, physiological and histochemical studies have suggested the possibility of cholinergic vasomotor control in the brain, heart, lung, kidney, skeletal muscle and uterus (Schenk and El Badawi, 1968; Bell, 1969; Iwayama *et al.*, 1970; Borodulya and Pletchkova, 1973). Thus, some vascular beds possess a dual adrenergic and cholinergic innervation.

Cholinergic nerves in the blood vessels of skeletal muscle originate from ganglia of the sympathetic chain, while those of the uterine artery arise from the paracervical ganglion. The origin of the cholinergic supply to the intracranial blood vessels is not yet known. Vessels of the gastrointestinal tract receive their cholinergic nerve supply from the intramural ganglia (Owman, 1983).

(c) Peptidergic Innervation of the vasculature

In addition to the sympathetic adrenergic and parasympathetic cholinergic divisions of the autonomic nervous system, a network of peptidergic nerves which innervate the vasculature of a range of mammals, including man, has been shown

to exist (Furness *et al.*, 1984). A variety of small peptides, with up to about 40 amino acid residues, have been found in peripheral nerves which supply autonomically innervated organs (Hökfelt *et al.*, 1980; Furness *et al.*, 1982; Furness and Costa, 1982). Within the cardiovascular system, the distribution of the peptides appears to vary from bed to bed and from one species to another. While many of the peptides have been found in the cardiovascular system, those most commonly involved are substance P (SP), calcitonin gene-related peptide (CGRP), neuropeptide Y (NPY) and vasoactive intestinal polypeptide (VIP).

Substance P

Among the peptides listed, SP has perhaps the longest history. It was first described as a biologically active compound in crude gut extracts by von Euler and Gaddum in 1931. It was not until 1971 that Chang and co-workers isolated SP in its pure form from bovine hypothalamus and established the eleven amino acid sequence of the peptide. Shortly thereafter the peptide was synthesized successfully (Tregear *et al.*, 1971). Then, the availability of a pure and chemically defined SP made it possible to generate specific antibodies to the peptide for radioimmunoassay and immunohistochemistry.

Substance P has a widespread distribution in the central and peripheral nervous systems in a variety of species, including man (Furness *et al.*, 1984).

Extensive studies have established the presence of SP in most parts of the central nervous system of all mammals. Detailed radioimmunoassay studies have indicated that the highest concentrations are present in the mesencephalon, hypothalamus and preoptic area, while the cerebellum contains insignificant amounts of SP (Brownstein *et al.,* 1976; Kanazawa and Jessell, 1976; Gale *et al.,* 1978; Emson *et al.,* 1980; Cooper *et al.,* 1981). Immunohistochemical mapping has so far identified SP in over 30 areas of the brain including the brain stem and spinal cord. For a thorough account of studies which have been done to localize SP in the central nervous system the reader is referred to Pernow (1983).

In the periphery substance P is mainly in sensory nerves with a widespread distribution to the heart and most vascular beds. In some animals such as the guinea-pig, virtually all vascular beds are supplied with SP-containing nerve fibres. The presence of SP fibres has been demonstrated in the guinea-pig heart (Wharton *et al.,* 1981a), rat carotid sinus (Helke *et al.,* 1980), and in the arteries and veins of the tracheobronchial tissue of the guinea-pig (Sundler *et al.,* 1977). Immunohistochemical studies have also identified substance P-like immunoreactive (SPLI) nerves in the guinea-pig aorta and pulmonary artery (Reinecke *et al.,* 1980) and in the vessels of the urinary and reproductive systems (Alm *et al.,* 1978; Wharton *et al.,* 1981b). Substance P fibres have been shown to innervate the cerebral (Liu-Chen *et al.,* 1986), coronary (Brum *et al.,* 1986) and mesenteric vascular beds (Scott *et al.,* 1989).

Both vasomotor and sensory functions have been suggested to account for the presence of SP-fibres in blood vessels. In coronary, pulmonary, mesenteric, renal and cerebral blood vessels SP has been shown to cause vasodilation (Maxwell, 1968; Hallberg and Pernow, 1975; Eklund *et al.*, 1977). Vasoconstriction by SP has been reported in the hepatic portal vein of the rat (Hellstrand and Jarhult, 1980; Mastrangelo *et al.*, 1980) and the anterior mesenteric vein of the rabbit (Berube *et al.*, 1978). A sensory function for SP in vascular nerve fibres has also been suggested, since pretreatment with capsaicin eliminates SP-like immunoreactivity from these fibres (Furness *et al.*, 1982; Matthews and Cuello, 1982).

Calcitonin Gene-Related Peptide

Calcitonin gene-related peptide (CGRP) is a 37-amino acid peptide resulting from alternative processing of the RNA during expression of the calcitonin gene (Amara *et al.*, 1982; Rosenfeld *et al.*, 1983). The existence of CGRP was first predicted by analysis of a new RNA in a rat cell line. A similar peptide was subsequently isolated from human medullary carcinoma of thyroid tissue and sequenced in 1984 (Morris *et al.*, 1984). CGRP has been shown to have strong vasodilatory actions (Brain *et al.*, 1985). It exerts its vasodilatory effect both through direct influence on vascular smooth muscle and by inhibition of neurogenic vasoconstriction (Brain *et al.*, 1985; Fischer *et al.*, 1983; Hanko *et al.*, 1985).

Immunohistochemical studies have shown a widespread distribution of CGRP in sensory neurones and nerve fibres of the cardiovascular system of several species (Wharton and Gulbenkian, 1987). Many studies have been done in guinea-pig where perivascular CGRP fibres have been seen in all vascular beds. In an extensive study by Uddman and co-workers (1986), the distribution of perivascular CGRP nerve fibres in the guinea-pig was described in detail. Their findings indicate a substantial CGRP nerve supply to the heart and coronary blood vessels. The carotid arteries and thoracic aorta receive numerous CGRP fibres, while the brachial and femoral arteries to the limbs contain few fibres. In the guinea-pig brain, the cerebral arteries were shown to be surrounded by a dense network of perivascular CGRP fibres, whereas the small pial arteries were accompanied by few fibres. Vessels of the respiratory and genitourinary tracts received a moderate supply of CGRP fibres. In the gastrointestinal tract the mesenteric and gastroepiploic arteries had a dense supply of CGRP-containing nerve fibres. In contrast, small blood vessels of skeletal muscle received a very scarce supply of CGRP fibres. Studies in the rat have also indicated a widespread distribution of CGRP-fibres in the cardiovascular system (Mulderry *et al.*, 1985). There are, however, some regional and species variations regarding the distribution of CGRP-immunoreactive nerve fibres (Wharton *et al.*, 1986).

The distribution of CGRP- and SP-immunoreactive fibres is very similar and several studies have now demonstrated that they are frequently co-localized in sensory nerves (Lundberg *et al.*, 1985; Uddman *et al.*, 1986; Wanaka *et al.*, 1986).

Neuropeptide Y

Neuropeptide Y (NPY) is a 36-amino acid peptide isolated from porcine brain tissue (Tatemoto *et al.*, 1982). The peptide has been sequenced (Tatemoto, 1982) and synthesized by a solid phase method (Balasubramaniam *et al.*, 1987; Yamamoto *et al.*, 1984). NPY has been found to be rich in tyrosine and is structurally similar to pancreatic polypeptide (PP) and peptide YY (PYY; Tatemoto *et al.*, 1982). NPY is widely distributed in the central and peripheral nervous systems and has been shown to be one of the most potent vasoconstrictor peptides isolated so far (Emson and DeQuidt, 1984).

In the periphery, NPY has been localized in perivascular nerve fibres of several species, including man (Pernow *et al.*, 1987). It is often closely associated with or co-stored with noradrenaline (Ekblad *et al.*, 1984). In the rat, Allen and co-workers (1985) have identified high concentrations of NPY throughout the heart and within the major blood vessels, in particular in the renal and superior mesenteric arteries. This study showed a similar pattern of innervation by NPY- and NA-containing nerve fibres, which was depleted after treatment with 6-hydroxydopamine

(6-OHDA; Allen *et al.*, 1985). In the guinea-pig, NPY-immunoreactive nerve fibres have been found to be widely distributed in the vascular system (Uddman *et al.*, 1985). In general, NPY fibres were more numerous around arteries than in veins of the guinea-pig. The heart and coronary vessels, like the rat, were shown to have a rich NPY-innervation. A well developed NPY plexus was visualized around large elastic and muscular arteries such as the thoracic aorta and common carotid arteries. Perivascular NPY fibres were numerous in arteries of the respiratory, gastrointestinal and genitourinary tracts. Techniques of surgical and chemical sympathectomy, as well as sequential immunohistochemical staining, revealed the coexistence of NPY with NA in perivascular nerve fibres of the guinea-pig (Uddman *et al.*, 1985).

Vasoactive Intestinal Polypeptide

Vasoactive intestinal polypeptide (VIP) is a 28-amino acid peptide which was originally isolated from porcine intestine and named for its potent vasodilatory actions (Said and Mutt, 1970). VIP was first synthesized in 1973 (Bodansky *et al.*) and has since become commercially available. This peptide, which is structurally related to secretin and glucagon, was originally thought to be limited to the gastrointestinal tract. However, radioimmunoassay and immunohistochemical studies have revealed that VIP has a widespread distribution in the body, localized in neurons (Fuxe *et al.*, 1977; Larsson *et al.*, 1976).

Perivascular nerve fibres containing VIP-immunoreactivity have been found mainly in regional vascular beds rather than in the larger conducting blood vessels (Wharton and Gulbenkian, 1987). In the cat, VIP-containing nerve fibres were found to be numerous around arteries of the upper respiratory, gastrointestinal, and genitourinary tracts (Uddman *et al.*, 1981). Very few fibres were found in blood vessels of skeletal muscle and none were found in coronary arteries. VIP fibres were sparse around large arteries and veins and appeared to be absent entirely from blood vessels of the liver, spleen and kidney. In the guinea-pig, similar results have been reported (Della *et al.*, 1983). This study demonstrated a sparse supply of VIP-immunoreactive nerve fibres to the heart and major distributing arteries: aorta, subclavian, carotid, femoral and pulmonary. Of the vascular beds receiving VIP fibres, the most densely supplied arteries were the mesenteric and uterine arteries. VIP innervation of cerebral vessels varied from the most dense in the anterior and middle cerebral arteries to very little in the basilar artery. As in the cat, arteries running to skeletal muscle and major organs were less densely supplied. Throughout the body veins of the guinea-pig were sparsely supplied with VIP nerve fibres.

Evidence for the coexistence of VIP with ACh has been summarized by Lundberg (1981). In addition it has been shown that VIP-containing fibres were not depleted when noradrenergic nerves were degenerated by 6-OHDA or when SP nerves were disrupted by capsaicin (Della *et al.*, 1983). This suggests that VIP nerves

innervating the vasculature form a population distinct from substance P or sympathetic nerves.

In summary, it can be seen that the innervation of the vasculature is more complex than was commonly accepted until recently. It now appears that three types of perivascular nerve plexus exist: the catecholaminergic system which has NPY co-localized with NA in nerve terminals, a system of peptidergic nerves containing SP and CGRP, and the system of cholinergic and VIP-containing nerve fibres.

1.1.2. Circulating and Local Factors

There are many factors which contribute to the overall control of the cardiovascular system, some to a greater degree than others. In the circulation there are agents such as peptides and hormones which have a widespread influence on vascular tone. Local factors such as flow, pH, P_{O_2} and P_{CO_2} as well as the concentrations of certain ions in the blood can determine the functional state of a vascular bed.

(a) Circulating Factors which Regulate Blood Flow

There are certain naturally occurring vasoactive amines, peptides and lipids which circulate in the blood and can be grouped under the term local hormones or autacoids. Among these are acetylcholine and catecholamines which may be formed extraneuronally and released into the blood. Acetylcholine has been shown to cause relaxation of arteries, which is dependent upon the presence of intact endothelium (Furchgott *et al.*, 1981). Little effect has been observed in veins. Adrenaline, secreted from the adrenal medulla, can cause vasoconstriction by its action on α-adrenoceptors or vasodilation by an action on β-adrenoceptors of vascular smooth muscle.

Histamine is one of the most potent of the endogenous biogenic amines. Its actions on blood vessels are complicated, since it acts on two receptor subtypes and may also interact with the sympathetic nervous system (Brody, 1980). In a number of species histamine has been shown to produce vasoconstriction mediated by H_1 receptors, and vasodilation mediated by H_2 receptors (Owen, 1977). Serotonin is a vasoactive amine identified as 5-hydroxytryptamine (5-HT). In the blood, 5-HT is contained in platelets and is involved in the clotting process. It is a potent vasoconstrictor except on blood vessels of skeletal muscle and coronary vessels of the heart, which are dilated (Cohen *et al.*, 1981; Van Nueten *et al.*, 1984).

Adenosine and its phosphorylated derivatives, AMP, ADP and ATP have vasodilator activity in the vascular beds of skeletal muscle and the coronary vessels of the heart, and have thus been shown to produce a fall in blood pressure upon intravenous injection. In many blood vessels ATP and ADP have been shown to cause endothelium-dependent relaxations while relaxations to adenosine and AMP were independent of the presence of endothelial cells (Vanhoutte and Rimele, 1983; reviewed by Furchgott, 1984).

The polypeptide family of kinins includes substance P, kallidin and bradykinin, and angiotensin. Substance P has already been reviewed. The substances kallidin and bradykinin are closely related peptides of which bradykinin has been the most studied. Bradykinin has been shown to have a potent vasodilatory action (Fox *et al.*, 1961; Haddy *et al.*, 1970). This is an endothelium-dependent action which will be discussed later. Studies have demonstrated that this action is important in the maintenance of normal blood pressure (Gavras *et al.*, 1987). Kallidin is thought to have the same actions and similar potency as bradykinin. Renin is an enzyme, secreted by the kidneys, which activates the renin-angiotensin system to generate angiotensin II. While angiotensin II is a direct vasoconstrictor agent, it also elicits the release of aldosterone from the adrenal cortex. Aldosterone regulates sodium and potassium excretion by the kidneys and thus contributes to the control of arterial blood pressure (Reviewed in Bowman and Rand, 1980).

Vasopressin is a peptide hormone released from the neurohypophysis into the circulation in response to nerve impulses. It is also known as antidiuretic hormone (ADH). ADH is a potent vasoconstrictor of all types of blood vessel, especially capillaries and venules. A major role played by ADH is in the response to haemorrhage. Atrial natriuretic factor (ANF) is another peptide hormone belonging to a recently discovered group of peptides (de Bold *et al.*, 1981). ANF is released from granules in the atria of mammals and has been shown to be a potent vasodilator in pre-contracted blood vessels (Currie *et al.*, 1983).

Prostaglandins are another group of local hormones that exert an effect at or distal to the site of their synthesis (Vane and McGiff, 1975). Prostaglandins are released from a variety of tissues including blood vessels themselves (Tuvemo and Wide, 1973). Prostaglandins have a wide variety of cardiovascular actions including reduction of blood pressure, vasodilation of many vascular beds and constriction of cerebral blood vessels.

(b) Local Factors Which Regulate Blood Flow

In addition to circulating amines and peptides in the blood, there are certain local factors which may influence the circulation (Haddy and Scott, 1968). The balance between dissolved oxygen (O_2) and carbon dioxide (CO_2) can have both a direct and indirect effect on vascular tone. When levels are elevated, CO_2 acts

directly to dilate blood vessels and depress cardiac function (Price, 1960). Through reflex actions, CO_2 causes the release of adrenaline from the adrenal medulla to result in peripheral vasoconstriction and an increase in blood pressure. Similarly, studies have shown that a reduction in oxygen tension of the perfusing blood lowers the resistance to flow in the coronary, hindlimb, forelimb, intestinal, gracilis and renal vascular beds (Berne *et al.*, 1957; Daugherty *et al.*, 1967; Attinger *et al.*, 1967; Skinner and Powell, 1967).

The pH of blood is another factor involved in local regulation of flow. Although the blood is a buffered solution, metabolic changes during exercise can cause slight variations in hydrogen ion concentration. Studies have shown that local administration of acid produces changes similar to those which occur during increased CO_2 tension. In fact some studies indicate that the action of CO_2 is mediated via the hydrogen ion (Hilton and Eichholtz, 1925; McElroy *et al.*, 1958). It has thus been shown that the hydrogen ion is a vasoactive agent, with high concentrations causing dilation and low concentrations causing constriction.

The concentration of potassium ion in the blood can also influence vascular tone. It has long been known that a small increase in potassium in the blood or in a perfusing solution causes vasodilation (Dawes, 1941). It seems, however, that the effect of potassium on the tone of vascular smooth muscle depends on its concentration. Konold and co-workers (1968) found a vasodilatory response up to a potassium concentration of about 35 mM but at higher concentrations a contractile

response was obtained. Vasoconstriction was also observed upon decreasing potassium concentration below normal (2.68 mM).

The dilator response of conduit arteries to an increase in blood flow was first observed in 1933 (Schretzenmayr, A: quoted by Pohl *et al.*, 1986). More recently the vasodilator response to increased flow has been shown to be mediated by the endothelium (Pohl *et al.*, 1986; Rubanyi *et al.*, 1986). Bevan and Joyce (1988a) have shown in rabbit ear artery that increased flow can cause contraction of an intact segment with or without the presence of endothelium. In the same preparation, they have shown flow induced vasodilation after active tone had been induced by noradrenaline (Bevan and Joyce, 1988b).

Many of the factors involved in the control of vascular tone depend on or are influenced by the endothelium. While this cell layer does act as a barrier between the blood and vascular smooth muscle, it is also an active tissue involved in the synthesis and release of compounds which act to contract or dilate the blood vessel.

1.2. The Endothelium

Endothelial cells form a continuous layer a single cell thick that lines the entire circulatory system. These cells act not only as a barrier between the blood and the vascular wall, but as an active tissue receiving a variety of signals, both blood-borne and tissue-derived. Vasoactive compounds interact with endothelial cells to cause the release of prostacyclin (Crutchley *et al.*, 1983), platelet activating factor (McIntyre *et al.*, 1985), endothelium-derived vasodilators (reviewed by Furchgott, 1984) and endothelium-derived vasoconstricting factors (reviewed by Rubanyi, 1988). Thus, the endothelium plays an important functional role in the control of cardiovascular tone.

1.2.1. Receptors on the Endothelium

A variety of receptors have been characterized on the intimal endothelial surface of blood vessels by the use of pharmacological techniques and autoradiography. It is through the mediation of these receptors that many vasoactive agents act.

Several studies have shown that the presence of a functional endothelium limits the vasoconstrictor response to adrenergic agonists such as noradrenaline (Cocks and Angus, 1983; Miller and Vanhoutte, 1985; Angus *et al.*, 1986). These authors proposed that α-adrenergic agonists stimulate the release of an endothelium-derived

relaxing factor (EDRF) through an α_2-receptor located on the endothelial cell surface. The vascular endothelium has also been shown to have β-adrenoceptors, stimulation of which leads to elevation of cyclic AMP (Schafer *et al.*, 1980). These β-receptors are of the β_2 subtype and do not mediate the release of EDRF when stimulated (MacDonald *et al.*, 1987).

The release of EDRF from endothelial cells in response to ACh has been shown to be mediated by a muscarinic receptor on the endothelium (Furchgott and Zawadzki, 1980a). Furchgott and Cherry (1984) could find no evidence to indicate that this muscarinic receptor was of a novel subtype in their rabbit aortic ring preparations. On the basis of affinity for pirenzepine, a selective muscarinic antagonist for the M_1 subtype, the muscarinic receptor of the endothelium which mediates vasodilation has been classified as M_2 (Hynes *et al.*, 1986, Choo *et al.*, 1986). However, more recently it has been suggested that two subtypes of muscarinic receptor may be responsible for the release of different types of EDRF (Rubanyi *et al.*, 1987). Horst and colleagues have also suggested this possibility and have indicated that further studies with new and novel M_2 antagonists may clarify the situation (Horst *et al.*, 1987).

Receptors for substance P have also been localized on the intimal surface of blood vessels. Substance P relaxed preparations of preconstricted porcine coronary artery only when an intact endothelium was present, thus suggesting the presence of SP receptors on the endothelial cells (Gulati *et al.*, 1986). In dog carotid artery,

autoradiographic techniques have been used to demonstrate the presence of SP receptors on the endothelium (Stephenson *et al.*, 1986).

The action of bradykinin in canine and human arteries has been shown to depend on the presence of intact endothelium (Altura and Chand, 1981; Cherry *et al.*, 1982). These studies suggest that the vasodilatory effect of bradykinin in these vessel beds is the result of an interaction with some receptive substance of the endothelium to elicit the release of EDRF.

Histamine receptors have been studied in a variety of blood vessel preparations. In rat thoracic aorta, H_1-receptors were found to be responsible for mediating the release of EDRF in response to histamine (Van de Voorde and Leusen, 1983). Radioligand binding studies indicate a differential receptor population between arteries and veins. In guinea-pig aorta, H_1-receptors were found to be more concentrated on the endothelium than on the smooth muscle (Hide *et al.*, 1988). The population of H_2-receptors was found to be of higher density on the endothelium in venules of mouse diaphragm than in arterioles or capillaries (Antohe *et al.*, 1986).

Studies with ATP have indicated that subtypes of receptors for purines exist on the vascular endothelium. The P_2-receptor which responds to the purine nucleotides, ATP and ADP, has been differentiated into 2 subtypes on the basis of affinity for the antagonists, reactive blue 2 and α,β-methylene-ATP (Houston *et al.*, 1987). These experiments indicate that it is the P_{2Y} subtype which is located on the endothelium and is responsible for the release of EDRF elicited by ATP and ADP.

Similarly, Hopwood and Burnstock (1987) have found coronary vasodilation to be mediated by the P_{2Y}-purinoceptor subclass.

Although some agents act directly on the vascular smooth muscle to elicit their response, it is evident that receptors on the intimal surface of the endothelium play an active role in mediating vascular reactivity to a wide variety of vasoactive substances.

1.2.2. Endothelium-Derived Relaxing Factor

It was first discovered in 1980 that an intact endothelium was required for the relaxation of isolated arteries to occur in response to ACh (Furchgott and Zawadzki, 1980a, 1980b; Furchgott *et al.*, 1981). This finding explained why ACh, a potent vasodilator of arteries *in vivo*, often produced no relaxation or even contraction of isolated preparations of arteries *in vitro* (Furchgott, 1981; Furchgott, 1982). In those tissues which failed to respond to ACh, the endothelium was often damaged or stripped from the vessel wall during preparation. In their original report of these findings, Furchgott and Zawadzki (1980a) proposed that ACh was acting on a muscarinic receptor on the endothelium to stimulate the release of one or more factors which caused relaxation of vascular smooth muscle. The term endothelium-derived relaxing factor(s) (EDRF) was coined for this substance(s) (Cherry *et al.*, 1982).

(a) Characterization and Identification of EDRF

These original findings by Furchgott and Zawadzki prompted many investigations into the identity and mode of action of EDRF. Early studies demonstrated the lack of effect of cyclooxygenase inhibitors on the relaxing action of ACh indicating that EDRF was not a prostaglandin (Furchgott and Zawadzki, 1980a). EDRF is a labile factor with measured half lives of 6 to 49 seconds (Griffith *et al.*, 1984; Förstermann *et al.*, 1984; Rubanyi *et al.*, 1985; Angus and Cocks, 1987). Like the nitrovasodilators, such as nitroglycerin and sodium nitroprusside (SNP), EDRF has been shown to cause vasodilation by stimulation of the soluble guanylate cyclase of vascular smooth muscle, resulting in elevated cyclic GMP levels (Förstermann *et al.*, 1986; Holtzmann, 1982; Ignarro *et al.*, 1984; Rapoport and Murad, 1983a). Cyclic GMP then mediates relaxation, probably by multiple actions on the control of intracellular free calcium (Collins *et al.*, 1986). EDRF and the nitrovasodilators also inhibit platelet aggregation (Ayuma *et al.*, 1986), presumably via a similar cyclic GMP-mediated action.

In the search for the identity of EDRF, several agents and conditions which inhibit endothelium-dependent relaxations were found. It was discovered that the actions of EDRF on vascular smooth muscle are potentiated by superoxide dismutase and cytochrome C (Gryglewski *et al.*, 1986a; Rubanyi and Vanhoutte, 1986; Moncada *et al.*, 1986) and are inhibited by Fe^{2+} (Gryglewski *et al.*, 1986b), hydroquinone

(Griffith *et al.*, 1984; Moncada *et al.*, 1986) and by pyrogallol (Moncada *et al.*, 1986). This led to the suggestion that these agents inactivate EDRF via the generation of superoxide anions (Moncada *et al.*, 1986). Haemoglobin also inhibits the actions of EDRF (Griffith *et al.*, 1984; Martin *et al.*, 1985) probably through a different mechanism of action involving the binding of the molecule (Cocks and Angus, 1985; Martin *et al.*, 1986).

Finally, as the actions of EDRF were found to be quite similar to those of the nitrovasodilators, it was suggested by Furchgott (1988) that EDRF might simply be nitric oxide (NO). Nitric oxide is thought to be the active metabolite through which nitrovasodilators stimulate soluble guanylate cyclase. Evidence has been given by Palmer, Ferrige, and Moncada (1987) to show that NO released from endothelial cells is indistinguishable from EDRF in terms of biological activity and stability, as well as susceptibility to inhibition by haemoglobin and enhancement by superoxide dismutase. Since this first report, further confirmation that EDRF is NO has been provided by studies of the comparative pharmacology of the two agents (Hutchinson *et al.*, 1987; Radomski *et al.*, 1987). Mechanisms other than the release of NO may also play a role in endothelium-dependent relaxation. Mechanisms have been suggested including mediators such as ammonia (NH_3; Vanhoutte, 1987a), or the involvement of ion channels which may regulate EDRF release or propagate endothelial signals via gap junctions (Olesen *et al.*, 1988; Lansman *et al.*, 1987). An endothelium-dependent transient hyperpolarization of vascular smooth muscle

cells, which may be independent of NO, has also been suggested (Vanhoutte, 1987a). These observations must be fully investigated before it is known if non-prostanoid endothelium-dependent mediators other than NO exist.

(b) Agents that Produce Endothelium-Dependent Relaxation

Although acetylcholine was the first agent shown to elicit endothelium-dependent vasodilation, a variety of agents, both humoral and synthetic, have also been shown to cause the release of EDRF. Some of these agents also cause the release of prostaglandins from endothelial cells. However, prostaglandins do not appear to have a significant role as mediators of these relaxing effects. In addition, with some of these agents, endothelium-dependent relaxation of isolated arteries has been limited to certain species. In some cases it is limited to specific arteries in a particular species.

Acetylcholine was first shown to cause relaxations which were dependent on the presence of intact endothelium in isolated rings of rabbit thoracic aorta using isometric tension measurements (Furchgott, 1981; Furchgott and Zawadzki, 1980a, 1980b; Furchgott *et al.*, 1981). In addition to the actions of ACh on rabbit aorta, it was found that ACh caused endothelium-dependent relaxations in isolated arteries from all mammalian species. These tissues included: rabbit superior mesenteric, pulmonary, and ear arteries; rat thoracic aorta; guinea-pig thoracic aorta; cat thoracic

and abdominal aortae, superior mesenteric, pulmonary, and external iliac arteries; and dog circumflex and left anterior descending coronary arteries (Furchgott and Zawadzki, 1980a). Other investigators have found relaxations to ACh in canine renal (Chand and Altura, 1981) and femoral arteries (De Mey and Vanhoutte, 1981), rat (Davies and Williams, 1984) and pig aorta (Gordon and Martin, 1983), as well as cat cerebral arteries (Lee, 1981) and bovine coronary arteries (Holtzmann, 1982). Studies on human blood vessels have shown that ACh causes relaxations in the presence of intact endothelium in isolated renal and peripheral arterial rings (Luscher *et al.*, 1987a) and in isolated renal, splenic, gastric, pulmonary, brachial, transverse cervical and coronary arterial rings (Thom *et al.*, 1985). The endothelium-dependent vasodilatory effect of ACh is much less pronounced in isolated venous preparations (De Mey and Vanhoutte, 1982).

A synthetic compound, the calcium ionophore A23187 is even more potent than ACh in its endothelium-dependent vasodilatory actions (Zawadzki *et al.*, 1980). Against high levels of tone, the maximal relaxation by A23187 is always greater than that by ACh (Furchgott *et al.*, 1983). A23187 has been shown to cause endothelium-dependent relaxations in rabbit thoracic aorta (Furchgott, 1981; Zawadzki *et al.*, 1980; Singer and Peach, 1982), rat thoracic aorta (Rapoport and Murad, 1983b), pig aorta (Gordon and Martin, 1983) and in a variety of human arteries (Thom *et al.*, 1985; Thom *et al.*, 1987a). It is interesting to note that in the presence of a maximally effective concentration of A23187, additions of ACh and other endothelium-

dependent vasodilators produce no relaxation of contracted arteries (Furchgott *et al.*, 1983). This has been attributed to A23187 fully activating the mechanism for production and release of EDRF in endothelial cells.

Noradrenaline and selective α_2-adrenoceptor agonists can cause relaxation of canine and porcine coronary and systemic arteries and canine pulmonary arteries and veins if the endothelium is present (Cocks and Angus, 1983; Miller and Vanhoutte, 1984). In the canine coronary artery the relaxing effects of the β-adrenergic agonists, noradrenaline and isoproterenol are reduced following removal of the endothelium (Rubanyi and Vanhoutte, 1985a). It is not certain whether this greater effect is due to an action on β-receptors on the endothelium or if relaxation is caused by the basal release of EDRF. The presence of α_2-inhibitory receptors on the endothelium does help to explain why the contractile response to nonselective α-adrenoceptor agonists is enhanced upon removal of the endothelium.

The purines ATP and ADP, which are released by platelets during aggregation, also induce endothelium-dependent relaxations in arteries. Removal of endothelial cells was shown to markedly reduce the graded concentration-dependent relaxations to ATP or ADP in isolated preparations of rabbit aorta (Furchgott, 1981; Furchgott *et al.*, 1983; Furchgott and Zawadzki, 1980c), dog femoral artery (De Mey and Vanhoutte, 1981) and pig aorta (Gordon and Martin, 1983). Vasodilation by adenosine and AMP in rabbit aorta and dog femoral artery were shown to be independent of the presence of endothelial cells (De Mey and Vanhoutte, 1981;

Furchgott *et al.*, 1983; Furchgott and Zawadzki, 1980c). However, in pig aorta a significant part of the relaxation by adenosine and AMP has been reported to be endothelium-dependent (Gordon and Martin, 1983). Relaxations to adenosine in canine coronary artery are also mediated by the endothelium (Rubanyi and Vanhoutte, 1985a).

Substance P is one of the most potent endothelium-dependent vasodilators studied (Zawadzki *et al.*, 1981). SP has been shown, using an electromagnetic flowmeter technique, to increase blood flow in the aorta, carotid, hepatic, superior mesenteric, and femoral arteries of the dog (Hallberg and Pernow, 1975). Subsequently SP has been shown to give endothelium-dependent relaxation in isolated rings of rabbit aorta and dog superior mesenteric arteries which had been preconstricted with noradrenaline (Furchgott *et al.*, 1983). The threshold concentration for relaxation by SP was 10 pM in rabbit aorta and 1 pM in dog superior mesenteric artery. In these experiments a desensitization to SP at 1 nM occurred within 10 minutes. This was overcome by washout of the preparation and did not have any effect on relaxation to ACh or A23187. Endothelium-dependent vasodilation by SP has also been studied in isolated dog (D'Orleans-Juste *et al.*, 1985) and pig (Gulati *et al.*, 1987) coronary arteries. In the dog, SP relaxed isolated coronary arterial rings which had been preconstricted with noradrenaline (20 nM) at a threshold concentration of 65 pM. In isolated rings of pig coronary artery; which, like human coronary artery, contracts to acetylcholine (50 μM), the threshold

for endothelium-dependent relaxation to SP was 30 pM. Substance P has also been shown to cause vasodilation upon intravenous administration in the preconstricted pulmonary vasculature of the dog (Archer *et al.*, 1986). In humans, SP has been shown to increase blood flow in the skin and skeletal muscle of the forearm upon close arterial infusion of 1-2 ng/minute (Eklund *et al.*, 1977). Experiments on isolated branches of human mesenteric arteries have shown that relaxations by SP are strictly dependent on the presence of endothelial cells (Furchgott *et al.*, 1983).

Bradykinin is also known as a potent vasodilator. Bradykinin has been shown to produce concentration-dependent relaxation of canine intrapulmonary and renal arteries which is dependent on the presence of an intact endothelium (Altura and Chand, 1981). Removal of the endothelium resulted in either a contractile response to bradykinin or no response at all. In isolated rings of dog carotid arteries preconstricted with noradrenaline (20 nM), bradykinin elicits concentration-dependent relaxations at a threshold concentration of 2 nM (D'Orleans-Juste *et al.*, 1985). These relaxations occurred only in the presence of an intact endothelium. The lack of inhibition of this vasodilatory response to bradykinin by cyclooxygenase and lipoxygenase inhibitors indicated that it was not mediated by prostaglandins. An extensive study by Cherry and co-workers (1982) evaluated the actions of bradykinin in isolated arterial rings from cats, dogs and rabbits. In the superior mesenteric and celiac arteries from rabbit and cat removal of endothelial cells did not consistently result in loss of the relaxations to bradykinin. This relaxation was blocked by

cyclooxygenase inhibitors and thus in these vessels the action of bradykinin appears to be mediated by prostaglandins. In contrast, all arteries tested from the dog: splenic, gastric, celiac, femoral, renal, coronary, pulmonary, and superior mesenteric, showed a requirement for endothelial cells in the relaxation by bradykinin. Cyclooxygenase inhibitors did not interfere with the relaxation response and so bradykinin is thought to act via EDRF in blood vessels of the dog. Bradykinin was also studied on rings of mesenteric artery from humans and found to elicit relaxations with similar mechanism to those in dog arteries. In addition, Gordon and Martin (1983) have reported that bradykinin induces relaxations of pig aorta which are also endothelium-dependent and prostaglandin-independent.

Vasoactive intestinal polypeptide is a potent vasodilator which acts by different mechanisms in a variety of species. In rat aorta VIP caused relaxations which were endothelium-dependent (Davies and Williams, 1984). In a study of human blood vessels, VIP produced endothelium-dependent relaxation in coeliac branch arteries, but in isolated pulmonary arteries its relaxing effect was independent of the presence of endothelial cells (Thom *et al.*, 1987a). Another group has also reported an endothelium-independent mechanism for relaxation of human pulmonary arteries by VIP (Barnes *et al.*, 1986). VIP has also been shown to relax human gastric and transverse cervical arteries in an endothelium-dependent matter (Thom *et al.*, 1987a). The vasodilation of bovine pulmonary artery and canine coronary artery has also been reported to be independent of the presence of endothelial cells (Griffith *et al.*, 1985).

Calcitonin gene-related peptide is another peptide which has vasodilator activity in man and other mammals. In a strip preparation of rat thoracic aorta CGRP gave dose-dependent relaxations of noradrenaline-induced tone which were dependent on the presence of endothelial cells (Brain *et al.*, 1985). This relaxation was partially suppressed by cyclooxygenase inhibitors and so prostaglandins may be responsible for some of the response to CGRP. In this study CGRP also caused vasodilation of the cutaneous microvasculature of rabbit and man in *vitro*. CGRP was 1000 times more potent than ACh, ATP, ADP, adenosine, 5-HT and SP in this action. Another study has confirmed endothelium-dependent relaxations to CGRP in rings of rat thoracic aorta (Kubota *et al.*, 1985). Recently CGRP has been shown to relax preconstricted segments of human radial, coronary, gastric and cerebral arteries in an endothelium-dependent manner (Thom *et al.*, 1987b). Cyclooxygenase inhibitors did not block the responses to CGRP and thus EDRF is thought to be the mediator of these relaxations.

Histamine is a vasodilator which has been shown to act via EDRF in certain isolated blood vessel preparations. It was first demonstrated to cause a dose-dependent relaxation in rings of rat thoracic aorta, precontracted with noradrenaline (Van de Voorde and Leusen, 1983). This relaxation to histamine (10-1000 μM) was dependent on the presence of an intact endothelium and was not mediated by prostaglandins. The use of specific histamine receptor antagonists indicated that histamine was acting on an H_1 receptor of the endothelium. Rapoport and Murad

(1983) have reported similar results for histamine in rat aorta. Histamine also produces endothelium-dependent relaxation of guinea-pig pulmonary artery (Satoh and Inui, 1984). This response is thought to involve the H_1 receptor on the endothelium in this tissue as well. It is also of interest to note that histamine causes the release of EDRF from human umbilical blood vessels via an H_1 receptor subtype (Van de Voorde *et al.*, 1987). This is a tissue which does not release EDRF in response to ACh.

Thrombin, which is generated during the cascade reactions of coagulation, causes endothelium-dependent relaxation in basilar, coronary, femoral, saphenous, splenic, and pulmonary arteries of the dog (De Mey *et al.*, 1982; De Mey and Vanhoutte, 1982; Katusic *et al.*, 1984) and in aorta of the rat (Rapoport *et al.*, 1984). Relaxation was not inhibited by inhibitors of cyclooxygenase and prostacyclin synthetase, but was inhibited by heparin. Similar findings have been made on thrombin-induced endothelium-dependent relaxation of isolated canine coronary arteries (Ku, 1982). In these arteries, the relaxation to each addition of thrombin was transient, and was counteracted by a direct contractile action on the smooth muscle.

Platelet activating factor (PAF) is released during platelet aggregation and has been shown to cause vasodilation in a number of animal models (Vargaftig and Benveniste, 1983). At very high concentrations, PAF induces endothelium-dependent

relaxations in isolated canine coronary and femoral arteries and rat aortae (Kamitani *et al.*, 1984; Kasuya *et al.*, 1984).

Serotonin is also released during platelet aggregation. Precontracted canine coronary arteries have been found to relax to serotonin only if endothelial cells are present (Cohen *et al.*, 1983). In the absence of endothelium, serotonin produces only a contraction in the canine coronary arterial rings. Endothelium-dependent relaxation by serotonin has not been reported for any other arteries.

Hydralazine is an antihypertensive drug which produces its relaxant effect on isolated rings of rabbit aorta mainly through an endothelial cell-dependent mechanism (Spokas *et al.*, 1983). This effect occurs at relatively low concentrations of 0.1-1 μM. This is of interest because most other antihypertensive drugs; nitroglycerin, nitroprusside, minoxidil and diazoxide, are thought to act directly on the vascular smooth muscle, independent of the presence of endothelial cells.

Endothelium-derived relaxing factor is also released from arteries in response to increments in flow (Holtz *et al.*, 1984a). Effluent from perfused rabbit aorta (Griffith *et al.*, 1984), or canine femoral artery preparations with endothelium (Rubanyi *et al.*, 1986), in response to increased flow, caused relaxations of bioassay coronary arteries without endothelium. Increments in flow through arteries *in vivo* (Gerova *et al.*, 1983; Hintz and Vatner, 1983; Holtz *et al.*, 1984a; Kaiser *et al.*, 1985; Rubanyi *et al.*, 1986) or *in vitro* (Holtz *et al.*, 1984b) induce vasodilation, which cannot be prevented by cyclooxygenase inhibitors (Holtz *et al.*, 1984a), but can be abolished

by removal of the endothelium (Holtz *et al.*, 1984b) or depressed by methylene blue (Kaiser *et al.*, 1985). Thus, the EDRF released by large arteries in response to flow has characteristics very similar to that released by ACh.

It is evident that a wide range of agents cause the release of a relaxing substance from the endothelium. Some of these agents are found in perivascular nerve fibres and some are endogenous to the circulation under various conditions, while some compounds which elicit the release of EDRF are synthetic. It seems that even the flow rate of blood through the vasculature is involved in the control of smooth muscle tone through the mediation of the endothelium. Whether all these agents release nitric oxide or other types of a non-prostanoid EDRF is not known. However, based on common susceptibilities to inhibitory agents it is likely that many of these agents act to release the same relaxing factor that ACh produces (Furchgott *et al.*, 1983; Furchgott, 1984).

1.2.3. Endothelium-Derived Contracting Factor

In addition to the vasodilator substances which are released by the endothelium, evidence has been given for the release of one or more vasocontractile factors from vascular endothelial cells. It was first reported in 1982 that an intact endothelium was necessary for contraction of canine systemic and pulmonary veins in response to arachidonic acid (De Mey and Vanhoutte, 1982). Vasoconstriction,

dependent on or enhanced by an intact endothelium, has been observed in response to various chemical and physical stimuli (reviewed by Vanhoutte, 1987b; Vanhoutte and Katusic, 1988).

(a) Characterization and Identification of EDCF

Part of the evidence supporting the release of vasoconstrictor substances from the endothelium comes from experiments in which anoxia produced endothelium-dependent contractions of peripheral, coronary, and cerebral arteries (Rubanyi and Vanhoutte, 1985b; De Mey and Vanhoutte, 1983; Katusic and Vanhoutte, 1986). These responses were not sensitive to inhibition of cyclooxygenase and thus were attributed to an unidentified vasoconstrictor substance termed $EDCF_1$ (Vanhoutte 1987b). It has also been demonstrated that cultured endothelial cells produce a vasoconstrictor peptide (Hickey *et al.*, 1985; Gillespie *et al.*, 1986), which has recently been characterized and named endothelin (Yanagisawa *et al.*, 1988). Endothelin has, upon further study, been shown to consist of a group of three structurally distinct isopeptides which have different pharmacological profiles (Inoue *et al.*, 1989). Since the production of endothelin and its contractile effect do not depend on cyclo-oxygenase activity, it has been proposed that $EDCF_1$ may be endothelin. There is, however, evidence to indicate that endothelin is unlikely to be the mediator of the so-called hypoxic response of vascular smooth muscle (Vanhoutte and Katusic, 1988).

Another series of experiments has led to the discovery of endothelium-dependent contractions that are sensitive to inhibitors of cyclooxygenase. These studies began with the findings of De Mey and Vanhoutte (1982) that arachidonic acid caused an endothelium-dependent augmentation of noradrenaline-induced contraction in system and pulmonary veins of the dog. Further studies revealed endothelium-dependent contractions in canine veins and cerebral arteries and in the aorta of spontaneously hypertensive rats to a variety of stimuli, including arachidonic acid, acetylcholine, A23187, serotonin and sudden stretch (Miller and Vanhoutte, 1985a; Luscher and Vanhoutte, 1986a; Luscher and Vanhoutte, 1986b; Katusic *et al.*, 1987; Katusic *et al.*, 1988). The mediator of these endothelium-dependent contractions was suggested to be an unidentified product of the cyclooxygenase pathway, possible thromboxane A_2, and was tentatively referred to as $EDCF_2$ (Vanhoutte, 1987b). Vanhoutte and Katusic have noted that superoxide anion causes contractions in canine basilar artery and that superoxide dismutase plus catalase abolish endothelium-dependent contractions to A23187 in the same preparation (Vanhoutte and Katusic, 1988). On the basis of this and other studies with cyclooxygenase (Kontos *et al.*, 1985; Rosenblum, 1982) it has been suggested that $EDCF_2$ is the superoxide anion (Vanhoutte and Katusic, 1988).

Thus, it can be seen that the endothelium can be responsible for mediating both vasodilation through the release of nitric oxide and possible other dilator substances, and vasoconstriction through the release of endothelin peptides,

thromboxane A_2, superoxide anion and possible additional vasoconstrictor substances. The physiological significance of these processes is beginning to be understood and the endothelium is now recognized as an important contributor to the control of vascular tone.

This review has attempted to encompass the wide number of components involved in the control of the cardiovascular system. From the direct control of vascular and cardiac muscle provided by the autonomic nervous system, to the influence of factors in the circulation, the rate of flow of blood depends on the end result of a balance between vascular contraction and relaxation. Although much progress has been made toward an understanding of the mechanisms involved in this control, much remains to be studied with respect to cardiovascular control in disease states such as atherosclerosis, diabetes and hypertension.

1.3. Objectives of the Study and Review of Methods

The objective of this study was to test the hypothesis that the ability of vascular endothelium to release EDRF in response to stimulation by vasoactive agents is related in some way to the pattern of perivascular innervation. From this work it is hoped to determine what influence, if any, the perivascular innervation of a vascular resistance bed has upon the ability of endothelial cells to cause relaxation of medial smooth muscle. In disease states such as hypertension and diabetes, changes occur in the structure and function of the vasculature (Scott and Pang, 1983; Brody *et al.*, 1980; Gabbay, 1971; McMillan, 1975). Information about the mechanisms involved in the control of the vasculature may be utilized in the development of pharmacological agents used to treat cardiovascular disease.

1.3.1. The McGregor Preparation

The tissue chosen for this study was the McGregor preparation of the isolated perfused mesenteric arterial bed of the rat (McGregor, 1965). There are several reasons for this choice. First, an isolated tissue was chosen so that cardiovascular reflex mechanisms did not contribute to the measured result. Some whole animal preparations have been used to investigate the effects of vasoactive agents on blood flow of vascular beds, but these results can be complicated by regulatory mechanisms

in situ (Hallberg and Pernow, 1975; Smiesko *et al.*, 1985; Angus *et al.*, 1983; Archer *et al.*, 1986). Second, an intact arterial resistance bed was studied in order to simulate the *in vivo* situation in the determination of the effect of the vasoactive agents used. While there are problems inherent in the setting up and maintaining of an isolated vascular bed, this tissue is a comparatively simple preparation of perfused mesenteric blood vessels. The majority of the studies of endothelium-dependent relaxation have been carried out on isolated ring or strip preparations of large conduit arteries (Furchgott and Zawadzki, 1980a; reviewed by Furchgott, 1984). Although these preparations are easily set up and respond well to vasoactive agents, the entire tissue is bathed in the administered drug solutions. In the isolated perfused mesenteric arterial bed, only the intimal surface of the blood vessel is exposed to administered agents. In addition, the role of the conduit artery in the contribution to blood pressure is thought to be less important than that of the smaller resistance vessels of the vascular beds (Prewitt *et al.*, 1987; Longhurst *et al.*, 1986). Ring and helical strip preparations of resistance blood vessels, including superior mesenteric artery, have also been studied (Toda, 1974; Furchgott and Zawadzki, 1980a; reviewed by Furchgott, 1984). However, the smaller diameter of these vessels makes it difficult to avoid endothelial damage during preparation. These tissues are also completely bathed in the agent being tested.

The choice of the mesenteric bed of the rat was based on the body of anatomical and histological information already available in this laboratory (Scott,

Robinson and Foote, 1986, 1989). There is also extensive information in the literature about nutrition, endocrinology, metabolism, and physiology of the rat which, along with its size, make it a useful animal for cardiovascular research (reviewed by Gill *et al.*, 1989).

The McGregor preparation of the isolated perfused mesenteric arterial bed is, therefore, a suitable tissue in which to study the action of vasoactive agents on the endothelium in a vascular resistance bed. However, in an isolated vascular bed such as this, there are many components which react to vasoactive substances to contribute to the overall measured changes in perfusion pressure. It is possible that in pharmacological experiments with this tissue the variation in measurements may be high.

1.3.2. Immunohistochemistry

The perivascular innervation of the mesenteric arteries was visualized using immunohistochemical staining to verify the presence or absence of nerve fibres containing SP or TH. The method of immunohistochemical staining was a three step peroxidase anti-peroxidase (PAP) procedure which utilizes diaminobenzidine to produce a colored reaction product which can be viewed at a later date with light microscopy. This method has been routinely used in this laboratory (Scott, Robinson, and Foote, 1987) and is quite specific for the neuropeptides being visualized.

Although primary and linking antibodies are readily available for this method of staining, these materials can be expensive. It was also possible to do immunohistochemical staining using primary antibodies which are linked to a fluorescent marker. This method is also specific for the neuropeptides but is more expensive and the tissues must be viewed immediately as the fluorescence may fade.

1.3.3. Electron Microscopy

Transmission electron microscopy was utilized to check the integrity of the endothelial lining of the blood vessels of the tissue preparations. This type of electron microscopy allowed us to visualize a cross section of even the smallest of the jejunal arteries. While each 1 μm section is only a very small sample of the vessel bed, a large number of samples were taken randomly. In this way it was possible to determine if endothelial cells were being removed from any vessels in the preparation. There are other methods for viewing the endothelial surface of blood vessels. Scanning electron microscopy can also be used to check the endothelium. As well, a method of *en face* silver staining allows visualization of the endothelium by light microscopy (Poole *et al.*, 1958). Neither of these methods is suitable for the small jejunal vessels of the mesenteric bed as the vessels must be cut lengthwise to visualize the intimal surface.

1.3.4. Methods of Chemical Denervation

6-Hydroxydopamine

Development of the sympathetic nervous system was prevented in a group of rats for this study by neonatal administration of 6-hydroxydopamine (6-OHDA), an isomer of noradrenaline. This treatment was used to provide an experimental model of blood vessels with an altered sympathetic nerve fibre plexus.

In 1959, while studying the enzymatic conversions of dopamine to noradrenaline in rat tissue homogenates, Senoh and co-workers discovered and isolated the metabolite, 6-OHDA. The initial biological effects of 6-OHDA were reported shortly thereafter (Porter *et al.*, 1963; Porter *et al.*, 1965; Stone *et al.*, 1963; Stone *et al.*, 1964). These experiments demonstrated that 6-OHDA produced depletion of noradrenaline in the hearts of mice and dogs and that this effect lasted longer than that caused by other agents. It was also found that certain other agents could antagonize the effect of 6-OHDA, indicating that uptake into the noradrenergic neuron was necessary for this action of 6-OHDA. Next came the discovery that 6-OHDA caused an actual destruction of the terminal endings of the sympathetic neurons (Tranzer and Thoenen, 1967, quoted by Kostrzewa and Jacobowitz, 1974). This result was later defined as "chemical sympathectomy" (Tranzer and Thoenen, 1968). Since these original findings, a vast amount of information has been

accumulated on 6-OHDA and its pharmacology. For detailed reviews the reader is referred to Thoenen and Tranzer (1973) as well as Kostrzewa and Jacobowitz (1974).

The sequence of events which lead to the destruction of noradrenergic fibres after administration of 6-OHDA has been well described, beginning with the active uptake into sympathetic neurons. 6-OHDA is actively transported to intraneuronal sites where it acts as a false transmitter, displacing noradrenaline (Thoenen and Tranzer, 1968). When critical intraneuronal concentrations of 6-OHDA or metabolites are attained, destructive processes begin and cellular enzymes and elements of the respiratory electron transport chain are destroyed. It is at this point that the sympathetic nerve terminals lose their ability to conduct action potentials but may still have a relatively intact monoamine uptake mechanism (Haeusler, 1971). Because of the internal destruction, noradrenaline is released into the synapse, resulting in a host of sympathomimetic effects (Stone *et al.*, 1964). After a period of time, the nerve terminals may be completely destroyed and there will be a marked reduction of noradrenaline, tyrosine hydroxylase activity, and monoamine uptake capacity in these tissues (Iversen and Uretsky, 1970).

The action of 6-OHDA is selective for terminal fibres of noradrenergic sympathetic nerves. Electron microscopic studies have indicated that cholinergic and noradrenergic neurons, myelinated axons, smooth muscle cells, Schwann cells and endothelial cells appear normal while noradrenergic neurons are in the process of

degeneration (Bloom *et al.*, 1969; Tranzer and Thoenen, 1967, 1968). However, it was discovered that after a period of weeks the adrenergic terminal plexus appeared to regenerate in tissues in which the NA content had been markedly reduced by 6-OHDA treatment (Thoenen and Tranzer, 1968). Thus, while destruction of noradrenergic terminals had been achieved, the perikarya appeared intact to bring about the regeneration of fibres. This reversibility was found to be related to the age of the animals at the time of 6-OHDA treatment. When 6-OHDA is administered to newborn animals its neurotoxic effects are accentuated. Angeletti and Levi-Montaïcini (1970) found that when 6-OHDA was given to newborn mice or rats it caused extensive lesions, resulting in the destruction of the sympathetic perikarya in the superior cervical, stellate, celiac, mesenteric ganglia and all of the thoracic paravertebral chain. Thus the distinction was made between the "reversible chemical sympathectomy" in adult animals and the "irreversible chemical sympathectomy" in newborn animals after treatment with 6-OHDA (Thoenen, 1971).

While 6-OHDA has proven to be a valuable pharmacological tool, there has been some controversy as to its usefulness in cardiovascular research. Some studies indicate that 6-OHDA does not produce a complete sympathectomy of the vasculature (Berkowitz *et al.*, 1972; Finch *et al.*, 1973a, 1973b; Kurnjek *et al.*, 1984). However, the degree of chemical sympathectomy attained depends upon a variety of factors. While the age at which treatment is initiated is clearly important, the dose of 6-OHDA, its route and frequency of administration, as well as the tissue

being examined are all important factors which contribute to the effectiveness of 6-OHDA treatment.

Capsaicin

Capsaicin treatment was used in this study to destroy peptidergic peripheral innervation containing the peptides SP and CGRP. This treatment produced a group of rats with an altered peptidergic nerve fibre plexus.

Capsaicin is the trivial name for 8-methyl-N-vanillyl-6-nonenamide, the major pungent component of hot peppers of the genus *Capsicum*. Hot peppers have been used as food additives and preservatives, as medicines and in social rituals for centuries. In the late 1940's an extensive characterization of the pharmacological effects of capsaicin and its congeners on sensory processes in mammals was undertaken by Jancso and colleagues in Hungary. These studies revealed that most of the biological effects of capsaicin result from an initial intense excitation of certain sensory neurons, followed by a prolonged period of insensitivity to physicochemical stimuli. For reviews of this early research on capsaicin the reader is referred to Jancso (1968), Virus and Gebhart (1979) and Szolcsanyi (1982).

The observation that capsaicin depletes fluoride-resistant acid phosphatase activity, known to be associated with central terminals of some primary afferent neurons from the dorsal horn of the spinal cord, suggested that capsaicin acts on

the biochemical processes of these sensory neurons (Jancso and Knyihar, 1975). This had been suggested earlier by Gasparovic and co-workers (1964) who discovered that capsaicin treatment reduced the amount of bioassayable SP in the spinal cord but not in the brain. Further confirmation of the specificity of the effects of capsaicin for SP-containing primary afferent neurons came from the results of Jessel and co-workers (1978). They observed that systemic treatment of adult rats with a cumulative dose of capsaicin depleted SP from the dorsal horn of the spinal cord without a general effect on spinal neurons and without destruction of primary afferent neuron terminals in the cord. Numerous studies have confirmed by immunohistochemical or radioimmunoassay techniques, the SP depleting actions of capsaicin in laboratory animals. In particular, a study by Furness and co-workers (1982) found that capsaicin markedly depleted SP from nearly every vascular bed of the guinea-pig. The exception to this was a group of SP-containing fibres on arteries to the distal colon and rectum. These investigators also confirmed the depletion of SP in the ureter, atrium and superior mesenteric artery of capsaicin-treated animals (Murphy *et al.*, 1982). These results indicate that the neurochemical effects of capsaicin are specific for primary afferent neurons.

Since the original research on the denervation of SP-containing sensory nerves with capsaicin, further studies using anatomical, histochemical and immunological methods have revealed the presence of a variety of peptides in afferent neurons (reviewed by Holzer, 1988). Functional evidence indicates a possible mediator or

transmitter role for some of these substances. CGRP is one such peptide which has been shown to coexist with SP in primary afferent capsaicin-sensitive fibres (Lundberg *et al.*, 1985; Skofitsch and Jacobowitz, 1985). Another peptide, VIP has also been localized in sensory afferent fibres but is not generally associated with SP. Usually VIP is found in ACh-containing nerve fibres with the exception of the cat, where evidence has been found for its coexistence with SP (Leah *et al.*, 1985).

Most studies with capsaicin have been carried out on small rodents such as rats, mice and guinea pigs. At low doses, in the μg/kg range, capsaicin causes a powerful excitatory effect which is confined to small unmyelinated afferent nerve fibres or C-fibres (Kenins, 1982; Buck and Burks, 1986; Szolcsanyi, 1977). This excitation is followed by a desensitization to capsaicin and blockade of impulse conduction. Systemic administration of high doses, in the mg/kg range, has a neurotoxic effect on sensory neurons. The extent of this toxicity depends upon the dosage, route of administration, species and age of the animals. The most extensive and consistent lesions are produced by systemic treatment of newborn animals. A single dose of 50 mg/kg results in permanent degeneration of 50% - 90% of all unmyelinated afferent fibres in newborn rats with no significant change in the myelinated afferents (Jancso *et al.*, 1977; Nagy *et al.*, 1981, 1983). Specifically, in guinea pigs capsaicin causes a marked depletion of SP and CGRP from cardiac SP- and CGRP-containing neurons, while cardiac adrenergic and VIP-containing neurons remain intact (Della *et al.*, 1983; Lundberg *et al.*, 1985; Papka *et al.*, 1981; Saito *et*

al., 1986). At doses higher than 50 mg/kg a reduction in the number of small myelinated fibres is also seen (Nagy *et al.*, 1983). Administration of 50 mg/kg capsaicin to adult rats results in less pronounced effects with no degeneration or partial degeneration of unmyelinated sensory afferents (Jancso, 1981; Jancso *et al.*, 1985). In the guinea pig, which is more sensitive to capsaicin than the rat, the same dose causes rapid degeneration of apparently unmyelinated fibres (Papka *et al.*, 1984).

It has been summarized that there are three major actions of capsaicin on primary afferent neurons (Buck and Burks, 1983, 1986). The first effect is an excitation which probably occurs when capsaicin, a lipophilic molecule, dissolves in the plasma membrane of the free ending of the nerve fibre. Capsaicin is presumed to affect the fluidity and possibly the ion permeability of the plasma membrane. These changes result in the flow of Ca^{2+} and other ions across the membrane as well as the release of SP. The second effect is a desensitization of the primary sensory afferent neurons to the peripheral stimuli. It is thought that capsaicin results in a long lasting plasma membrane perturbation that locks the membrane in a depolarized state. It is this inability for further depolarizations to be initiated or propagated that results in the sensory deficit which has been observed. Finally, capsaicin produces a depletion of most of the SP contained in the primary afferent neurons. This is caused in part by the release of SP that occurs during the initial membrane excitation. However, the neuron is also unable to replenish its peripheral and central SP stores because capsaicin has a disruptive effect on the intracellular organelles including the

microtubule system. Any remaining vesicular SP may also be lost due to capsaicin-induced disruption of the storage vesicles. It is not clear if capsaicin produces all these effects by more than one mechanism of action. It has been suggested from structure-activity studies that the initial excitation and SP release in primary afferents are one action and that the subsequent desensitization and sensory deficits produced by capsaicin are a separate action. There is as yet little data to support this idea.

Capsaicin was chosen as a tool in this study for depletion of the SP-CGRP afferent nerve fibre plexus in the mesenteric arterial bed of the rat. It is a useful agent because of its specificity of action and long lasting effect when given in a 50 mg/kg dose to newborn rats at 2 days old. However, although many studies have measured the effect of capsaicin treatment on nerve fibres, very little is known about its effect on other tissues such as endothelium and vascular smooth muscle. Studies on the acute effects of capsaicin on blood vessels have indicated that it can have two different effects. Capsaicin, when administered *in vitro*, causes a vasodilation which is independent of the presence of the endothelium but does not occur in vessels from animals which have been treated with capsaicin *in vivo* to remove sensory afferent innervation (Duckles, 1986; Donnerer and Lembeck, 1982, 1983). Thus, this vasodilatory effect is thought to be mediated by an unknown transmitter released from sensory afferents by capsaicin. In systemically capsaicin-treated animals a contractile response to capsaicin applied *in vitro* is observed. This is thought to be a direct excitatory action on vascular smooth muscle. The chronic effects of

capsaicin administration on vascular smooth muscle and endothelium are unknown. In addition, the effects of the vehicle treatment on vascular tissues have not been described. The most common vehicle used for capsaicin injection, and also used in this study, is a mixture of 10% ethanol and 10% Tween 80 in isotonic saline (Jancso *et al.*, 1977). However, in many studies the effects of capsaicin treatment are compared with a vehicle-treated control only. An untreated animal is rarely used as a control for the vehicle treatment. Since the effects of this vehicle treatment are unknown, it is suspect and must be considered to have potential effects of its own. Capsaicin was therefore used in this study, along with a vehicle and an untreated control, to cause permanent degeneration of the primary sensory afferent nerve fibre plexus in the mesenteric arterial bed of the rat. As with many pharmacological agents, the effectiveness of treatment is known to vary with the age of the animal at treatment, the dose administered and the route of administration.

MATERIALS AND METHODS

In this study, dose response relationships were determined for substance P and acetylcholine in the presence of methoxamine in the isolated perfused mesenteric arterial bed of the rat. These experiments were carried out on tissues from untreated and vehicle-treated control animals as well as groups of animals which had been treated with 6-hydroxydopamine or capsaicin to provide a chemically denervated model.

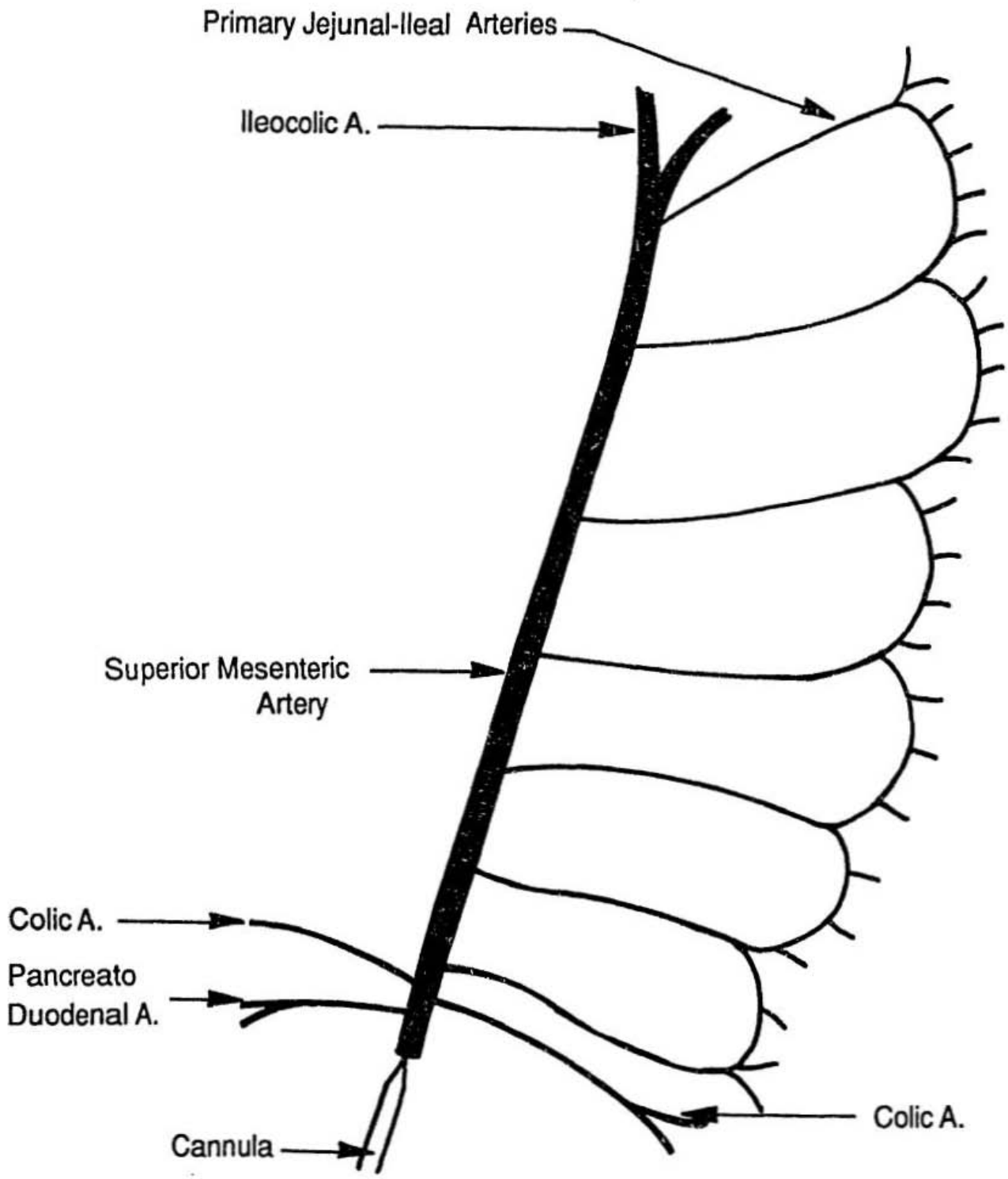
2.1. Tissue Preparation

The tissue chosen for this study was the McGregor preparation (McGregor, 1965) of the isolated perfused mesenteric arterial bed of the rat with some modifications. Adult male Sprague-Dawley rats (12 weeks, Charles River Canada Inc.) were anaesthetized with sodium pentobarbital (Somnotol, M.T.C. Pharmaceuticals) 35 mg/kg intraperitoneal. A longitudinal midline incision was made through the abdominal wall and the mesenteric bed was spread onto a gauze square dampened with buffer solution. The superior mesenteric artery was dissociated from the surrounding tissues and ligated proximal to the abdominal aorta. A cannula of PE-90 polyethylene tubing, (Intramedic, Becton Dickinson & Co.) which had been heated with a flame and drawn out to a tapered end, was inserted distally into the

superior mesenteric artery above the ligation and secured with 5-0 silk sutures (Ethicon). At this point the superior mesenteric vein was cut just proximal to the portal vein and the bed was flushed gently to prevent clotting.

Then the colic and ileo-colic branches of the superior mesenteric artery were quickly ligated (Figure 1).

Figure 1. The mesenteric arterial bed of the rat.



In addition, all primary jejunal arteries except the four most distal to the cannula were tied off in groups and the mesenteric vein was tied off with the pancreato-duodenal vessels. The tissue was removed from the animal by severing the vascular pedicle.

Finally, the intestine was separated from the mesentery by cutting close to the intestinal border, leaving only the cut ends of the tiny vessels on the intestinal margin of the mesentery for the perfusate to flow from.

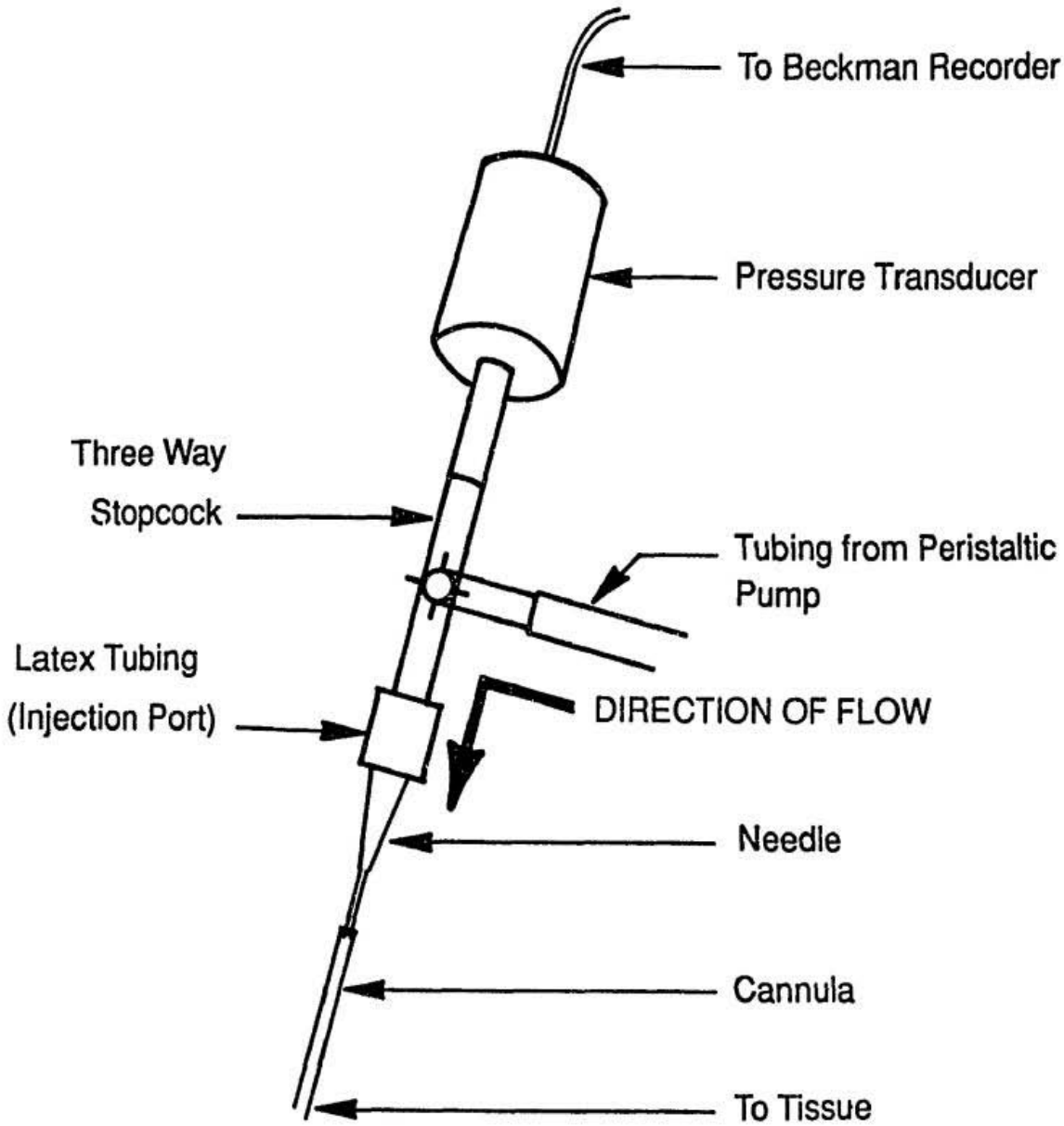
The tissue was suspended in a jacketed glass bath at 37°C and perfused at a constant rate of 2 mL/minute with a Krebs bicarbonate buffer (Hynes and Duckles, 1987).

The composition of the buffer solution was as follows (millimolar): Na^+, 147.6; K^+, 6.4; Ca^{2+}, 1.6; Mg^{2+}, 1.2; Cl^-, 130; HCO_3^-, 26; SO_4^{2-}, 1.2; $H_2PO_4^-$, 1.2; glucose, 11. Disodium ethylenediamine tetraacetate (EDTA) was not used. The buffer was incubated at 37°C in a thermostated water bath and was aerated with a gas mixture of 95% O_2/5% CO_2. Pressure measurements were made using a Gould Statham P23Dc pressure transducer and a Beckman Type R411 Dynograph recorder.

2.2. Apparatus

In these experiments perfusion pressure was measured from the isolated mesenteric arterial bed. A schematic diagram (Figure 2) is presented to illustrate the apparatus used.

Figure 2. A schematic diagram to illustrate the perfusion apparatus.



During the dissection the tissue was attached to a 10 mL syringe (Luer Lok, Becton Dickinson & Co.) with a 20 gauge 1 inch needle (Precision Glide, Becton Dickinson & Co.) which had been filed down and inserted into the blunt end of the polyethylene cannula. This syringe was filled with warmed oxygenated buffer to flush the tissue during the dissection. When isolated from the animal, the tissue was attached to the perfusion and detection apparatus by inserting the fitting of the needle into a 2 cm piece of latex tubing (4.8 x 2.4 mm, Scimatco, Fisher Scientific) which was connected to one port of a three way stopcock (Monoject). The second port of the stopcock was attached to a Gould Statham P23Dc pressure transducer and a Beckman Type R411 Dynograph recorder for the measurement of perfusion pressure. The third port of the stopcock was attached with silicon rubber tubing (2.9 mm I.D., 4.9 mm O.D., LKB) to an LKB 2132 microperpex peristaltic pump which supplied the tissue with buffer at a constant rate of 2 mL/minute. Both the buffer and the jacketed tissue bath were kept at 37°C by a Haake immersion circulator (model D1, Fisher Scientific).

2.3. Drugs and Chemicals

The methoxamine used in this study was a gift from Burroughs Wellcome Inc., Kirkland, Quebec. Stock solutions of 10^{-2} M were made up in water, aliquoted and

frozen until used for an experiment. Solutions were never refrozen for repeated use.

Acetylcholine chloride was obtained from Sigma Chemical Company, St. Louis, Missouri. Stock solutions were made up in water, fresh for every experiment, and were stored on ice during the experiments.

Substance P triacetate pentahydrate was purchased from Sigma Chemical Company, St. Louis, Missouri. Solutions of each concentration to be tested were made up in distilled water, aliquoted and frozen immediately. Individual aliquots were thawed just prior to injection. Substance P solutions were never refrozen for further use.

The collagenase used for removal of the endothelium was obtained from GIBCO laboratories, Burlington, Ontario. This preparation of clostradiopeptidase A was a crude extract. Collagenase was used at a concentration of 0.2 mg/mL, weighed freshly and made up in regular buffer solution for each experiment.

Sodium nitroprusside was purchased from Sigma Chemical Company, St. Louis, Missouri. Stock solutions of 10^{-2} M were made up in water fresh for each experiment. The solutions were stored on ice and protected from light during the experiment.

Angiotensin II was obtained from Sigma Chemical Company, St. Louis, Missouri. A 1 mg quantity was dissolved in 841 μL of water to achieve a 10^{-3} M stock solution for each experiment.

2.4. Animal Models

In this study the action of two vasoactive agents, acetylcholine and substance P were studied in the isolated perfused mesenteric arterial bed of the rat. In order to determine if the perivascular nerve fibres contribute to the development of the ability of the blood vessels to respond to these agents, experiments had to be carried out on tissues from animals with and without the normal nerve fibre plexus. In total, five groups of animals were studied. The experimental groups consisted of two models of chemically denervated adult male Sprague-Dawley rats. The control groups were untreated adult male Sprague-Dawley rats, as well as two groups of vehicle-treated animals to correspond with the experimental models.

The first experimental group was a model of chemical sympathectomy. Sprague-Dawley rats were treated from birth with 6-hydroxydopamine (6-OHDA) to prevent the development of the sympathetic nervous system. The 6-OHDA was dissolved in a solution of phosphate buffered saline which had been boiled and bubbled with nitrogen gas to remove dissolved oxygen. This solution also contained ascorbic acid (5 mg/mL) as an antioxidant and was adjusted to pH 5 with 1 M HCl to further protect the 6-OHDA. Rats were given a subcutaneous injection of 0.1 mL of this solution containing a dose of 60 mg/kg of 6-OHDA. Injections were administered on day 0, 1, 3, 5, 7, 9, 11 and weekly thereafter until the age of 12 weeks.

The second experimental group consisted of male Sprague-Dawley rats which had been treated with capsaicin from birth to destroy peptidergic peripheral innervation containing the peptides substance P and CGRP. Capsaicin was dissolved in absolute ethanol (2 mL) and a mixture of Tween-80 (2 mL) in 0.9% saline (16 mL) was added. This solution needed warming to dissolve the capsaicin completely. Rats were given a subcutaneous injection of 0.1 mL of this solution. Injections were administered on day 2, 3, 4, 11 and 25, followed by two further monthly intervals until the rats were 12 weeks old. On day 2 the injections contained a dose of 25 mg/kg of capsaicin, with subsequent doses containing 50 mg/kg.

Control experiments were done on untreated and vehicle-treated rats. The vehicle injections consisted of the same solutions, volumes and intervals as described for the experimental models, with the 6-hydroxydopamine or capsaicin omitted.

2.5. Experiment Protocols

2.5.1. Methoxamine Concentration Response Experiment

Methoxamine is an α_1 adrenoceptor agonist which was utilized as a pressor agent throughout this study. In this experiment eight rats were used to determine the concentration response relationship for methoxamine (gift from Burroughs Wellcome Inc., Canada) in the mesenteric bed.

Stock solutions of 1×10^{-2} M methoxamine were made up in water, fresh for each day. After dissection, each tissue was allowed to equilibrate for 60 minutes. Then, methoxamine was added cumulatively to the perfusing buffer solution in a concentration range from 1×10^{-7} to 5×10^{-5} M. After the tissue had reached a maximal plateau in pressure, ACh from a 10^{-2} M stock solution in water, was added to the perfusing solution to achieve a solution concentration of 1×10^{-5} M. This was done to determine the functional state of the endothelium in the preparation.

2.5.2. Acetylcholine Experiments

In this study concentration response relationships were determined for ACh, in the presence of tone induced by methoxamine, in the mesenteric bed preparation of five groups of rats.

The experimental groups consisted of rats treated with 6-OHDA or capsaicin, as previously described, to provide models of chemically denervated animals. The control groups were normal adult male Sprague-Dawley rats, as well as vehicle-treated animals to correspond with the two experimental groups.

In these experiments the mesenteric bed preparation was dissected and set up as described previously. The tissue was allowed to equilibrate for 60 minutes before it was challenged with 1×10^{-5} M methoxamine in the perfusing buffer for a 5-7 minute exposure. After a further 30 minute equilibration period of perfusion with regular buffer, the tissue was again exposed to methoxamine in the buffer at a concentration of $1\text{-}3 \times 10^{-5}$ M to induce a stable plateau of pressure of 75 to 120 mm Hg above the basal pressure. Then, cumulative additions of ACh were made to the buffer in the range from 1×10^{-9} to 1×10^{-5} M stepwise. Only one dose response experiment was completed for each tissue.

The dose-dependent reductions of pressure to acetylcholine were calculated as percent reductions of methoxamine induced pressure.

2.5.3. Substance P Experiments

A series of experiments were carried out to investigate the action of substance P in the isolated perfused mesenteric arterial bed of the rat. Dose response relationships were studied for substance P in the presence of methoxamine in six

groups of Sprague-Dawley rats. As for the acetylcholine study, rats were treated with 6-OHDA or capsaicin from birth to provide two models of chemical denervation. Control groups consisted of normal adult rats as well as the two corresponding vehicle-treated groups. In addition, substance P was studied in the presence of methoxamine in preparations which had been denuded of endothelium. Subsequently, experiments were done to further investigate the mechanism of action of substance P in this preparation. These studies involved the administration of substance P before methoxamine exposure, as well as substance P in the presence of Angiotensin II or elevated potassium concentration in the buffer.

It was determined in preliminary experiments that SP is susceptible to hydrolysis in a heated buffer solution such as that used to administer the methoxamine and ACh. This was not found to occur with ACh or methoxamine over the time course of the experiments. It also required a substantial amount of the synthetic peptide to add it to the perfusing buffer solution, which could be extremely expensive. However, when injections of a 10 μL dose of stock solutions ranging from 10^{-2} to 10^{-5}M were given at the injection port (figure 2, page 54) pressor responses were obtained. An injection artifact was ruled out by injecting 10 μL of the solvent (H_2O) and observing no response in the tissue. Higher volumes ($\geq$100μL) were found to give a transient artifact pressure response. Therefore SP was administered as an injected bolus dose in contrast to the cumulative concentrations of ACh in the perfusing buffer.

(a) Substance P in the Presence of Methoxamine

In this group of experiments the mesenteric bed was dissected and set up as previously described. After an equilibration period of 60 minutes the tissue was challenged with 1×10^{-5} M methoxamine in the perfusing buffer for a period of 5-7 minutes. The tissue was then perfused with regular buffer and allowed to equilibrate for a further 30 minutes. At this time the tissue was again exposed to 1×10^{-5} M methoxamine in the buffer. As soon as the stable plateau of pressure was reached, a control injection of 10 μL of water was delivered with a Hamilton syringe through the latex tubing which attached the tissue to the recording and perfusion apparatus. Then, at five minute intervals, two further injections of 10 μL of SP dissolved in water were made at the same site of injection. Finally, after the second response to SP was obtained, a 10 μL injection of 10^{-2} M ACh was made to indicate the functional state of the endothelium.

Concentrations of SP from 10^{-12} M to 10^{-2} M were tested and a range from 10^{-5} to 10^{-2} M was chosen for measurement of the dose response relationship. Doses of SP were calculated based on the 10 μL volume of injection of the 10^{-5} to 10^{-2} M solutions. Finally, to avoid desensitization, only two doses of SP were given to each tissue. The dose response data was expressed as both the actual change in perfusion pressure and as the percentage increase in the methoxamine induced pressure measured just prior to the injection of SP.

(b) Substance P in Endothelium-Denuded Preparations

To determine if the endothelial cells lining the blood vessels of the mesenteric bed were necessary for the action of substance P in this tissue, it was of interest to study tissues which had been denuded of endothelium. Removal of the endothelium was attempted by: flushing the tissue rapidly with buffer solution during dissection, allowing air to perfuse through the tissue for 5 minutes, and by a combination of a treatment with collagenase in the buffer and an air embolism. Electron microscopic examination of preparations treated by these methods indicated incomplete removal of the endothelium for the first two methods. The combined treatment with collagenase and air resulted in a tissue which was free of endothelial cells.

Six normal adult male rats were used for these experiments. The mesenteric bed was dissected and set up as previously described. After equilibration with normal buffer for 60 minutes, the tissue was challenged with 1×10^{-5} M methoxamine in the buffer and was tested for functioning endothelium with 10^{-5} M ACh in the buffer. After a wash out period of 5 minutes the tissue was exposed to collagenase 0.2 mg/mL in the perfusing buffer for 60 minutes. Following this treatment, an air bubble was allowed to perfuse through the tissue. The preparation was allowed to equilibrate with regular buffer for 15 minutes and was then exposed to 1×10^{-5} M methoxamine in the buffer. When a stable plateau of pressure had been reached, two 10 μL injections of SP from 10^{-6} to 10^{-2} M were given, followed by 10^{-5} M ACh

in the perfusing buffer. In addition a 10 μL injection of 10^{-2} M ACh was given to test for relaxations. Finally sodium nitroprusside was given, 10^{-5} M in the perfusing buffer, as well as a 10 μL injection of 10^{-2} M to ensure that the preparation could relax to a direct stimulus.

The responses to SP determined in the preparations which had been denuded of endothelium were expressed as the control data for SP had been.

(c) Substance P Before Methoxamine

To further investigate the mechanism of action of substance P, experiments were done in which SP was administered before the tissue had been exposed to methoxamine. These experiments involved the use of six normal adult male rats.

Tissues were dissected and set up as previously described. After an equilibration period of 60 minutes, a control injection of 10 μL of water was given. This was followed, after five minutes, by an injection of 10 μL of 10^{-2} M SP. After an additional five minutes, the tissue was exposed to 1×10^{-5} M methoxamine in the buffer. When a stable plateau of pressure was reached, the tissue was given two 10 μL injections of 10^{-2} M ACh.

(d) Substance P in the Presence of Angiotensin II

To further study the mechanism of action of substance P in this tissue, it was necessary to determine the response to SP in the presence of a pressor agent which activates a different receptor system. For this purpose, Angiotensin II was used. The experiment was carried out using six normal adult male rats.

The tissue was again dissected and set up as previously described. After 60 minutes of equilibration the tissue was exposed to 1×10^{-5} M Angiotensin II in the perfusing buffer. A control injection of 10 μL of water was given to the tissue, followed by an injection of 10 μL of 10^{-2} M SP. Finally, a 10 μL injection of 10^{-2} M ACh was given to check the integrity of the endothelium.

The responses of the tissue to this dose of SP were expressed as absolute changes in perfusion pressure.

(e) Substance P in the Presence of Depolarizing Buffer

The response of the bed to substance P in the presence of a depolarizing buffer was studied in this series of experiments. The direct substitution of potassium chloride for sodium chloride in the perfusing buffer gave a solution which would elicit a pressor response by acting directly on the smooth muscle cell membrane to cause a depolarization. Six normal adult male rats were used for this study.

The mesenteric bed was dissected and prepared as previously described. After a period of 60 minutes of equilibration with regular buffer, the buffer was changed to a similar solution with an increased potassium concentration of 40 mM and a decreased sodium concentration of 86.8 mM. This buffer solution was also warmed to 37 °C and oxygenated. After a stable pressor response was attained, the tissue was given a control injection of 10 μL of water which was followed, at five minute intervals, by a 10 μL injection of 10^{-2} M SP and 10 μL injection of 10^{-2} M ACh.

The responses to SP were measured and expressed in terms of actual change in perfusion pressure.

2.5.4. Immunohistochemical and Electron Microscopic Study

In addition to recording responses of the McGregor preparation to pharmacological agents, this study examined the tissue preparation at both the light and electron microscopic level to verify the presence or absence of the perivascular nerve fibres and the endothelium.

Tissues were fixed after the experiments with Zamboni's fixative (Zamboni and De Martino, 1967). The mesenteric bed preparations were flushed gently with fixative from a syringe and then immersed in the solution for 1 hour. Tissues were then stored in phosphate buffered saline (PBS) and the arterial bed was dissected out of the surrounding fat and connective tissue with the aid of a dissecting

microscope. The arterial bed, which now included the superior mesenteric artery and four primary jejunal vessels with their subsequent branches, was subdivided for immunohistochemical processing or processing for electron microscopy.

(a) Immunohistochemistry

Tissues were stained using immunohistochemical techniques to visualize the perivascular nerve fibre plexus at the adventitial-medial border of the blood vessel wall. Sections of the mesenteric bed which contained a segment of superior mesenteric artery with at least one attached primary jejunal artery with its branches intact were processed whole using a three step peroxidase anti-peroxidase (PAP) procedure. Vessels were washed in a solution containing phosphate buffered saline (PBS), 1% Triton X-100, and 1% normal goat serum for one hour before incubation in primary antiserum. This solution was used as an antibody diluent as well as for rinses. The primary antisera used were anti-substance P (1:2000; Immunonuclear) or anti-tyrosine hydroxylase (1:10,000; Eugene Tech). The tissues were incubated in the primary antisera for 36 hours at 6°C. After three 15-minute washes, the vessels were incubated in a linking antibody, goat anti-rabbit (1:50 Sternberger) for 2 hours and, following a further three washes, the tissues were incubated in rabbit PAP complex (1:300; Sternberger) for 2 hours. Then, after the final three washes, the tissues were reacted with a solution containing diaminobenzidine (50 mg) in

distilled water (50 mL) with 0.2 M phosphate buffer (50 mL) and 30% hydrogen peroxide (33 μL). After the reaction was completed (1-2 minutes) the tissues were dehydrated with 75% ethanol, absolute ethanol, and xylene for 15 minutes each and mounted on slides as whole mounts with Eukitt mounting medium.

The presence of a nerve plexus, specific to the primary antibody used, was determined by light microscopy.

Controls for the immunohistochemical staining method were carried out as described above with one of the steps altered or omitted. The procedure was carried out with primary antisera which had been preadsorbed with antigen, with primary antisera omitted, or with the PAP linking antibody omitted to ensure that the reaction product was due to specific labelling.

(b) Electron Microscopy

Tissues selected for electron microscopy were placed in individual labelled vials and kept on a rotating stirrer to ensure complete mixing throughout the processing. They were immersed in sodium cacodylate buffer (0.1 M) for 30 minutes, and then postfixed with osmium tetroxide (1% in cacodylate buffer) for 1 hour. After en bloc staining with uranyl acetate (saturated in 50% ethanol) for 30 minutes, the tissues were put through a dehydration sequence, beginning with 70% and 95% ethanol for 30 minutes each. Then, the tissues were further dehydrated with 3

changes of absolute ethanol and cleared with two changes of absolute acetone for 10 minutes each. The tissues were infiltrated in a 50:50 mixture of resin and acetone overnight at 6°C. The resin was made up using 16 g of Epon 812, 8 g of DDSA (Dodecenyl Succinic Anhydride), 8 g of MNA (Methyl Nadic Anhydride) and 0.4 g of DMP 30 (2,4,6-tri[dimethylaminomethyl] phenol). In the morning the tissues were immersed in fresh resin for 4 hours on the rotating stirrer and polymerised in Epon resin in flat embedding molds overnight at 60°C. Prior to embedding and polymerisation, the superior mesenteric bed and its branching jejunal arteries were separated and labelled.

Sections 1 μm thick were cut and stained with toluidine blue. Thin sections were stained with lead citrate and examined in a Philips EM300 electron microscope. The presence of intact endothelium and perivascular nerve fibres was determined from examination of the thin sections. With the exception of the intentionally air embolized tissues, data was not used for tissues which were found to have damaged or removed endothelial linings.

2.5.5. Statistics

Statistical analysis of the data was completed using the SPSS-X package on the VAX/VMS computer at Computing Services, Memorial University of Newfoundland. A oneway analysis of variance (ANOVA) was carried out on the

data from each animal group to determine if a dose response relationship exists for the acetylcholine or substance P experiments. The Scheffe test for significance was used in these procedures ($P<0.05$ was considered significant).

From the means and standard errors of the means, calculated by the ANOVA procedure, dose-response curves were constructed for each group using the Sigmaplot package on a Tatung TCS-7000 personal computer. The EC_{50} values were obtained by graphically calculating the concentration of agonist producing 50% of its maximum response using the method of Fleming *et al.* (1972). To test for differences in the dose-response relationships for an agonist with the chemical denervation animal treatments, a twoway ANOVA procedure was used. Differences indicated by the ANOVA were considered to be significant for $P<0.05$.

In experiments where an ANOVA was not used, the means and standard errors of the means were calculated using a Hewlett Packard HP-11C calculator.

RESULTS

3.1. Immunohistochemistry Results

Upon examination by light microscopy, the mesenteric arterial bed preparations of the untreated animal groups were found to exhibit a nerve fibre plexus which was disposed in two planes. Bundles of large diameter fibres appeared to run along the outer adventitia, while at the adventitial-medial border a plexus of finer beaded fibres occurred. This layering of the perivascular nerve fibre plexus has been previously described (Scott *et al.*, 1987). Although the form and extent of the fibre plexus was different for SP and TH, both appeared to exist in two planes with the outer fibres appearing smooth and the deep plexus displaying varicosities.

The tissues from the capsaicin-treated group did not display SP immunoreactivity at either layer in the mesenteric arterial bed. However, the preparations stained for TH revealed nerve fibres at both layers in all vessels of the bed. The density of these fibres was not measured.

Tissues from 6-OHDA-treated rats stained positively for the SP-containing nerve fibres at both levels. However, tissues stained for TH did not have the fine deep fibre plexus observed in the controls but some large fibres were still present in the outer adventitia.

3.2. Methoxamine Concentration Response Experiment

Methoxamine was shown to exhibit a graded concentration response relationship in the isolated perfused mesenteric bed (Figure 3). In the ACh experiments a concentration of $1\text{-}3x10^{-5}$ M methoxamine was used to achieve a stable plateau of pressure from which the relaxations to ACh could be measured. In the concentration response curve to methoxamine this concentration range can be seen to give a submaximal to maximal pressor effect in this preparation.

3.2.1. Methoxamine Response in Capsaicin-treated Rats

It was found in this study that the response of the mesenteric arterial bed to methoxamine was increased in tissues from capsaicin rats (Figure 4). At a concentration of 1×10^{-5} M methoxamine in the perfusing buffer the pressor response was significantly higher in tissues from capsaicin-treated rats. A change in reactivity of the preparations from capsaicin vehicle-treated rats to methoxamine was not observed.

Figure 3. Concentration response curve for methoxamine in the isolated perfused mesenteric bed of untreated rats. Standard errors are indicated (n=8).

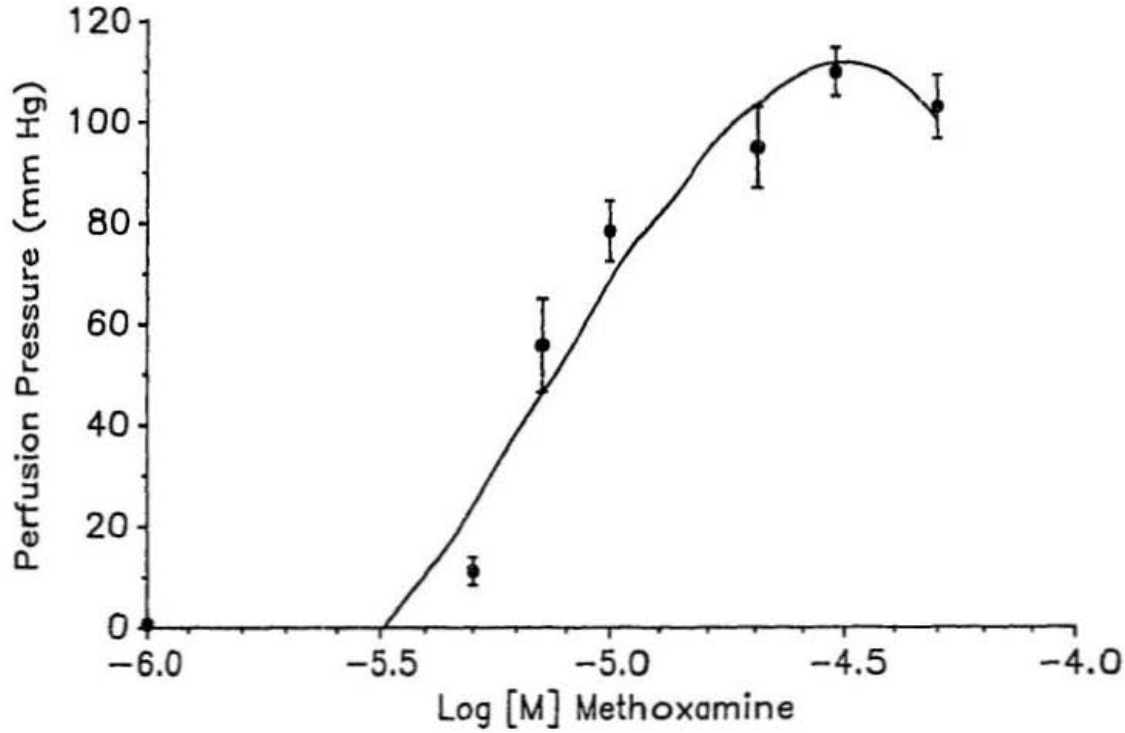
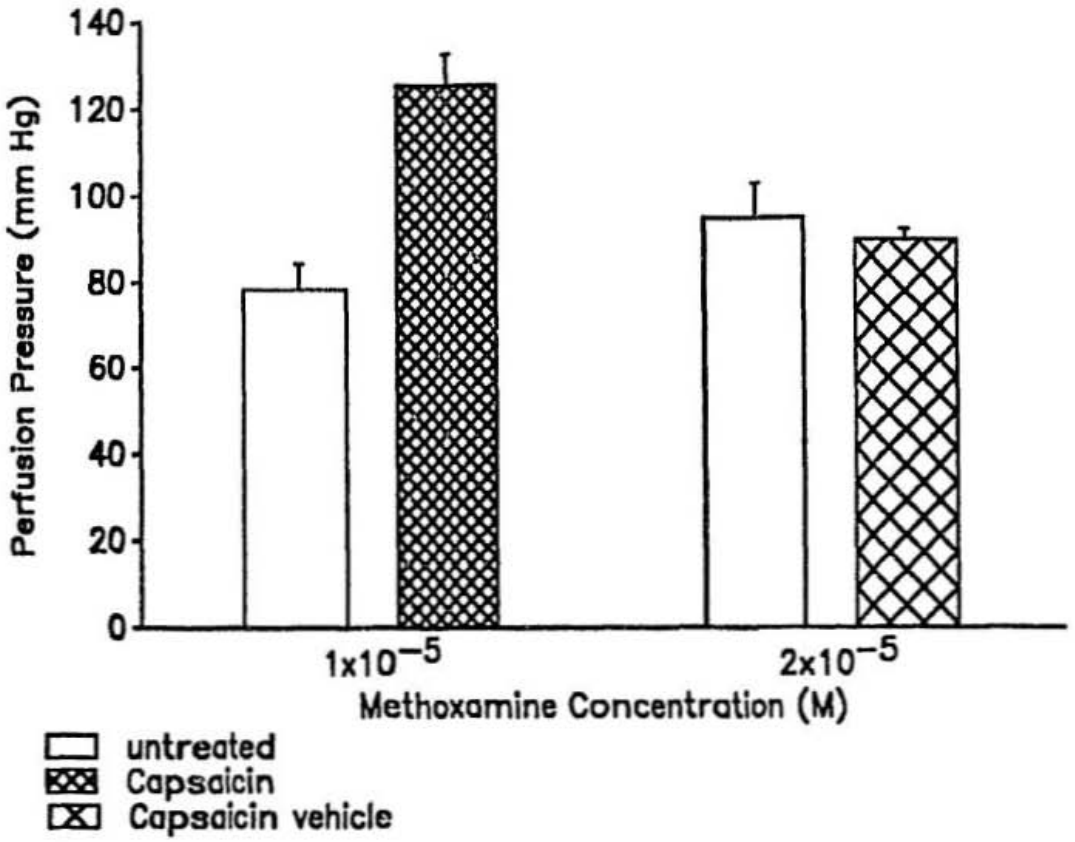


Figure 4. The response of the mesenteric arterial bed to methoxamine in tissues from capsaicin-treated rats. Standard errors are indicated (n=10).



3.2.2. Methoxamine Response in 6-OHDA-treated Rats

A decreased reactivity of the mesenteric arterial bed to methoxamine was noticed in preparations from 6-OHDA-treated rats in this study (Figure 5). The pressor response to 3×10^{-5} M methoxamine in the perfusing buffer was significantly lower in tissues from 6-OHDA-treated rats than those from untreated or vehicle-treated groups.

3.3. Acetylcholine Experiments

Acetylcholine caused a concentration dependent reduction in the methoxamine induced pressure in the mesenteric bed preparation. Figure 6 shows the graded concentration response relationship for ACh in the untreated animal group.

3.3.1. Capsaicin-treated Animals

A significant decrease in the sensitivity of tissues to ACh was detected by the two way ANOVA for the capsaicin-treated group of animals. The concentration response curve (Figure 7) is shifted to the right 1.2 log units. The corresponding EC_{50} values are shown in Table 1.

Figure 5. The response of the mesenteric arterial bed to methoxamine in tissue from 6-OHDA-treated rats. Standard errors are indicated (n=6).

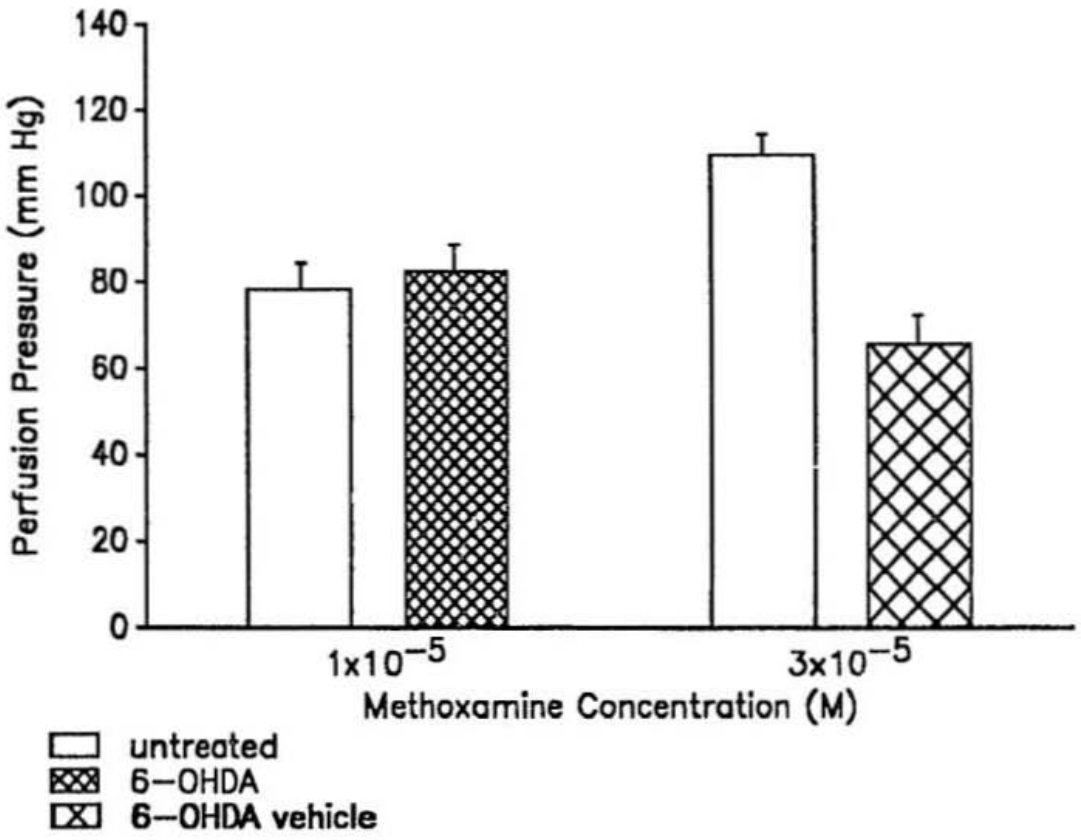


Figure 6. Concentration response curve for acetylcholine in tissues from untreated rats. Standard errors are indicated (n=10).

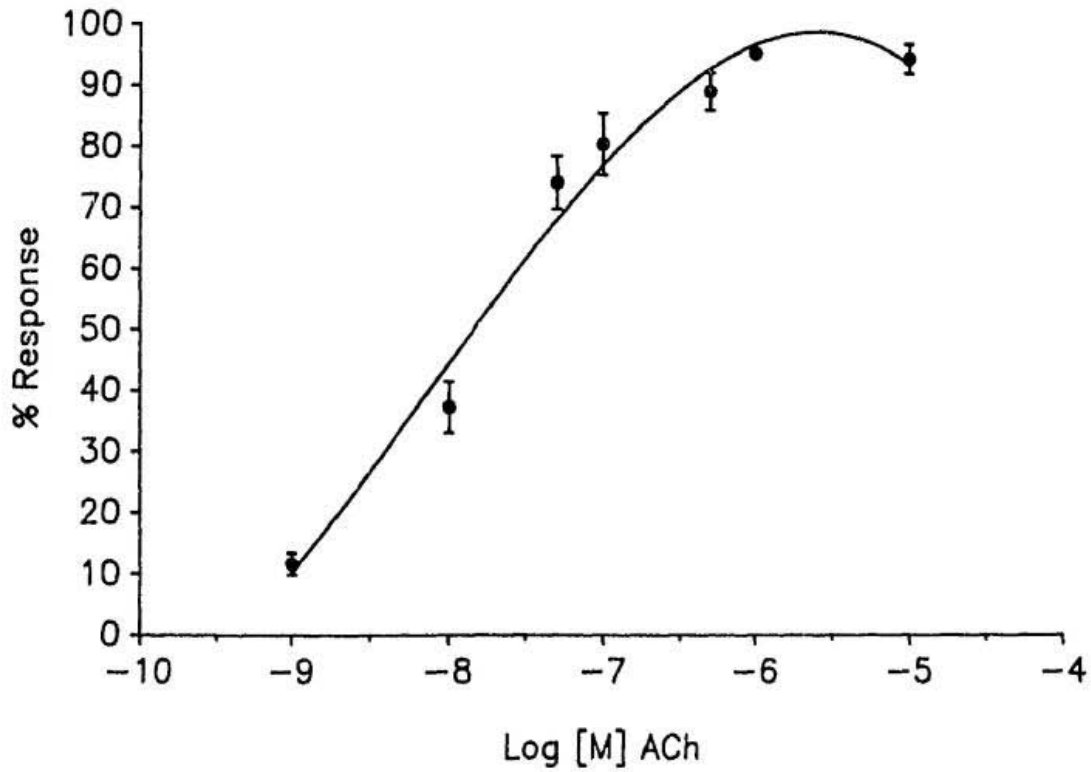


Figure 7. Concentration response curves for acetylcholine in tissues from untreated and capsaicin-treated rats. Standard errors are indicated (n=9).

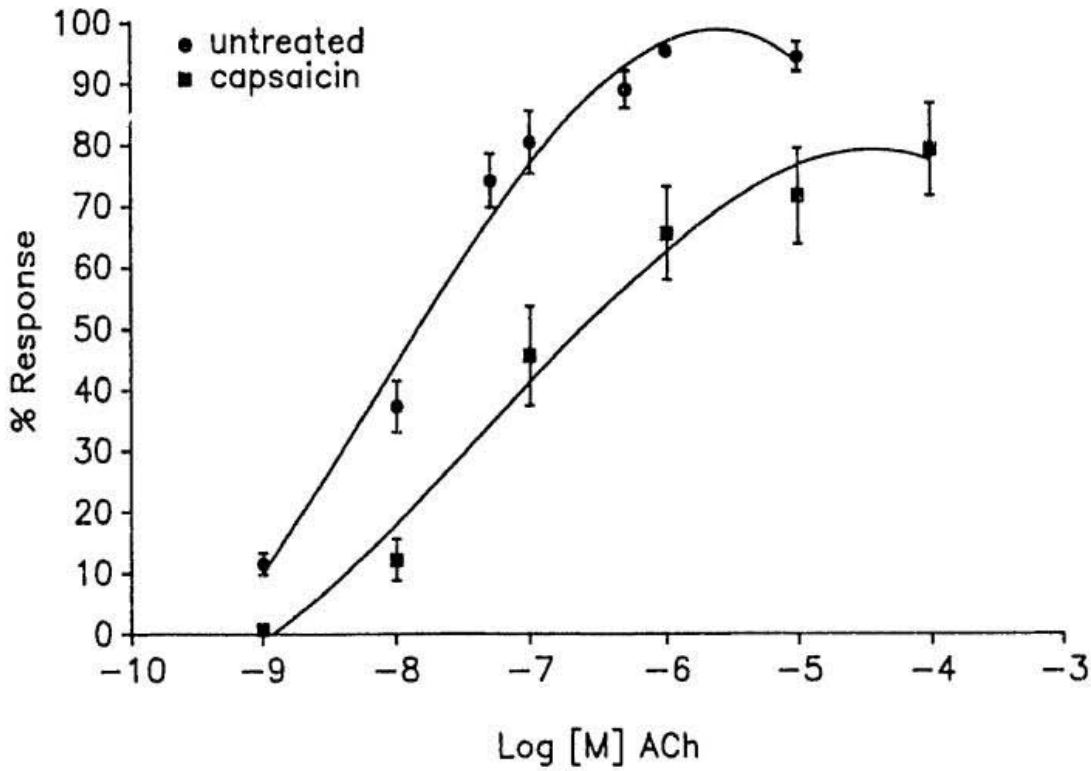


Table 1. EC_{50} Values for Acetylcholine

Treatment	EC_{50} [M]
untreated	1.45×10^{-8}
capsaicin	2.34×10^{-7} *
6-OHDA	1.29×10^{-8}
capsaicin vehicle	3.89×10^{-8}
6-OHDA vehicle	7.24×10^{-9}

* This value was found to be significantly different at $P<0.05$.

The EC_{50} for ACh in the capsaicin vehicle-treated animal group is not significantly different from the untreated group. Figure 8 shows the concentration response relationship for ACh in the untreated, capsaicin-treated and capsaicin vehicle-treated animal groups.

3.3.2. 6-Hydroxydopamine Treated Animals

No significant change in the sensitivity of tissues to ACh was detected by the two-way ANOVA for the 6-OHDA-treated group of animals. In addition, no difference could be shown between the responses in the 6-OHDA vehicle-treated group and the untreated animals. The concentration response curves for the three groups are shown in Figure 9.

3.4. Substance P Experiments

Substance P was shown to cause a dose dependent augmentation of the methoxamine induced pressure in the isolated perfused mesenteric bed of the rat (Figures 10, 11). In preliminary experiments on untreated rats, SP concentrations as low as 1pM did not result in relaxations in the tissues. In these experiments the tissues did exhibit reductions of methoxamine induced pressure in response to ACh (10^{-2} M). This response was not changed upon removal of the endothelium (Figures 10, 11).

Figure 8. Concentration response curves for acetylcholine in the mesenteric bed of untreated, capsaicin-treated and capsaicin vehicle-treated rats. Standard errors are indicated (n=9).

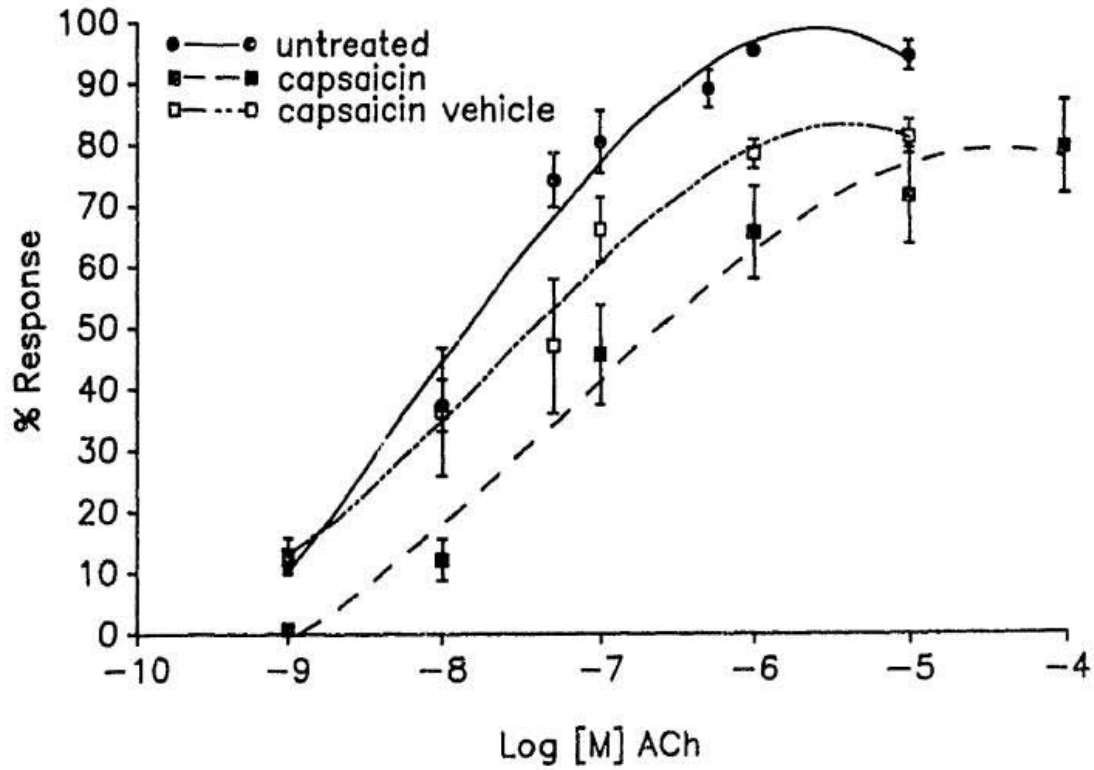


Figure 9. Concentration response curves for acetylcholine in tissues from untreated, 6-OHDA-treated, and 6-OHDA vehicle rats. Standard errors are indicated (n=8).

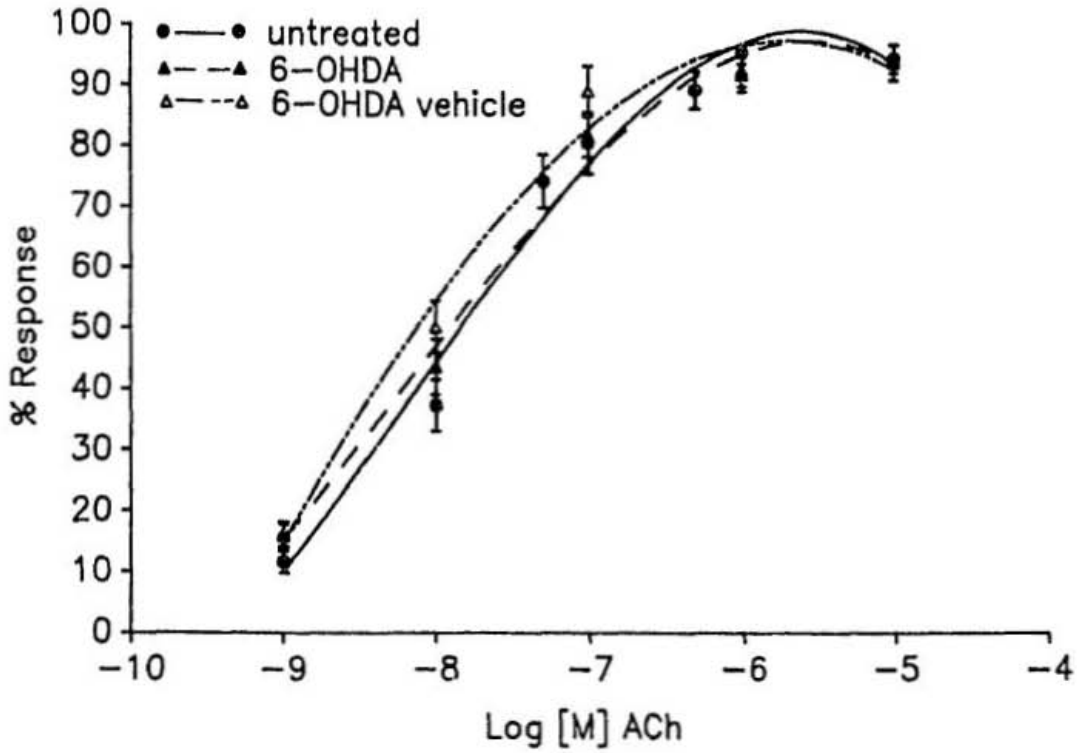


Figure 10. The dose response relationship for substance P in untreated and air embolized tissues. Data is expressed as absolute change in perfusion pressure (mmHg). Standard errors are indicated (n=6).

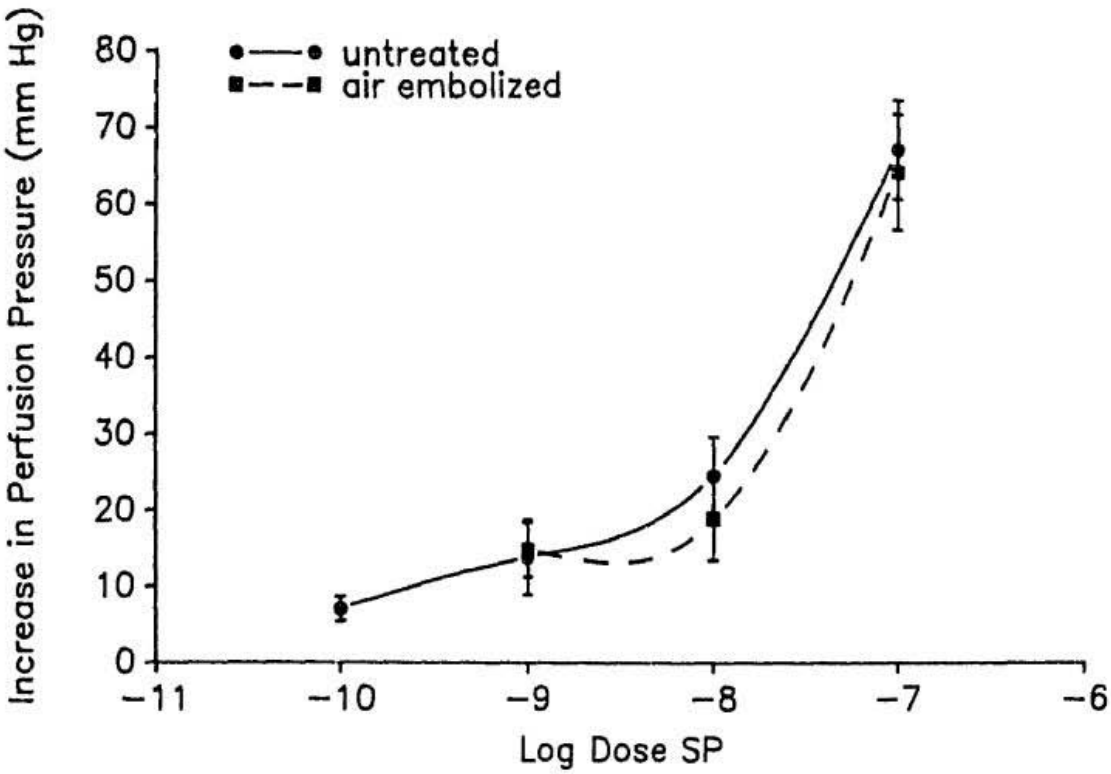
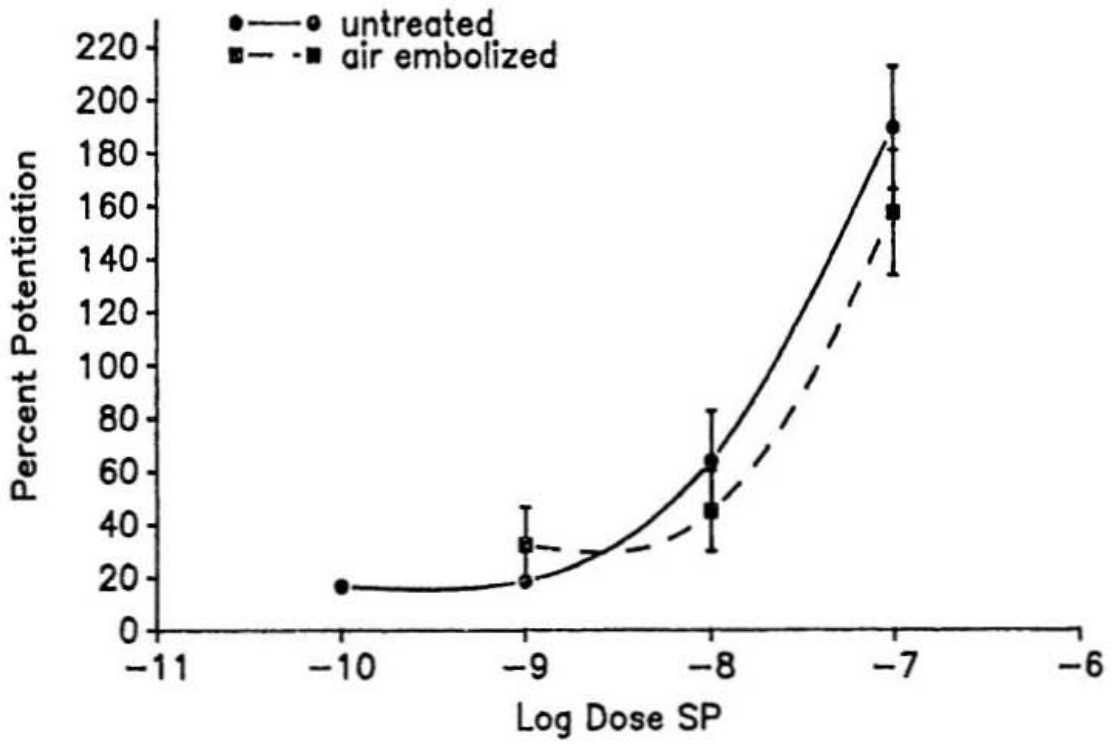


Figure 11. The dose response relationship for substance P in untreated and air embolized tissues. Data is expressed as the percentage increase in methoxamine-induced pressure (mmHg). Standard errors are indicated (n=6).



Since this appeared to be a potentiation of the adrenergic vasoconstriction the pressor response to SP was calculated as a percentage increase in the methoxamine induced pressure measured just prior to the injection of SP. When expressed as an absolute change in perfusion pressure the SP dose response relationship is not altered in the treated animal groups (Figures 12, 13). However, when the dose response data is expressed as a percentage potentiation of the methoxamine induced pressure 6-OHDA treatment results in an increase in the pressor response at the 10^{-7} mole dose (Figure 14). No significant change is seen with capsaicin treatment although the response at the 10^{-7} mole dose appears to be depressed (Figure 15).

3.4.1. Substance P Before Methoxamine

When a 10^{-7} mole dose of SP was given to tissues before methoxamine buffer no measurable response was seen. These tissues did respond normally to methoxamine and ACh after the SP injection.

Figure 12. Dose response relationships for substance P in tissues from untreated, capsaicin-treated and capsaicin vehicle-treated rats. Data is expressed as absolute change in perfusion pressure (mmHg). Standard errors are indicated (n=6).

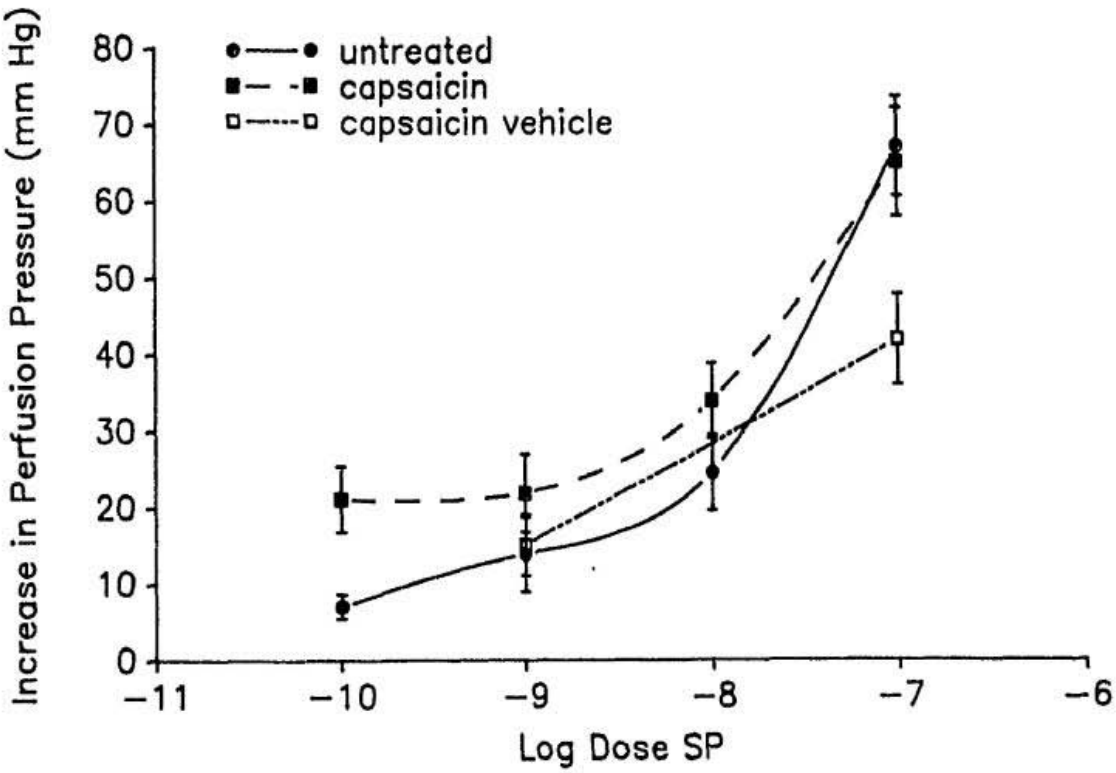


Figure 13. Dose response relationships for substance P in tissues from untreated, 6-OHDA-treated and 6-OHDA vehicle-treated rats. Data is expressed as absolute change in perfusion pressure (mmHg). Standard errors are indicated (n=12).

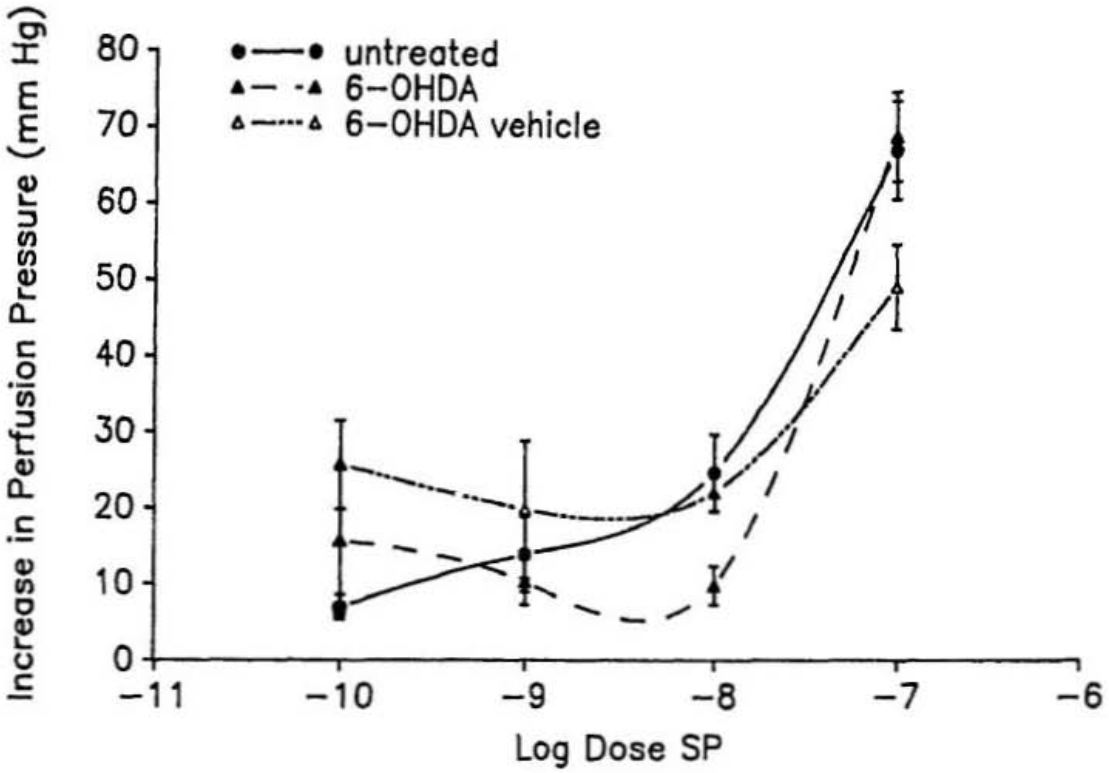


Figure 14. Dose response relationships for substance P in tissues from untreated, 6-OHDA-treated and 6-OHDA vehicle-treated rats. Data is expressed as the percentage increase of methoxamine-induced pressure (mmHg). Standard errors are indicated (n=12).

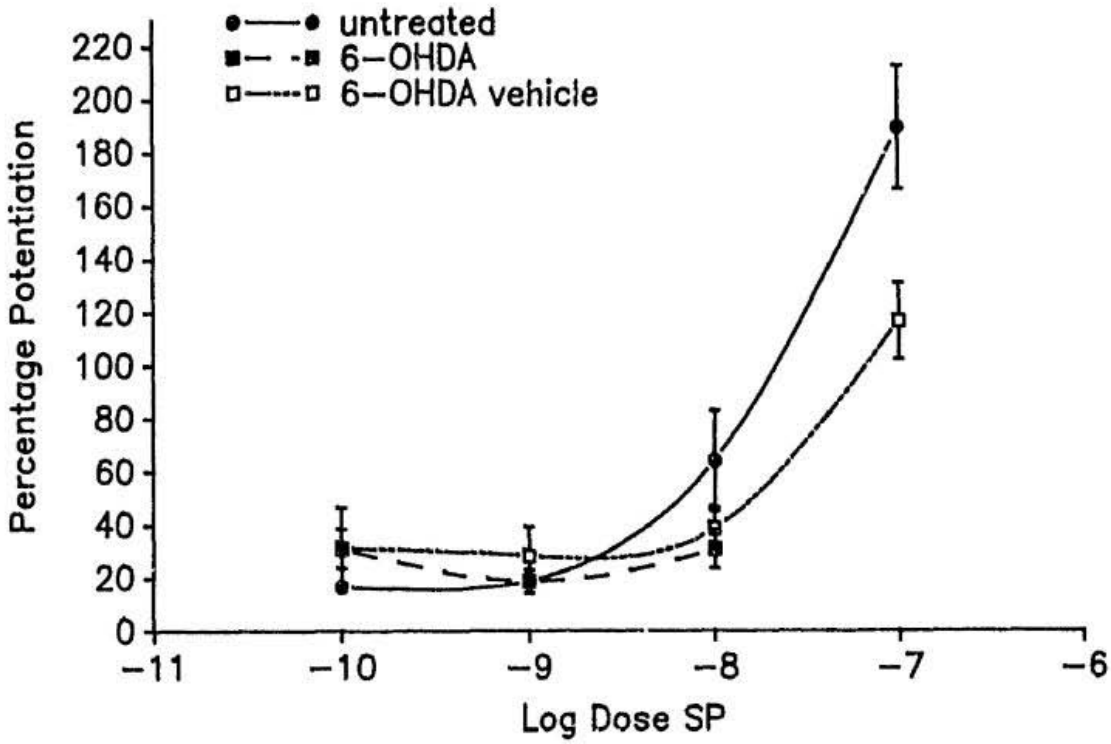
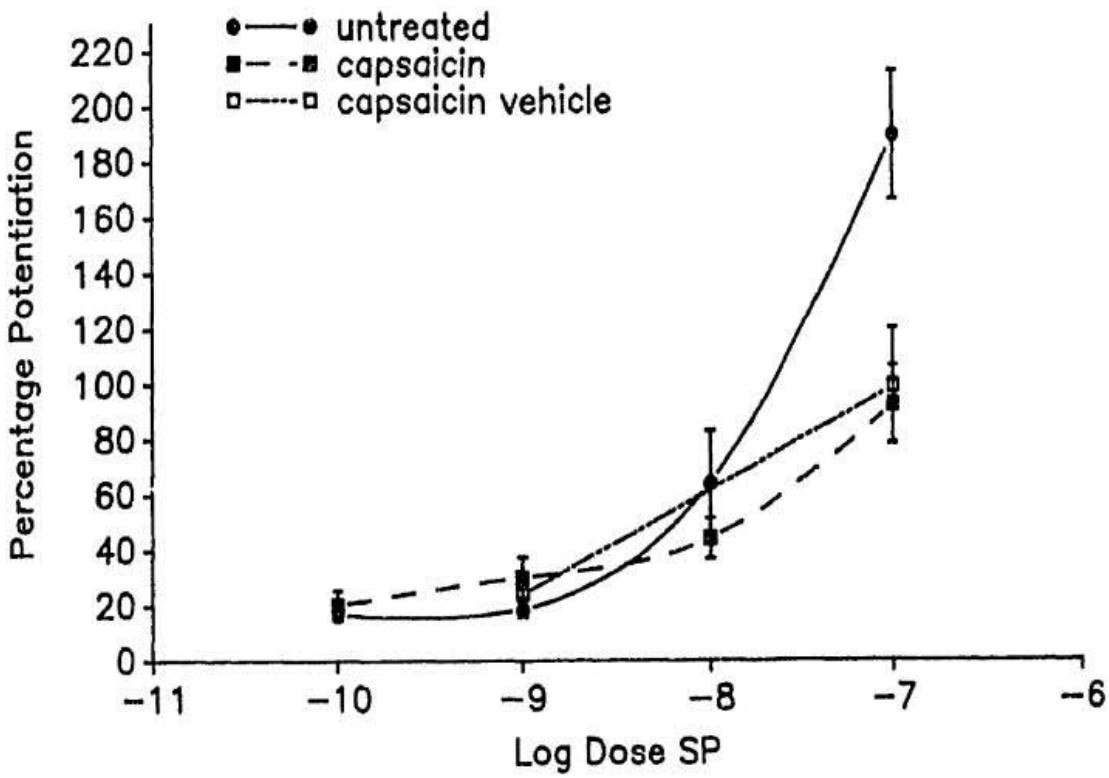


Figure 15. Dose response relationships for substance P in tissues from untreated, capsaicin-treated and capsaicin vehicle-treated rats. Data is expressed as the percentage increase of methoxamine-induced pressure (mmHg). Standard errors are indicated (n=6).



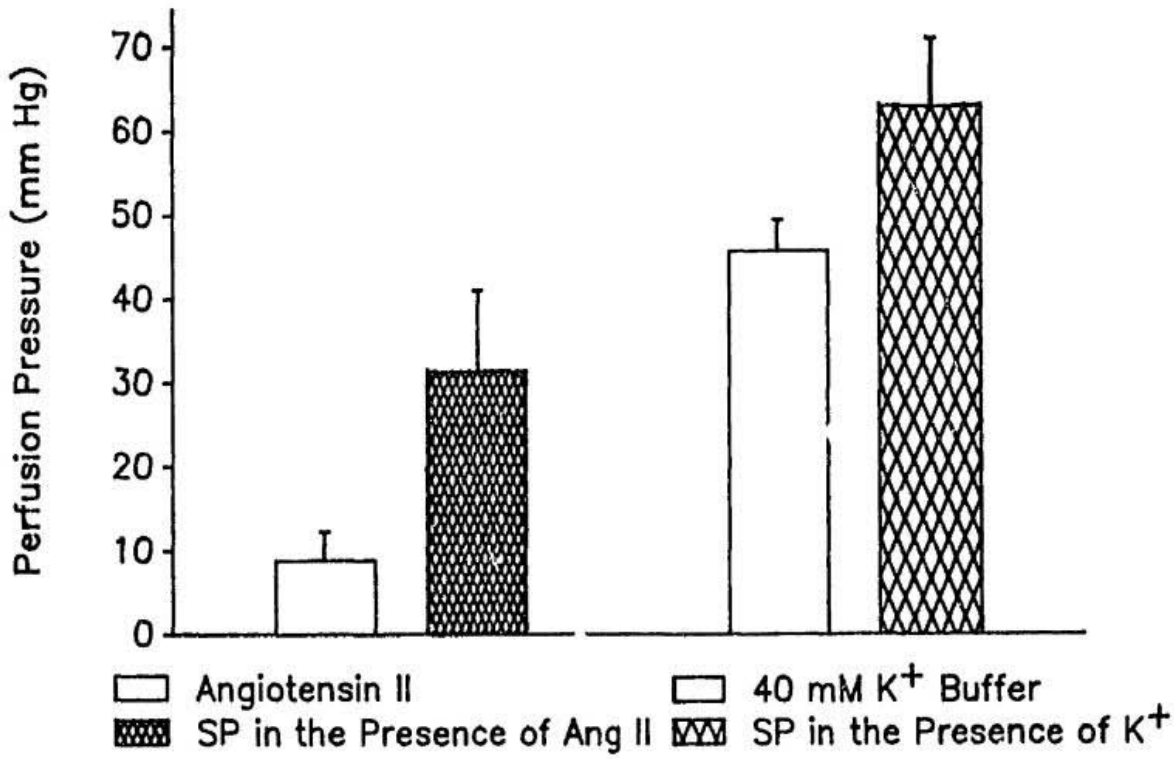
3.4.2. Substance P in the Presence of Angiotensin II

When a 10^{-7} mole dose of SP was injected into tissues perfused with buffer containing 10^{-5} M Angiotensin II as a pressor agent, a small but measurable pressor response was obtained (Figure 16). This response was highly variable.

3.4.3. Substance P in the Presence of Depolarizing Buffer

When a 10^{-7} mole dose of SP was injected into tissues perfused with 40 mM potassium depolarizing buffer, a small but measurable pressor response was achieved above that caused by the buffer (Figure 16). This response was also highly variable.

Figure 16. The response to substance P in the presence of Angiotensin II and depolarizing (K^+) buffer in the isolated perfused mesenteric arterial bed of the rat. Standard errors are indicated (n=6).



DISCUSSION

This study has found that chronic capsaicin treatment causes a decreased sensitivity to ACh in the mesenteric arterial bed of the rat which is indicated by a shift in the concentration response curve for this endothelium-dependent vasodilatory response (Figure 7, page 77). No significant change in sensitivity of the preparation to ACh was noticed with capsaicin vehicle, 6-OHDA, or 6-OHDA vehicle-treated groups (Table 1, page 78). In this same tissue preparation SP caused a pressor response which was independent of the presence of endothelial cells (Figures 10, 11, pages 82, 83). When expressed as a percentage potentiation of the methoxamine response, 6-OHDA treatment resulted in an increased SP response (Figure 14, page 87).

4.1. Consideration of Denervation Treatments

To compare the responses to ACh and SP of the mesenteric bed preparations between tissues from untreated, capsaicin and 6-OHDA-treated animals a consideration of the success of these treatments must be undertaken.

Capsaicin treatment was found to remove the SP-immunoreactive nerve fibre plexus completely in the mesenteric arterial bed in this study. This is not surprising since the treatment was initiated at 2 days after birth and was quite rigorous. Studies

have shown that it is possible to completely remove SP innervation from the mesenteric arterial bed of the rat with this sort of capsaicin treatment (Murphy *et al.*, 1982). Because the density of innervation was not measured, it is not known for sure if an increase in TH-containing adrenergic fibres occurred with capsaicin treatment. However, it has been reported that this can occur (Woolgar and Scott, 1989).

The 6-OHDA treatment was shown to remove the fine beaded TH-containing fibre plexus at the adventitial-medial border. Since this is thought to be the terminal plexus of the sympathetic nerves in the blood vessel wall, a functional sympathectomy was achieved. However, large TH-containing fibres remained in the outer adventitia of many 6-OHDA-treated preparations. Although these fibres do not appear to have varicosities from which transmitter would be released, their presence means that NA is present in the blood vessel wall during development. The complete removal of sympathetic innervation with 6-OHDA is difficult to achieve in the mesenteric arterial bed but studies have shown that it is possible with neonatal treatment (Angeletti and Levi-Montalcini, 1970). Treatment with 6-OHDA did not appear to inhibit the development of the SP nerve fibre plexus. Whether SP perivascular innervation was increased in these tissues was not measured.

4.2. Factors Contributing to the Alteration of the ACh Response

The decreased sensitivity to ACh in this tissue with chronic capsaicin treatment has not been previously described. Although a high variability in measurements in this preparation has been encountered, it is unlikely that the observed shift in the ACh concentration response curve is an artifact. A substantial number of animals were used so that reliable means for the responses were obtained. Statistical analysis of data using an ANOVA indicated that a significant difference ($P<0.05$) exists for the capsaicin-treated group but not for the 6-OHDA or vehicle-treated groups. Therefore a change in one or more elements of the blood vessel wall must be assumed to have caused the observed shift of the ACh response. What components of the mesenteric arteries might be involved in such a functional change? The decreased sensitivity to intralumenally administered ACh could have resulted from:

(1) a change in the muscarinic receptor density

(2) a change in intracellular events in the endothelial cells

(3) a change in the ability of EDRF to diffuse to its site of action

(4) changes in the smooth muscle relaxing ability

4.2.1. Changes in Muscarinic Receptor Density

A decreased EDRF response of the preparation to ACh would occur if the muscarinic receptor population of the endothelium was decreased. The muscarinic receptor density was not directly measured in this study. However, the use of agonists which activate other receptor systems on the endothelium to produce EDRF would provide data for a comparison of receptor effects. The original use of SP in this project was as an agonist which causes endothelium-dependent relaxations by activating a different endothelial receptor population (Zawadzki *et al.*, 1981). Other agonists which might be useful for further study into this question of muscarinic receptor population change are bradykinin and A23187. Bradykinin is another potent endothelium-dependent vasodilator which is thought to act via a receptive substance on the endothelium (Altura and Chand, 1981). The use of the ionophore, A23187, to produce endothelium-dependent relaxations in the treated and control groups of this study would provide data for comparison (Zawadzki *et al.*, 1980). If responses to A23187 were not altered with capsaicin treatment, then the shift in the ACh responses would most likely be due to a change in the density of muscarinic receptors.

A change in the muscarinic receptor population of vascular endothelium with removal of the SP- and CGRP-containing innervation is quite possible. It has been

reported that both SP and CGRP can have an influence on the development of ACh receptors (Boyd and Leeman, 1987; New and Mudge, 1986).

4.2.2. Changes in Intracellular Events of the Endothelium

A decrease in the ability of endothelial cells to generate and release EDRF would also result in a change in sensitivity of the mesenteric bed to ACh. Several observations strongly suggest the existence of more than one EDRF and as such more than one synthetic pathway is likely to be involved (Vanhoutte, 1987a). While products of arachidonic acid via the cytochrome P-450 pathway, or even ammonia, have been suggested as possible mediators of endothelium-dependent relaxation, NO is the only compound which has been positively identified as an EDRF so far (Palmer *et al.*, 1987). Palmer and colleagues have since reported evidence for the involvement of L-arginine as the endogenous substrate from which NO is generated in vascular endothelial cells (Palmer *et al.*, 1988). This pathway appears to involve two distinct steps, the initial mobilization of the substrate (L-arginine) and then its conversion to NO. These steps are both activated upon endothelial stimulation by bradykinin and A23187. If availability of endogenous substrate is altered, the NO-generating enzyme is capable of utilizing exogenous substrate effectively. The actual mechanisms by which release of L-arginine and activation of this NO-generating [illegible] occur has not yet been reported.

It is not known whether changes occurred in any of these EDRF generating pathways in this study. However, to fully investigate this possibility the use of a cascade system of arteries is required. Many studies of the mechanism of EDRF production and release have utilized a donor tissue with intact endothelium and a bioassay strip of artery from which the endothelium has been removed (Rubanyi *et al.*, 1985). The use of such a preparation would allow a separation of the effects of changes in several different components of the blood vessel preparation. If the bioassay strip relaxed to the same extent with equal concentrations of ACh in perfused mesenteric bed preparations from untreated and capsaicin-treated rats, then it might be assumed that the previously observed shift for ACh was due to changes in the ability of the smooth muscle to relax, rather than a loss of EDRF producing ability. However, if the same decreased sensitivity to ACh was observed, further experiments with an inhibitor such as indomethacin would be required to block the synthesis of prostaglandins and some of the effects of EDCF. This might determine if the vasodilator ability of the endothelium had been altered due to an increased basal release of contracting factors. Finally, although the enzyme system involved in the production of NO has not been fully characterized, the addition of L-arginine to the ACh solution might improve the ACh response if the mechanism by which the substrate for NO production is mobilized were altered by capsaicin treatment.

It is not known what effect long-term capsaicin treatment might have on the biochemical pathways involved in the production of EDRF.

4.2.3. Changes in the Diffusion of EDRF

It is possible that the diffusion of EDRF after its release from the endothelium might be limited by morphological changes in the intimal medial region of the blood vessel wall. This would also result in an altered response to ACh in the mesenteric arterial bed.

In this project, the endothelium was visualized with transmission electron microscopy. Electron microscopic examination of arteries from the mesenteric bed of the capsaicin-treated rats did not reveal any intimal thickening or ultrastructural damage. Thus, the decreased sensitivity of the preparation to ACh in the capsaicin-treated group is not likely to be due to a change in the ability for EDRF to diffuse to its site of action on the medial smooth muscle.

4.2.4. Changes in Smooth Muscle Cells

A major factor which may contribute to the decrease in vasorelaxation is a change in the ability of the smooth muscle cells to relax. The relaxation of vascular smooth muscle in response to EDRF is mediated by guanylate cyclase via the production of cyclic GMP and the activation of cyclic GMP-dependent protein kinase (Vanhoutte *et al.,* 1986; Rapoport and Murad, 1983a). This results in altered protein phosphorylation and in dephosphorylation of the myosin light chain which is though

to lead to relaxation. This action of EDRF on vascular smooth muscle is dependent on Ca^{2+} and is thought to be the mechanism by which NO and the nitrovasodilators act (Vanhoutte, 1987a) Another EDRF is thought to act via activation of the Na^+, K^+-ATPase and is thus K^+ dependent (Rubanyi and Vanhoutte, 1988). Thus, an alteration in these intracellular pathways would affect the ACh response.

In this study, ACh was the only vasodilator agent whose responses were compared in tissues from untreated and denervated animals. SNP was used as an endothelium independent vasodilator agent in some preliminary experiments in the ACh study and also in part of the SP study. Therefore, it is not known if capsaicin treatment affected the relaxing ability of the smooth muscle cells of the preparations. However, if SNP had been used at the end of the ACh concentration response experiment, it would be possible to determine if the tissue had relaxed fully. A comparison of the SNP induced maximal level of relaxation in untreated and capsaicin-treated animals would determine whether or not the relaxing ability of vascular smooth muscle had been altered.

It was also found in this study that the response to methoxamine in capsaicin-treated rats was increased. The perfusion pressure increase to methoxamine was significantly higher in tissues from capsaicin-treated rats than that for an equal concentration in untreated or vehicle-treated controls (Figure 4, page 73). What effect of capsaicin treatment could have cause this? It has been reported that peptidergic denervation with capsaicin may result in an increased density of catecholaminergic

innervation in the rat mesenteric vascular bed (Woolgar and Scott, 1989). It is likely that an increase in catecholaminergic nerve fibres during development will result in an increased reactivity to adrenoceptor agonists such as methoxamine. Whether this change in sympathetic innervation will contribute to changes in the EDRF response is unknown.

From these experiments it has been determined that long term capsaicin treatment results in a change in the endothelium dependent relaxations to ACh in the mesenteric arterial bed of the rat. From the present study it can be concluded that this decreased sensitivity was not due to the inability for EDRF to diffuse from the endothelium to the smooth muscle. However, it cannot be determined if the effect is a result of changes in muscarinic receptor density, alteration of endothelial cell biosynthetic pathways, or changes in the ability of vascular smooth muscle to relax.

The decreased EDRF response to ACh in the mesenteric arterial bed of the rat with long-term capsaicin treatment has not been previously reported. However, a depression of the EDRF system has been described in other situations, some of which are also associated with changes in perivascular innervation.

Hypertension is a disease with which many changes in vascular structure and function have been associated. In various models of hypertension, certain blood vessels show a depression of endothelium-dependent relaxations. Decreased EDRF responses have been reported in both aorta and small vessels and genetic hypertensive

rats (Konishi and Su, 1983; Winquist *et al.,* 1984; DeMey and Gray, 1985), aortic coarcted rats (Lockette *et al.,* 1986), mineralocorticoid hypertensive rats (Mayhan *et al.,* 1987), Dahl hypertensive rats (Luscher *et al.*, 1987), and in aortic coarctation hypertensive rabbits (Miller *et al.* 1987). Although the final measured result was a depression of relaxation in these experiments, several different mechanisms may be involved. In some cases an endothelium-derived contractile factor appeared to cause the depression, while in another experiment comparison with a SNP response suggested a lowered production of EDRF. It has been found that, although EDRF responses may be depressed in one vessel, an increased relaxation may occur in other vessels from the same animal (Konishi and Su, 1983). In estrogen treated rats, an increase in relaxation to ACh but not the ATP has been reported (Williams *et al.* 1988). Hypertension appears to have a selective effect on the action of some endothelium-dependent vasodilators but not others (Lamping and Dole, 1987).

In hypertension changes occur in the type and pattern of perivascular innervation. It has been reported that there is an increase in the density of catecholaminergic fibres in certain vascular beds during development of hypertension in the spontaneously hypertensive rat (Scott and Pang, 1983; Dhital *et al.*, 1988; Donohue *et al.*, 1986). Some changes have also been reported in peptidergic innervation of blood vessels in hypertension but these are not universal (Scott *et al.*, 1986; Lee *et al.*, 1988).

Diabetes is a disease which involves alterations in vascular structure and function. In diabetic rats, endothelium-dependent relaxation has been shown to be impaired (Durante *et al.*, 1988), although conflicting reports do exist (Oyama *et al.*, 1986; White and Carrier, 1986; Wakabayashi *et al.*, 1987). The variation in observations may be related to the experimental models of diabetes used (genetic, streptozotocin, alloxan) or they may simply reflect differences due to variation between different vessels. Durante and co-workers (1988) have concluded from their experiments with aorta of genetically diabetic rats that a change in the synthesis of EDRF has occurred, since the decreased vasodilation was the same for both ACh and A23187 and the reaction to SNP was unchanged. There are also species differences in the effect of diabetes on contractile responses of arteries (Head *et al.*, 1987) which may result in part from changes in endothelium-dependent mechanisms.

Autonomic neuropathy is a common complication in diabetes (Hosking *et al.*, 1978). However, changes in perivascular innervation have not been well documented. No change in catecholaminergic innervation was found in cerebral vessels of diabetic rats (Lagnado *et al.*, 1987). A substantial reduction of perivascular catecholaminergic fibres has been found in the optic nerve of diabetic rats, but this was accompanied by an increase in nerve density around blood vessels of the vagus and sciatic nerves and the sympathetic chain (Dhital *et al.*, 1986). Since changes in sensory neurons in diabetes have been reported (Sidenius and Jakobsen, 1980) it is likely that changes in SP- and CGRP-containing perivascular nerve fibres would occur. In cerebral blood

vessels from diabetic rats no change in NPY innervation was found (Lagnado *et al.*, 1987). A significant reduction of perivascular VIP innervation has been reported in certain vessel beds in diabetes. In streptozotocin induced diabetes in the rat, the density if VIP-immunoreactive nerve fibres was reduced in the internal carotid, middle cerebral and anterior cerebral arteries at 8 weeks after the onset of diabetes (Lagnado *et al.*, 1987). Reduction of VIP-immunoreactive nerves has also been found in penile vascular tissue from 8 week streptozotocin diabetic rats and from human diabetic patients with impotence (Crowe *et al.*, 1983).

Atherosclerosis is a disease in which many components of the vascular system may be affected, including the endothelium. Several studies have reported a depression of endothelial derived vasorelaxation resulting from cholesterol feeding. Various mechanisms for this observed change in EDRF response have been suggested. It was concluded from studies of human atherosclerotic arteries and rabbit aorta that the defect involved the muscarinic receptors of the endothelium (Bossaler *et al.*, 1987). Harrison and co-workers (1987) have made similar conclusions from studies of atherosclerosis in monkeys. Most studies claim that the alteration is either a reduction in EDRF synthesis, or in cases with severe intimal thickening a reduced diffusion of EDRF. A study by Jayakody and co-workers (1988) compared the effects of cholesterol feeding in young and old rabbits. Despite significant structural differences, there was a reduction in relaxation in both groups. It has also been suggested that the amount of depression of endothelium-dependent vasorelaxation is related to the

severity of intimal lesions (Verbreuren *et al.*, 1986). It is therefore difficult to conclude which of the vascular changes involved in atherosclerosis are responsible for the observed reduction of EDRF activity.

It appears that the reduction in the ability of ACh to produce endothelium-dependent relaxations in capsaicin-treated rats is related to a change in either the muscarinic receptor population, the ability to synthesize EDRF, or the ability of the smooth muscle to relax. While this decreased EDRF response has been observed in hypertension, diabetes and atherosclerosis where changes are also known to occur in perivascular innervation, it is not possible to determine at this time if a common mechanism such as denervation is responsible for this effect.

4.3. The Effect of 6-OHDA Treatment on the ACh Response

It has been shown in this study that chemical sympathectomy with 6-OHDA does not cause a shift of the ACh concentration response curve in the rat mesenteric arterial bed (Figure 9, page 81). The trophic influence of the catecholaminergic nerve fibre system on vascular tissues has been well documented (Aprigliano and Hermsmeyer, 1977; Fronek, 1983; Bevan and Tsuru, 1981; Lee *et al.*, 1987; Dimitriadou, *et al.*, 1988). It is surprising then that removal of this nerve fibre plexus did not affect the endothelium-dependent vasodilatory response to ACh. The effect

of sympathectomy on EDRF ability of blood vessels has not been well studied. Therefore there have been no reports as yet to substantiate this observation.

It was noticed in this study that the pressor response to methoxamine was altered after 6-OHDA treatment (Figure 5, page 75). The perfusion pressure attained with methoxamine in tissues from rats treated with 6-OHDA was significantly lower than that for an equal concentration in untreated or vehicle-treated controls. A similar decreased reactivity of the mesenteric arterial bed has been reported for noradrenaline in spontaneously hypertensive rats, sympathectomized with a combination treatment of guanethidine and anti-nerve growth factor (Lee *et al.*, 1987). This decreased reactivity to methoxamine is not thought to affect the relaxation response to ACh.

4.4. The Response to SP in the Mesenteric Bed

Substance P was originally used in this study as an additional agonist to produce an EDRF response in the mesenteric arterial bed. It was intended to compare the effects of 6-OHDA and capsaicin treatments on the vasodilatory response to SP with those of ACh. However, administration of SP resulted in an increased pressure above that induced by methoxamine in the buffer. This pressor response is a dose dependent effect in this tissue and is not affected by removal of the endothelium (Figures 10, 11, pages 82, 83). When this pressor response was expressed as a

percentage potentiation of the methoxamine response, 6-OHDA treatment caused an increase in the effect (Figure 14, page 87).

SP is known as a potent endothelium-dependent vasodilator agent in a variety of blood vessels (Zawadzki *et al.*, 1981; Furchgott *et al.*, 1983). Its threshold for activity in many isolated blood vessels is in the picomolar range (Furchgott *et al.*, 1983). In this study doses of SP from 1 pmole to 0.1 μmoles were tested in the methoxamine constricted preparations. Relaxations to SP were not seen at any dose, while these preparations did give an EDRF response to 10^{-5} ACh in the perfusing fluid. No more than 2 doses of SP were administered to a tissue to avoid receptor desensitization which could mask a potential response. Thus, the perfused mesenteric arterial bed did not relax in response to SP. Although SP has been reported to cause endothelium-dependent relaxations in ring preparations of superior mesenteric artery (Furchgott *et al.*, 1983), this effect might be hidden in this more complex perfused system. It has been reported that SP does not cause relaxations in a perfused mesenteric bed preparation (Gulati *et al.*, 1982). In addition, based on studies with a variety of neuropeptides, Gardiner and colleagues (1987) have demonstrated that different peptides can affect different vascular beds and that the same peptides can have different effects depending on the mode and route of administration.

4.4.1. The Mechanism of Action of SP in the Mesenteric Bed

Substance P has been shown to potentiate the vasoconstrictor responses induced by NA in the isolated perfused mesenteric arterial bed of the rat (Gulati *et al.*, 1983). In this study concentrations of SP from 4 x 10^{-9}M to 4 x 10^{-7}M caused a dose dependent potentiation of the NA induced vasoconstrictor response. This effect was blocked by the use of saralasin, a specific antagonist of angiotensin II, suggesting that SP might either simulate or be modulated by angiotensin II. The use of indomethacin in this study indicated that SP does not simulate or modulate the production of prostaglandins to achieve its NA potentiating effect. It is suggested that SP may play an important modulatory role in aorenergic vasoconstriction of the rat mesentery. This study did not rule out the possibility that SP might have a direct nonspecific post synaptic sensitizing effect on the vascular smooth muscle.

In this thesis the pressor response to SP was also found to be dose dependent. This response can be expressed as the absolute change in perfusion pressure or as a percentage potentiation of the methoxamine induced pressure. Since SP may play a modulatory role in adrenergic vasoconstriction (Gulati *et al.*, 1983), the pressor response was calculated as a percentage increase in the methoxamine induced pressure measured just prior to injection of SP. The endothelium did not seem to be involved in this response since no change was observed after its removal (Figures 10, 11, pages 82, 83).

In addition to the observed pressor response to SP this study has also found that neonatal 6-OHDA treatment results in an increase of this pressor response (Figure 14, page 87). How might 6-OHDA cause this effect? The desired effect of 6-OHDA treatment in this tissue is removal of sympathetic innervation. As a result there may be changes in the peptidergic innervation during development. An increase in peptidergic innervation has been reported for guanethidine induced sympathectomy by Aberdeen and co-workers (1987). As a result of these trophic interactions it is possible that the reactivity of the mesenteric bed may be altered. It has already been shown in this study that a decreased reactivity to methoxamine resulted from 6-OHDA treatment. It has also been shown that SP causes only a very small increase in pressure in the presence of K^+ depolarizing buffer, thus ruling out any major contribution of a nonspecific action on the smooth muscle membrane (Figure 16, page 90). The effect of SP is also unlikely to be an independent effect of activation of SP receptors on the smooth muscle, since SP caused no measurable response in the absence of induced tone in the preparation. Therefore, it is possible that SP may act on the α-adrenoceptor at a site different from that occupied by methoxamine to cause its modulatory effect. In the case of a decreased catecholaminergic innervation and an increased peptidergic innervation, this effect of SP might be enhanced, resulting in an increased importance of its adrenergic modulatory role. However, the possibility of SP acting via an angiotensin II mechanism cannot be ruled out as saralasin was not used in this study. Further studies with antagonists such

as saralasin and other agonists such as 5-HT would be required to fully characterize the pressor action of SP in the mesenteric arterial bed.

4.5. Conclusion

This thesis has examined the effects of catecholaminergic and peptidergic denervation treatments on the actions of ACh and SP in the mesenteric arterial bed of the rat. It has shown that alteration of the peptidergic perivascular innervation is accompanied by a decreased EDRF response to ACh and that changes in the catecholaminergic fibres occur together with an enhancement of the SP modulation of adrenergic vasoconstriction.

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