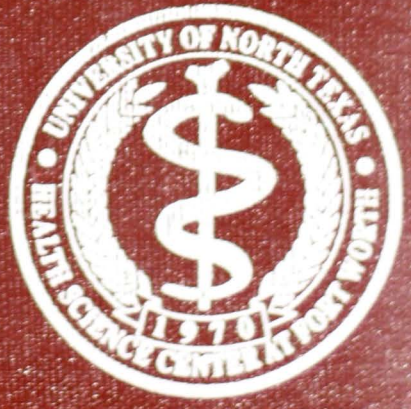
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FUNCTIONAL HETEROGENEITY IN CANINE

CORONARY RESISTANCE ARTERIES.

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FUNCTIONAL HETEROGENEITY IN CANINE CORONARY RESISTANCE ARTERIES

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Two thirds of the coronary vascular resistance resides in the smallest arteries and investigators have hypothesized that they may respond differently to endogenous vasoactive substances. The arterial responses to norepinephrine, acetylcholine, and adenosine were evaluated in large (> 700 μm, n = 24), intermediate (400 <> 600 μm, n = 24), and small arteries (< 300 μm, n = 24). Maximal vessel lumen diameter (D_{max}) was determined in Ca^{++} free medium. A reference diameter (84 ± 4.3 % of D_{max}) was established by re-equilibration in medium containing 2.0 mM Ca^{++}. Arterial maximal responses as a percentage of D_{max} to norepinephrine, acetylcholine, and adenosine are given in table 1:

TABLE 1

Agonists	Large % of D_{max}	Inter. % of D_{max}	Small % of D_{max}
Norepinephrine	41 ± 2.3	50 ± 4.2	83 ± 2.4
Acetylcholine	96 ± 2.7	88 ± 3.9	78 ± 1.9
Adenosine	71 ± 1.8	81 ± 4.2	96 ± 1.4

The sensitivity of the canine coronary arteries to norepinephrine, acetylcholine, and adenosine in terms of ED_{50}'s are given in table 2:

TABLE 2

Agonists	Large ED_{50} μM	Inter. ED_{50} μM	Small ED_{50} μM
Norepinephrine	0.037 ± 0.002	0.078 ± 0.004	no response
Acetylcholine	0.028 ± 0.003	0.087 ± 0.005	0.309 ± 0.03
Adenosine	0.295 ± 0.002	0.095 ± 0.004	0.035 ± 0.03

These data indicate that canine arterial responses to the native agonists norepinephrine, acetylcholine, and adenosine are heterogeneous and that neural control predominates in the larger "transport" arteries while local control predominates in the smaller "distributive" arteries.

Responses of small and intermediate isolated canine coronary arteries (lumen diameter 147±42μm, and 531±37 μm respectively) to norepinephrine were evaluated after pharmacological or mechanical interruption of endothelial relaxing activity. Following with the nitric oxide synthase inhibitor N-Nitro-L-Arginine Methylester (L-NAME) 10^{-5} M the small and intermediate vessels spontaneously constricted to 73±4.1% of D_{max} indicating a significant basal release of nitric oxide. After L-NAME or endothelial disruption graded additions of norepinephrine now reduced the vessel diameter in previously unresponsive small arteries. These data suggest that the weak and equivocal response of coronary resistance arteries to norepinephrine results from the competitive dilatory influence of endothelial derived nitric oxide production and not to the absence of norepinephrine receptors.

ACKNOWLEDGMENTS

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CHAPTER 1

INTRODUCTION TO THE STUDY

Introduction:

The coronary circulation has been the subject of concentrated study for many years due to the importance of supplying nutrients and removing metabolic waste to maintain the continued function of the heart. Coronary artery disease remains the major cause of death in the industrialized world. As a consequence we must understand the basic control mechanisms by which coronary blood flow is modulated. Investigators have defined five primary regulatory systems which modify coronary blood flow. These control systems are i) "local" or metabolic; ii) neural; iii) hormonal; iv) endothelial: and v) myogenic [47].

The most common result of coronary artery disease is myocardial ischemia, due to reduced blood flow, and a consequent imbalance between myocardial oxygen supply and demand. The prevailing cause of myocardial ischemia is the development of arteriosclerotic plaque within the coronary arteries. Arteriosclerosis not only physically restricts blood flow but also inhibits the functions of the coronary vascular endothelium [1, 3, 16, 18, 27]. Since the endothelium mediates coronary vascular dilation, impaired endothelial function may increase vascular reactivity during ischemia, thus decreasing blood flow even further to the already ischemic tissue. Chilian et. al. [12] demonstrated that the coronary vascular effects of serotonin, ergonovine, and phenylephrine were

potentiated in the atherosclerotic cynomolgus monkey heart compared to normal hearts. Kuo et. al. [52], working with isolated arteries from normal and atherosclerotic pig hearts, determined that the normal endothelial dependent vasodilatory effects of ATP, serotonin, and histamine were significantly attenuated and that normal endothelial dependent flow induced vasodilation was abolished in atherosclerotic porcine hearts.

Berne [4] proposed that during myocardial hypoxia cardiac myocytes release adenosine, a potent vasodilator which increased coronary blood flow. However, when coronary atherosclerosis is the cause, the ischemic myocardium cannot maintain normal contraction and cardiac output. The resulting decline in blood pressure activates high pressure baroreceptor reflexes and increases sympathetic activity producing a direct a-receptor mediated coronary vasoconstriction which competes with local metabolic vasodilatory mechanisms [25]. These examples illustrate the complex interaction among vascular control systems which may lead to impaired cardiac function when disturbed. Therefore, we must continue to pursue the basic mechanisms by which blood flow within the coronary circulation is modulated

Coronary blood vessels do not respond independently to signals from any one control system. The actual responses are the net result of a complex integration of multiple control systems. Thus, to study the effects of any one control system it must be effectively isolated from the other vascular controllers. One practical method of eliminating competing vascular control systems is to isolate an arterial segment in a tissue bath. Isolation of vessel segments effectively removes autonomic nervous, hormonal, and local myocardial control mechanisms. However, the vascular endothelium and smooth muscle

contribution remain intact necessitating the use of pharmacological blockade to effectively eliminate the endothelial influence and isolate the muscle response.

The coronary vasculature is innervated by both sympathetic and parasympathetic nerves [26, 25, 27]. Sympathetic activation releases the coronary vasoconstrictor norepinephrine. Norepinephrine vasoconstricts arteries by activation of α_1-receptors stimulating of the release of the second messenger IP_3 which increases intracellular calcium ion concentrations. Norepinephrine may also mediate vasoconstriction by activating α_2-receptors which reduces cyclic-AMP. When β_2-receptors are present, norepinephrine can vasodilate through the stimulation of adenylate cyclase. Norepinephrine also dilates vessels through vascular endothelial purinergic receptors activating nitric oxide synthase to produce nitric oxide. Release of nitric oxide from the vascular endothelium relaxes smooth muscle through the activation of cyclic-GMP. Finally, norepinephrine release from the sympathetic nerves causes β_1-receptor activation on cardiac myocytes to increase the myocardial force of contraction. Increased cardiac work increases myocardial oxygen demand resulting in the release of vasodilatory metabolites such as adenosine.

Parasympathetic activation leads to the release of the neurotransmitter acetylcholine. In the intact canine coronary vascular system acetylcholine produces an endothelium dependent vasodilation which utilizes nitric oxide as described above [27, 30-32, 45]. Acetylcholine can also constrict vascular smooth muscle directly through muscarinic M_2-receptors which act on membrane calcium channels [45]. The vascular endothelial response is mediated by muscarinic M_3-receptors. Finally, acetylcholine release from the parasympathetic nerves causes muscarinic receptor activation on cardiac myocytes to decrease the myocardial force of contraction. Decreased force of contraction by the

cardiac myocytes decreases myocardial work, and oxygen demand resulting in fewer vasodilatory metabolic byproducts such as adenosine and a secondary vasoconstriction.

Historical Perspective:

The first attempts to determine the effects vasoactive agents on the coronary vascular tree involved bolus injections of agents into coronary vessels *in vivo* while measuring coronary blood flow. This method continues to provide an overall *in vivo* picture with all vascular control systems functioning normally. When combined with pharmacological blockade of one or more vascular control systems, intraarterial injections can generate significant information about specific effects of the vascular agent under study. However, complete isolation of any vascular control system *in vivo* is difficult at best. For example, sympathetic release of norepinephrine increases during α-receptor blockade, and the vascular responses are complicated by both β_2-mediated dilation and the secondary consequences of β_1-mediated increases in myocardial metabolism [25].

In order to more directly examine the effects of the vascular control systems on the coronary vasculature, Furchgott [28, 29] developed the isolated arterial strip method which effectively removed neural, hormonal, and local metabolic control systems from consideration. The strip method consists of dissecting a spiral segment of the artery, suspending it in a tissue bath, and attaching it to a force transducer which measures the force of contraction exerted. Furchgott first demonstrated that the vascular endothelium exerted a significant influence on vascular control using strips [32]. He observed that acetylcholine relaxed strips of rabbit aorta with intact endothelial cell layers as

previously reported *in vivo* . However, whenever the endothelial cell layer was removed, he obtained an unexpected vasoconstrictive response to acetylcholine (see FIGURE 1) [32].

Despite the distinct advantages of arterial strips, this method does have a serious drawback in that the vessels do not function *in vivo* as strips, but as continuous cylinders. Furthermore, the act of cutting the vessel into strips disrupts the continuity of the endothelial cell layer. Although force of contraction indicates vasoconstriction or vasodilation, this remains an indirect measurement of vascular diameter changes. Furthermore, the longitudinal tension applied to vessel strips differs from the combined radial and tangential wall stresses which develop from intraluminally applied pressure. Finally, the strip method is limited to relatively large vessels which can be mechanically dissected into strips.

Bevan et. al. [10] developed the isolated "ring" method to avoid several of the drawbacks associated with strips. In the ring method, isolated arteries are cut transversely and mounted on parallel wires passed longitudinally through the vascular segment. The wires are reflected in opposite directions and attached to the bottom of a tissue chamber and to a force transducer (see FIGURE 2). This method corrects many of the problems associated with strips since the tension applied to the ring better approximates the normal forces. Although the wires placed through the vascular lumen of necessity do some damage, the ring method causes less damage to the endothelial cell layer than occurs during the preparation of arterial strips. However, the ring method still depends upon measuring developed tension rather than vessel diameter. In both the ring and arterial strip methods a vasoactive agent exposes both the lumenal and abluminal sides of the vessel simultaneously so differences in polarity are difficult to evaluate.

Halpern and Kelly [44] developed a method which permits investigators to study vessels under near physiological conditions while removing most control systems. The "Halpern myograph" overcomes most of the disadvantages associated with the previous isolated vessel methods. In the "Halpern myographic" method the vessels are cannulated at each end, placed within a physiological bath, and the lumenal pressure is adjusted as desired. This method is illustrated in FIGURE 3. As a result, the vessel can be perfused luminally at physiological pressures or superfused abluminally. The vessel assumes a normal round shape and develops spontaneous tone, exhibits myogenic responses to changes in lumen pressure, and the endothelium is undisturbed, [43]. The vessel sizes that may be used are only limited by the size of the cannulas; thus very small coronary resistance arteries may be investigated. Furthermore, the vessels can be returned to their normal *in situ* length [43]. Finally, vessel diameter and wall thickness are measured rather than developed tension. Thus, this method allows for an *in vitro* environment which much more closely resembles the normal physiological state of the vessel.

Rationale:

The purpose of this study is to investigate the roles of autonomic, local metabolic, and vascular endothelial influences in controlling blood flow through canine coronary arteries using the method described by Halpern [44] and Duling [24] . The use of isolated vessels and the pharmacological blockade of nitric oxide synthesis will allow for the independent study of vascular and endothelial control systems. The interaction of the vascular endothelium and the autonomic nervous system will also be studied.

The sympathetic neurotransmitter norepinephrine will be superfused through the abluminal chamber to examine the effects of the sympathetic nervous system on canine coronary epicardial arteries and arterioles. The sympathetic nerves release norepinephrine on the abluminal side of the vessel wall. Therefore, infusion of norepinephrine into the chamber perfusate will mimic normal norepinephrine release from the sympathetic nerve terminals. Abluminal infusion of norepinephrine may need to diffuse farther in larger vessels, due to the increase in wall thickness, resulting in lower concentrations as one approaches the endothelium. However studies by Halpern et al [44] have not demonstrated any significant differences between luminal or abluminal administration of norepinephrine in isolated rat mesenteric arteries under no flow conditions. However, when luminal flow was permitted the normal vasoconstrictive response to norepinephrine from either the luminal or abluminal side was inhibited [44]. This may have been due to shear stress mediated release of nitric oxide from the vascular endothelium [9, 53].

Norepinephrine activates coronary vascular α_1-receptors and β_2-receptors to produce vasoconstriction and vasodilation respectively. Several investigators have observed that small coronary arteries dilate in response to norepinephrine in *in vitro* preparations [16, 56, 70]. They proposed that this vasodilation was either the result of direct norepinephrine-mediated β_2-receptor activation [70] or the indirect result of suppressing norepinephrine release via prejunctional α_2-receptors [16]. However, the vasodilatory responses to norepinephrine in the coronary microvasculature may also be due to norepinephrine induced release of nitric oxide. This study will attempt to determine which of these hypothesis are true.

Chilian et. al. [17] has determined that approximately 60% of coronary resistance resides in the small resistance arteries. Since these small arteries do not constrict to norepinephrine, the adrenergic control of the coronary vasculature observed in intact animals may be limited to the control of the larger arteries and the remaining 40% of the coronary resistance.

Acetylcholine will be likewise introduced into the vessel chamber perfusate to determine the effects of parasympathetic stimulation on the canine coronary arterial tree. Acetylcholine activates endothelial muscarinic M_2-receptors to produce an endothelial dependent vasodilation within the coronary vascular system [23, 45]. In the absence of the vascular endothelium acetylcholine constricts coronary arteries through the activation of M_3 muscarinic receptors on vascular smooth muscle[45]. This would imply that the effect of acetylcholine on the vascular endothelium is opposed by the vascular smooth muscle effect. In *in vitro* systems isolated coronary vessels with intact endothelium consistently vasodilate to either luminal or abluminal administration of acetylcholine with no significant difference [44]. The lack of a significant difference between abluminal or luminal administration of acetylcholine would indicate that concentration differences due to diffusional direction is not significant.

Local or metabolic control is mediated, in part, through the myocardial release of adenosine [26, 25]. Therefore, adenosine infusion into the vessel chamber perfusate will be used to determine the influence of "local" metabolic stimulation. Adenosine is known to stimulate endothelial cell release of nitric oxide by activating A_2-adenosine receptors leading to an endothelial mediated vasodilation [5, 14, 25, 30]. Adenosine also acts directly on vascular smooth muscle A_1-adenosine receptors to cause relaxation leading to direct vasodilation [5, 14, 25, 30].

Changes in lumenal diameter will be monitored on a video dimension analyzer and recorded on video tape and a strip chart recorder. Resistance vessels from three size groups, larger arteries (> 700 μm in lumen diameter), intermediate arteries (400 <> 600 μm in lumen diameter), and small arteries (< 300 μm in lumen diameter) will be tested with norepinephrine, acetylcholine, and adenosine.

The effects of the endogenous vasoactive substances norepinephrine, acetylcholine, and adenosine may not be uniform throughout the coronary vascular tree but may vary instead with the function of the vessel involved. Previous reports have suggested the responses to vasoactive agents do not have uniform responses throughout the coronary vascular tree but rather that the response may vary with arterial size. A histochemical study by Cannon and Jones [11] found that the metabolic profile of large canine coronary arteries differed from that of the smaller arteries. The large arteries appeared more dependent on aerobic metabolism, while the smaller arteries were better adapted to anaerobic metabolism. Other workers have also reported similar changes in the metabolic profiles of large and small canine coronary arteries [20, 51]. Functionally, the large conduit arteries are responsible for the delivery of adequate quantities of blood to large areas of the myocardium. Therefore, the regulation of large arteries is more concerned with global myocardial needs. The smaller coronary arteries are then more responsive to local conditions and appear to satisfy local tissue nutrient needs. Furthermore, because large numbers of the small resistance arteries are arranged in parallel, changes in their lumen diameters would provide the largest component to the total coronary resistance [25, 73]. Therefore, isolated canine coronary arteries will be tested for

size dependent heterogeneity in response to the endogenous vasoactive agents norepinephrine, acetylcholine, and adenosine.

This study will utilize a Halpern isolated vessel myograph to investigate the effects of norepinephrine, acetylcholine, and adenosine on various size epicardial coronary resistance arteries in the dog to address the following hypotheses.

Hypotheses:

1. Canine coronary artery responses to the neurotransmitters norepinephrine and acetylcholine will be greater in large conduit arteries and less in small distributive arteries.

2. The response to the local metabolite adenosine will be greater in the small distributive arteries and less in the large conduit arteries.

3. Endothelial mediated responses will be evaluated in all vessels but the relative influence of the endothelium will be greatest in the smallest vessels.

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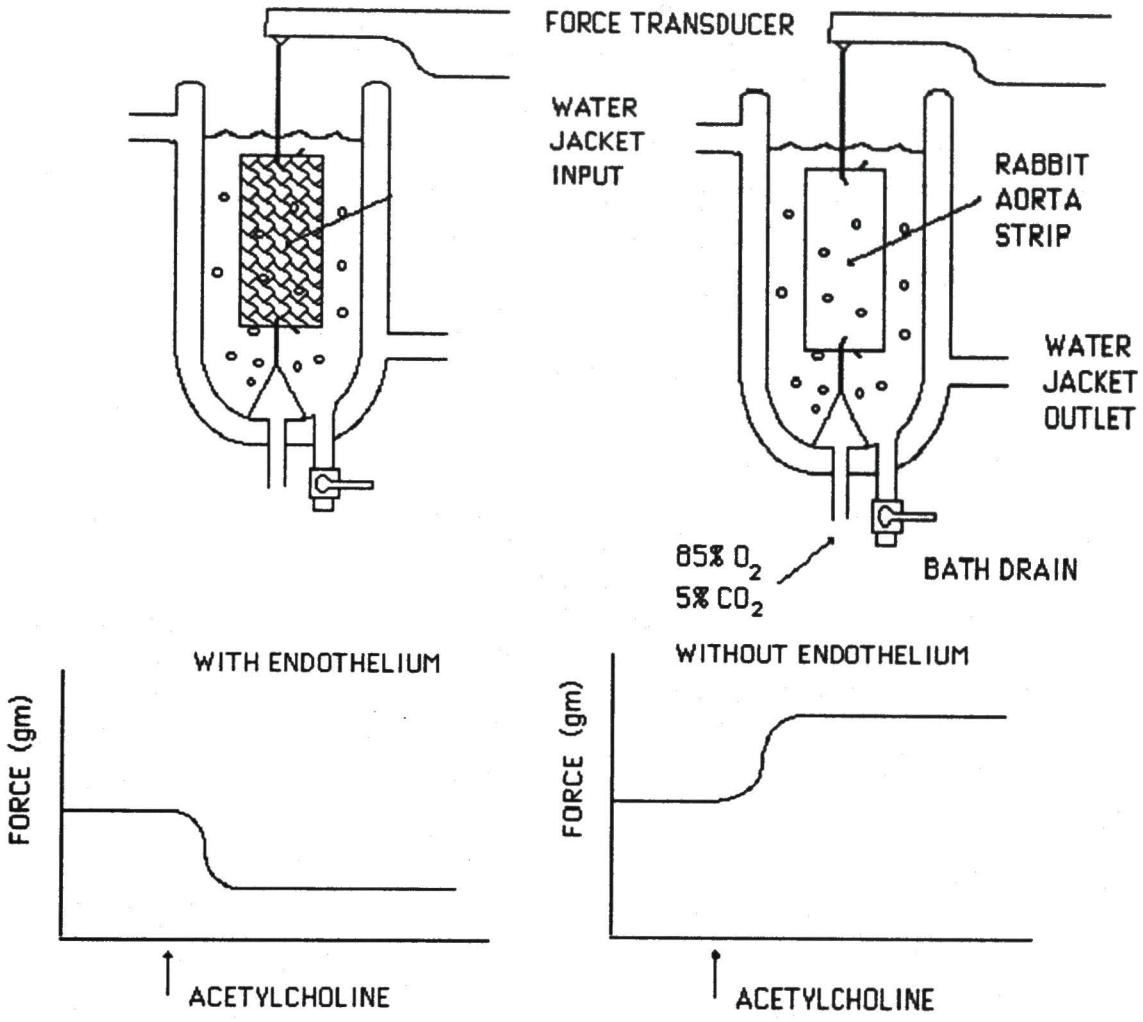
FIGURE LEGENDS

FIGURE 1 - This figure illustrates both the arterial strip method (top panel) and Furchgott's experimental results with acetylcholine (bottom panel). When acetylcholine is added to the tissue bath containing the rabbit aortic strip (left panel) with intact endothelium the strip relaxes and the measured force decreases. When acetylcholine is added to the tissue bath containing the rabbit aortic strip with the endothelial cell layer removed (right panel) the strip contracts and the measured force increases.

FIGURE 2 - This figure illustrates the ring method used in isolated vessel research.

FIGURE 3 - Illustrates the systems employed to measure vessel lumen diameter and wall thickness. The chamber perfusion pump and heating coil maintains the perfusate at 37^{o}C and allows for rapid exchange of perfusate and the introduction of agents. The servo pressure system maintains the intraluminal pressure as desired. The video dimension analyzer and microscope measure the vascular lumen and wall thickness from recorded TV images.


FORCE TRANSDUCER
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RABBIT AORTA STRIP
WATER JACKET OUTLET
85% O_2
5% CO_2
BATH DRAIN
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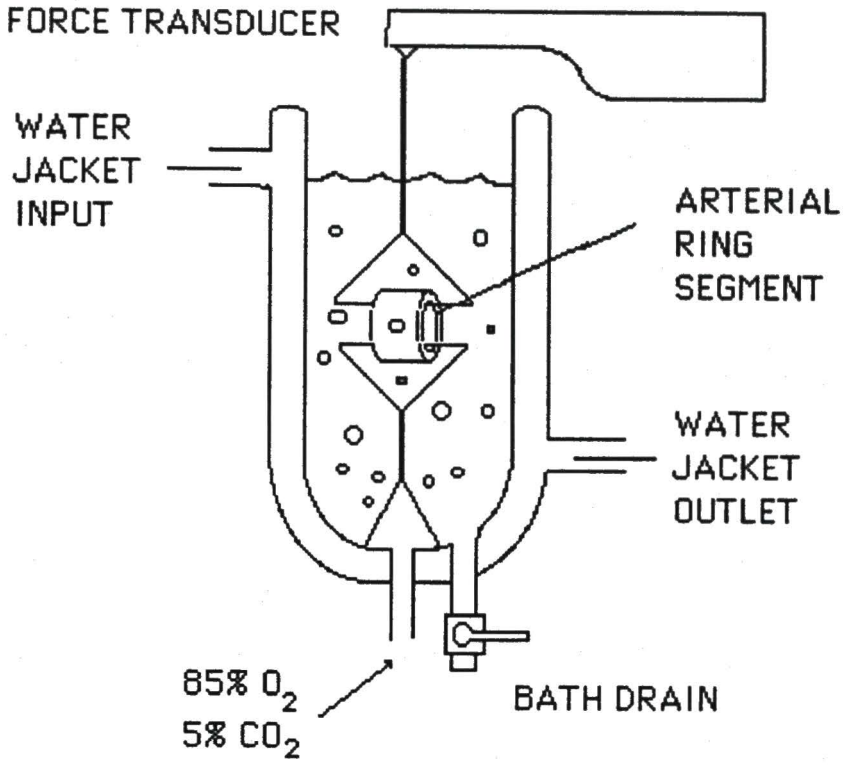
FORCE (gm)
ACETYLCHOLINE
WITHOUT ENDOTHELIUM
FORCE (gm)
ACETYLCHOLINE

FIGURE 1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WATER
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BATH DRAIN

FIGURE 2

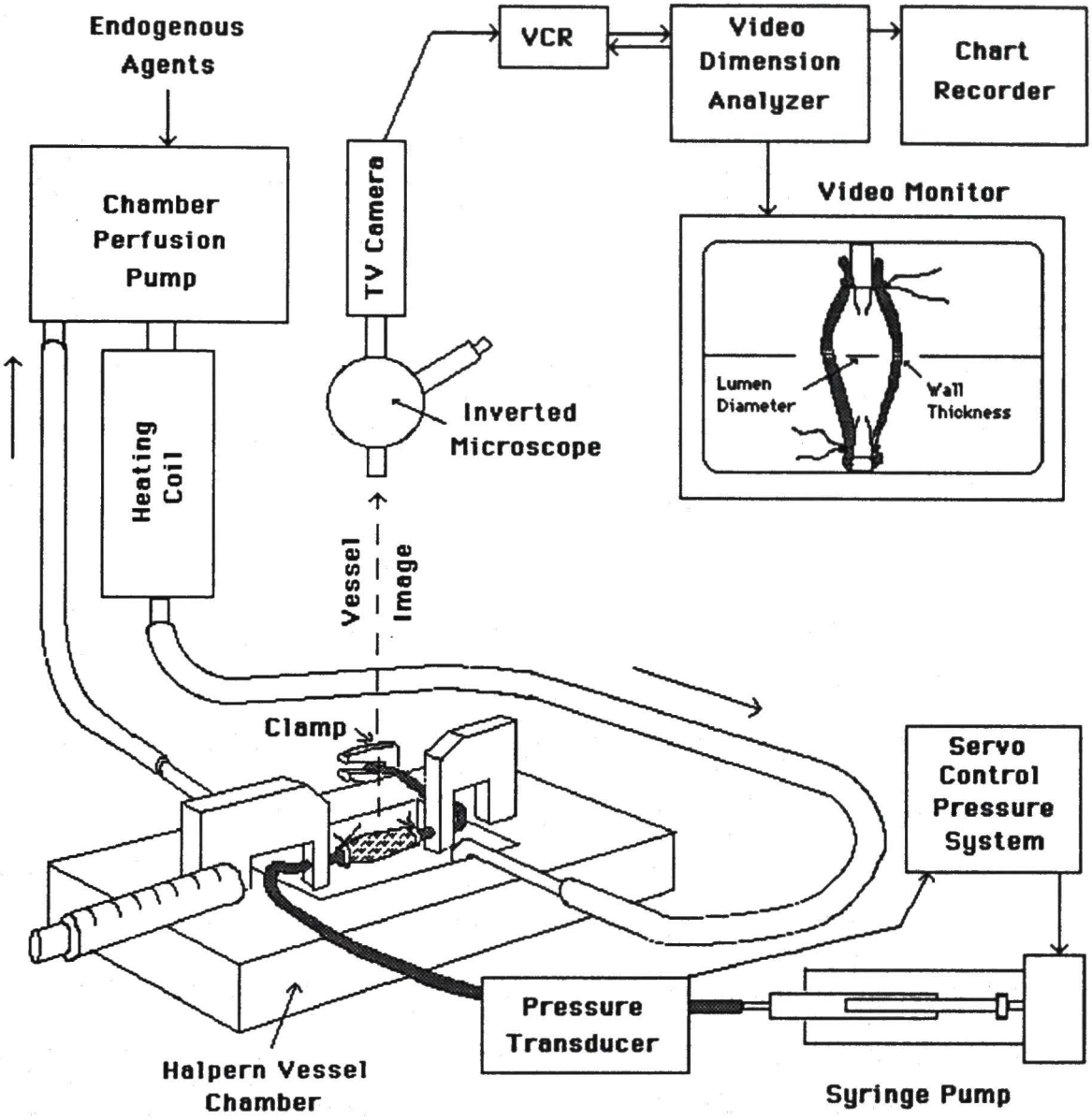
Endogenous Agents
Chamber Perfusion Pump
Heating Coil
TV Camera
VCR
Video Dimension Analyzer
Chart Recorder
Video Monitor
Lumen Diameter
Wall Thickness
Inverted Microscope
Vessel Image
Clamp
Servo Control Pressure System
Pressure Transducer
Halpern Vessel Chamber
Syringe Pump

FIGURE 3

CHAPTER 2

FUNCTIONAL HETEROGENEITY IN CANINE CORONARY RESISTANCE ARTERIES.

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Abstract:

Two thirds of the coronary vascular resistance resides in the smallest arteries and investigators have hypothesized that they may respond differently to endogenous vasoactive substances. The arterial responses to norepinephrine, acetylcholine, and adenosine were evaluated in large (> 700 μm, n = 24), intermediate (400 <> 600 μm, n = 24), and small arteries (< 300 μm, n = 24). Isolated arteries were mounted in a vessel chamber (Halpern), the lumens pressurized to 40 mmHg at zero flow and diameters were determined with a video dimension analyzer (Living Systems). Maximal vessel lumen diameter (D_{max}) was determined in Ca^{++} free medium. A reference diameter (84 ± 4.3 % of D_{max}) was established by re-equilibration in medium containing 2.0 mM Ca^{++}. Arterial vasoconstrictive responses to norepinephrine were greatest in the large (41 ± 2.3 % of D_{max}; ED_{50} = 0.037 ± 0.002 μM), intermediate in the intermediate (50 ± 4.2 % of D_{max}; ED_{50} = 0.078 ± 0.004 μM), and absent in the small arteries

(83 ± 2.4 % of D_{max}). Vasodilatory responses for acetylcholine and adenosine were determined in vessels precontracted to approximately 50% of D_{max} with KCl. The arterial vasodilatory response to acetylcholine was greatest in the large (96 ± 2.7 % of D_{max}; ED_{50} = 0.028 ± 0.003 μM), intermediate in the intermediate (88 ± 3.9 % of D_{max}; ED_{50} = 0.087 ± 0.005 μM), and least in the small arteries (78 ± 1.9 % of D_{max}; ED_{50} = 0.309 ± 0.03 μM). The arterial vasodilatory response to adenosine was least in the large (71 ± 1.8 % of D_{max}; ED_{50} = 0.295 ± 0.002 μM), intermediate in the intermediate (81 ± 4.2 % of D_{max}; ED_{50} = 0.095 ± 0.004 μM), and greatest in the small arteries (96 ± 1.4 % of D_{max}; ED_{50} = 0.035 ± 0.03 μM). These data indicate that canine arterial responses to the native agonists norepinephrine, acetylcholine, and adenosine are heterogeneous and that neural control predominates in the larger "transport" arteries while local control predominates in the smaller "distributive" arteries.

Key Words: Isolated vessel, Norepinephrine, Acetylcholine, Adenosine, Coronary arteries

Introduction:

Investigators have speculated that canine coronary arterial responses to endogenous vasoactive agents may not be uniform [3, 11, 13, 48, 67]. Support for this hypothesis has been provided by a number of observations *in vivo* where precise determination of vessel size was not possible [4-7, 26, 25, 36, 37, 39-41, 46, 48, 49, 66, 73, 74, 76, 79]. The interaction between added agonist and vessel size *in vivo* was complicated further by direct contributions from endogenous neurotransmitters and the local metabolic consequences of their indirect action on the myocardium.

The activation of the sympathetic nervous system releases the neurotransmitter norepinephrine which constricts coronary arteries through α-adrenoceptors primarily concentrated on vascular smooth muscle [1-3, 34]. The α-adrenoceptor-mediated vasoconstriction to norepinephrine is opposed by vascular β_2-receptors, the release of nitric oxide from the endothelium, and the secondary effect of adenosine release by the cardiac myocytes [26, 25, 59, 70]. Despite the assembly of opposing vasodilator influences, the net response to norepinephrine remains vasoconstriction [26, 36, 37, 59]. Even during exercise, persistent sympathetically mediated coronary vasoconstriction produced a limitation on myocardial performance which was effectively restored by the α_1-receptor antagonist prazosin [37]. Similar effects have been reported by others [2, 13, 21]. Exogenously administered norepinephrine consistently reduces coronary blood flow in intact canine hearts and this response can be eliminated by the α_1-receptor antagonist prazosin [19, 26, 37-39, 46, 48, 49, 77, 80-82]. Although small coronary arteries (< 300 μm) represent the majority of the coronary vascular resistance [17], recent observations suggests these vessels are largely unresponsive to norepinephrine [16, 63, 70, 82]. These observations imply that coronary arterial vasoconstrictive responses to norepinephrine in intact animals are mediated by large and intermediate coronary arteries. Since approximately 60 % of coronary resistance resides in small arteries which do not respond to norepinephrine [17], less than half of the coronary resistance may be available to direct neural control.

Coronary vessels are also innervated by parasympathetic nerve fibers [27], which release acetylcholine. Activation of the vagus nerve increases coronary blood flow presumably through the activation of endothelial M_2-muscarinic receptors, and the release of endothelial derived nitric oxide, a strong

vasodilator [45]. A direct cholinergic vasoconstriction mediated by M_3-muscarinic receptors is sometimes observed [45]. Like norepinephrine, acetylcholine produces responses which appear to depend on the size of the artery evaluated [63, 72]. Coronary cholinergic responses are also species dependent [61, 63, 72, 78]. Acetylcholine produces endothelium dependent vasodilation in rabbits, dogs, and rats [1, 32, 34], while the net balance favors vasoconstriction in pigs, sheep, and probably man [23, 34, 63].

The possibility that the effects of adenosine may also depend on vessel size has been extensively investigated [4-6, 14, 33, 71, 75]. In fact, early investigators speculated that the effect of adenosine would be greatest on the microcirculation due to the massive increase in blood flow observed [4-6, 14, 75]. Chilian and Layne [14] examined small coronary arteries (< 200 μm in luminal diameter) *in vivo* and reported that they are more sensitive to adenosine than intermediate arteries (300<> 600 μm in luminal diameter).

While most of these studies suggest that a size dependent functional heterogeneity exists in the coronary circulation they do not provide precise measures of vessel diameter under controlled conditions, nor do they categorize a variety of agonists in a full range of vessel sizes. Since both of these measures are needed to evaluate for functional transition zones, this study includes careful dose responses for norepinephrine, acetylcholine, and adenosine not only in small and large arteries but also in a spectrum of intermediate size arteries in between.

Materials and Methods:

Eighty four Mongrel dogs of either sex were anesthetized with sodium pentobarbital (34 mg/Kg i.v.) and the hearts removed through a left throacotamy. The hearts were immediately placed in cold, calcium free physiological salt solution (PSS) containing 1% albumin. The left anterior descending coronary artery (LAD) was cannulated, and perfused with a mixture of calcium free PSS-albumin, gelatin, and india ink at 30 to 35°C until all the surface arteries were clearly visible. The heart was replaced in iced PSS-albumin for approximately 5 minutes to allow the intraluminal solution to gel. The LAD region was cut free and immediately immersed in a dissecting dish containing calcium free PSS-albumin solution maintained at approximately 4°C. Constituents of the calcium free PSS solution were as follows (all quantities in mM): 145 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 1.2 NaH_2PO_4, 5.0 glucose, 2.0 pyruvate, 0.02 ethylenediaminetetraacetic acid, 3.0 3-(N-morpholino) propanesulfonic acid [54]. The pH was adjusted to 7.4 at the required temperature [53]. The PSS-gelatin-india ink suspension was prepared by mixing 10 ml of warm PSS, 0.2 ml of non-dialyzed and non-waterproof india ink (Koh-I-Noor), and 0.36 gm porcine skin gelatin [53]. The PSS, gelatin, india ink mixture forms a solid at temperatures below 20°C which facilitates microvessel dissection. The mixture liquefies again when physiological temperatures are reestablished and is easily flushed from the vessel.

Three groups consisting of 24 dogs in each group (total = 72 dogs) were each treated with one of the following vasoactive agents: norepinephrine, acetylcholine, and adenosine. Each of the treatment groups were randomly split into three subgroups (n = 8 in each group) consisting of small resistance arteries with lumen diameters between 60 and 200 μm, intermediate arteries with lumen

diameters between 400 and 600 μm, and large transport arteries with lumen diameters greater than 700 μm. Epicardial arteries were selected according to size, dissected free, carefully transferred in a Pasteur pipette to a lucite tissue chamber (Halpern) and mounted between two glass cannulas (O.D. 40 - 800 μm), as described by Osol and Halpern [68] and Duling et. al. [24]. The tissue chamber was transferred to a microscope stage equipped with a video dimension analyzer (Living Systems Inc.), a video tape recorder, and a strip chart recorder (see Figure 1). The arteries were distended with an intraluminal pressure of 40 mmHg which approximates intraluminal pressure estimates for coronary resistance arteries *in vivo* [17]. All studies were conducted at zero flow to prevent flow induced endothelial release of nitric oxide [9, 30, 44, 53]. The extravascular chamber was then perfused (12 to 16 ml/min.) with calcium free PSS-albumin and the temperature allowed to rise to 37°C, where it was maintained by a circulating heat exchanger. After equilibration of the isolated artery for one hour in calcium free PSS, the maximal vasodilation (D_{max}) was recorded. Calcium (2.0 mM) was added to the chamber perfusate and the vessel was again allowed to equilibrate for approximately one hour before measuring the resting or reference diameter.

Vessels were evaluated for viability using the criteria introduced by Duling et. al. [24] for microcirculatory vessels: i) development of spontaneous tone at 37°C, ii) spontaneous vasomotion (in PSS), iii) myogenic responses, and iv) responsiveness to vasoactive drugs. All vessels used satisfied all criteria except for the vasomotion requirements since vasomotion is not normally observed in arterioles with lumen diameters greater than 80 μm [22]. All vessels were myogenically active, developed spontaneous tone, and responded to

acetylcholine prior to use. Five out of the 84 vessels failed to meet these criteria and were rejected.

Cumulative dose responses to L-norepinephrine, acetylcholine or adenosine were conducted by increasing the concentration in the perfusate from 10^{-10} to 10^{-4} M. Dilatory responses to acetylcholine and adenosine were conducted in vessels precontracted to approximately 50% of D_{max} with KCl. After the initial dose responses, vessels were returned to calcium containing PSS-albumin and allowed to equilibrate until a steady state was observed (about 20 min.). To evaluate the functional integrity of the endothelium, vessels were preconstricted to approximately 50% of D_{max} with KCl and graded doses of acetylcholine were added 10^{-10} to 10^{-4} M. Vessels which failed to dilate in response to acetylcholine were removed from the study (n=7 out of 84).

Data Analysis:

Changes in vessel diameter were normalized as a percentage of the initial calcium free maximal lumen diameter (D_{max}). The mean maximal responses, and their respective ED_{50}'s were compared by analysis of variance. A $p < 0.05$ was accepted as significantly different, and multiple post-Hoc comparisons to identify which specific groups differed were made with Sheffe's test. Values were expressed as means ± SE.

Results:

All vessels were equilibrated in calcium free medium prior to determining their luminal diameter at maximal vasodilation (D_{max}). The mean luminal diameter for large arteries (> 700 μm) was 860±43 μm, n=24, for intermediate arteries (400<>600 μm) was 532±50 μm, n=24, and for small arteries (<300 μm) was 158±35 μm, n=24. The introduction of 2.0 mM Ca^{++} to the chamber

perfusate resulted in a mean vasoconstriction to 84±5.3% of D_{max} which was not significantly different among size groups.

Norepinephrine:

The response of isolated canine coronary arteries to norepinephrine was measured in larger arteries with a mean luminal diameter of 871±49 μm (n=8), intermediate arteries with a luminal diameter of 522±48 μm (n=8), and in small arteries with a luminal diameter of 147±35 μm (n=8). Isolated canine coronary arteries reacted to graded doses of norepinephrine with a significant ($p < 0.05$) constriction in both large arteries (maximal response 41±2.3% of D_{max}, with an ED_{50} of 0.037±0.003 μM) and intermediate arteries (maximal response 50±3.6% of D_{max}, ED_{50} of 0.078±0.004) (FIGURES 2 and 3). The dose response for large arteries was shifted to the left of that observed for intermediate arteries and the ED_{50} and maximal response are significantly different from one another. However, in the small resistance arteries the responses to norepinephrine were equivocal in nature, with some arteries dilating slightly (n=5) and some constricting slightly (n=3). The resulting maximal response of 83±2.4% of D_{max} was not different from control, and a meaningful ED_{50} could not be determined. Maximal constrictor responses to norepinephrine for individual vessels are plotted in Figure 4. These data provide support for the existence of three functionally distinct groups of vessels with average diameters clustered around 100, 500, and 800 μm. The data also suggest the existence of functional transition zones between each vessel group where vessel response vary rapidly with the diameter. The transition vessels appear to be clustered in ranges 300 - 400 μm and 600 - 700 μm.

Acetylcholine:

The response of isolated canine coronary arteries to acetylcholine was measured in large arteries with a mean luminal diameter of 852±51 μm (n-8), in intermediate arteries with a mean luminal diameter of 549±52 μm (n=8), and in small resistance arteries with a mean luminal diameter of 173±43 μm (n=8). Vessels were precontracted with KCl to 46±3.7% of D_{max}. The percent of precontraction was not significantly different among vessel sizes. The isolated canine coronary arteries responded to graded doses of acetylcholine with a significant ($p < 0.05$) vasodilation in all size groups (Figures 5). Acetylcholine produced the greatest response in large arteries (maximal response of 96±2.0% of D_{max} with an ED_{50} of 0.028±0.003μM), an intermediate response in intermediate arteries (maximal response of 88±3.6% of D_{max} with an ED_{50} of 0.087±0.05μM), and the least response in small arteries (maximal response of 78±1.7% of D_{max} with an ED_{50} of 0.309±0.003μM) (FIGURES 5 and 6). The dose response curve for each successively larger group of vessels was shifted further to the left and the ED_{50}'s and maximal responses for each were significantly different from each other. When individual vessel responses to acetylcholine are plotted versus vessel size (FIGURE 7), the same three functional groups and transition zones observed for responses to norepinephrine appear again for acetylcholine. The three groups of vessels display the same rank order of response to acetylcholine, and vessels in the transition zones between groups appear clustered again in the 300 - 400, and 600 - 700 μm ranges as observed in response to norepinephrine.

Adenosine:

The responses of isolated canine coronary resistance arteries to adenosine were measured in large arteries with a mean luminal diameter of 863±44 μm (n-8), in intermediate arteries with a luminal diameter of 529±48 μm (n=8), and in small arteries with a mean luminal diameter of 151±35 μm (n=8). Vessels were precontracted with KCl to 44±4.1% of D_{max}. The isolated canine coronary arteries responded to graded doses of adenosine with significant ($p < 0.05$) vasodilation in all size groups (FIGURE 8). However, adenosine was significantly less effective in large arteries (maximal response of 72±1.8% of D_{max} with an ED_{50} of 0.295±0.003μM) than in intermediate arteries (maximal response of 81±4.6% of D_{max} with an ED_{50} of 0.095±0.006μM) and most effective in small arteries (maximal response of 96±1.2% of D_{max} with an ED_{50} of 0.035±0.002μM) (FIGURES 8 and 9). As a result the rank order of responses was opposite to that observed for norepinephrine and acetylcholine with the adenosine dose response curves for intermediate and small vessels shifted progressively to the left of the larger vessels. In each case the ED_{50} for one size vessel was significantly lower than that for the next larger size group of vessels. Once again, when the individual vessel responses were plotted versus vessel diameter the same three functional groupings emerge from the data. Here the order is, however, reversed with the smallest vessels being the most responsive to adenosine. Transition zones also appear again to be positioned in the same size ranges, *i.e.* between 300 - 400 μm and between 600 - 700 μm.

Discussion:

This study demonstrated that canine coronary arterial responses to the endogenous vasoactive compounds norepinephrine, adenosine, and acetylcholine were heterogeneous in a size dependent manner. This consistent

heterogeneity in response suggests that these vessel groups may be controlled by different mechanisms and serve fundamentally different functions. If large resistance arteries are chiefly concerned with the rapid global delivery of O_2 and nutrients, then greater autonomic control of these arteries might be expected since the autonomic nervous system is generally more concerned with whole organ function rather than the local control of blood flow. In contrast, if the small resistance arteries are more concerned with local requirements, then they should respond to local myocardial conditions largely independent of general autonomic nervous input.

For instance, the larger arteries which respond well to norepinephrine and acetylcholine should be more responsive to neural control and, as a result, may provide for rapid and / or global redistribution of flow. In contrast the small arteries which are most responsive to adenosine and unresponsive to norepinephrine should respond well to local myocardial metabolic conditions and may serve primarily local distributive and nutritive functions.

The reproducible identification of transition zones in the same size ranges for all agonists reinforces the concept of functionally distinct resistance vessel categories and suggests a physical or biochemical basis for the differences observed. A study on the effects of serotonin and vasopressin in the open chest cat found that arteries larger than 90 μm constricted while arteries smaller than 90 μm dilated to serotonin. Arteries larger than 90 μm did not respond to vasopressin while arteries less than 90 μm constricted [55]. This study implies that distinct size related differences exist in the coronary arteries of the cat and that these differences occur at approximately the same size range for both serotonin and vasopressin [55]. Histochemical investigations indicate that the metabolic profile of large and small canine coronary arteries differed [11]. These

studies suggest that the large arteries are dependent on aerobic metabolism, while the smaller arteries are better adapted for anaerobic metabolism [11]. Evaluations of the enzymes of the canine arterial wall suggests that at about an arterial diameter of 500 to 800 μm anaerobic enzymes become more prominent while aerobic enzymes decline [51]. This is approximately the same transition zone as reported in this study separating intermediate and large coronary arteries. These metabolic differences suggest again that larger vessels may respond more to intravascular conditions where the PO_2 is high while smaller vessels are better adapted to local myocardial conditions where the PO_2 is considerably lower.

Since the coronary vascular endothelium responds to norepinephrine, acetylcholine, and adenosine by releasing nitric oxide, a powerful vasodilator [14-16, 53, 56, 57, 60-62, 67, 70], some responses may reflect the relative strength of endothelial and smooth muscle contributions to the final net response. As vessels enlarge one predicts that the endothelial influence should decline for two reasons. First large arteries have several layers of smooth muscle and the ratio of endothelium to muscle will decline progressively as the wall thickens. As a result, the constrictor capacity continues to increase while the opposing endothelial influence presumably remains constant. Second, as the vessel wall thickens the distances traversed by endothelial products increase with greater opportunities for degradation, thus, decreasing the local concentration of the endothelial secretions. Consequently the vascular smooth muscle in larger arteries should be increasingly independent of the endothelium.

Responses to norepinephrine or acetylcholine in specific size vessels represent net responses which result from varying degrees of opposing dilator and constrictor activities respectively. In this regard we reported that the

equivocal response to norepinephrine in the small resistance arteries results from the competing dilatory influence of endothelial nitric oxide [69]. If the synthesis of nitric oxide is blocked or the endothelium is mechanically removed these vessels contract much like their larger predecessors. This would imply that endothelial release of nitric oxide inhibits norepinephrine mediated vasoconstriction in the small canine coronary arteries. If the smallest resistance arteries already have high baseline rates of nitric oxide synthesis, then this may explain their relative insensitivity to added acetylcholine since this agonist depends on increasing endothelial derived nitric oxide production. Other investigators have also found adrenergically mediated vasodilatory responses in the small coronary resistance arteries [70]. They attributed the response to α-adrenergic receptor mediated release of endothelial nitric oxide and to the direct relaxation of smooth muscle by β-adrenergic receptors [70]. The responses observed in small isolated coronary arteries have been confirmed *in vivo* [16]. in the cat where vessels below 100 μm in diameter vasodilated in response to norepinephrine [16].

Investigators [17, 64] demonstrated that 60 to 70% of coronary resistance to blood flow resides in arteries below 200 μm in diameter in cat and rabbit hearts, respectively. We have assumed that the majority of vascular resistance in the canine heart resides in similar sized arteries. Arteries of this category have been shown to differ from the large arteries in their response to a variety of endogenous vasoactive substances [16, 50]. Since small arteries represent the majority of the vascular resistance these observations suggest that the coronary microcirculation should vasodilate in response to norepinephrine infusions. However, many investigators have demonstrated that infusions of norepinephrine decrease coronary blood flow, when secondary contractile demands are blocked [37, 46, 48, 49] suggesting that integrated responses *in vivo* are complex.

In summary, this study has demonstrated that the canine arterial response to endogenous vasoactive substances is heterogeneous. Norepinephrine and acetylcholine have greater effects on the large resistance arteries with reproducible declines in response within two transition zones occurring between 300 to 400 μm and 600 to 700 μm in diameter. The order of response to adenosine is reversed with greater effects on small resistance arteries with transition zones occurring in virtually the same size ranges as found for norepinephrine and acetylcholine. These data support the hypothesis that larger arteries (> 400 μm) in the coronary circulation are functionally distinct from their smaller (< 200 μm) successors. The differential quality of responses to neurotransmitters (norepinephrine, acetylcholine) and local metabolites (adenosine) are correlated with the hypothesis that the larger arteries are concerned more with the rapid neurally mediated redistribution of flow and the smaller arteries more concerned with responding to local metabolic requirements.

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FIGURE LEGENDS

FIGURE 1 - Illustrates the systems employed to measure vessel lumen diameter and wall thickness. The chamber perfusion pump and heating coil maintains the perfusate at 37°C and allows for rapid exchange of perfusate and the introduction of agents. The servo pressure system maintains the intraluminal pressure at 40 mmHg. The video dimension analyzer and microscope measure the vascular lumen and wall thickness from recorded TV images.

FIGURE 2 - Control and maximal constrictor responses to 10^{-4} M norepinephrine expressed as a percentage of maximal Ca^{++} free diameter. Values are means ± SE, * indicates responses are significantly different from control, $p < 0.05$. Control n = 24, large n = 8, intermediate n = 8, and small n = 8. # indicates responses are significantly different from each other.

FIGURE 3 - Dose responses to norepinephrine for large, and intermediate sized canine coronary arteries. The changes in the small resistance arteries were not sufficient to produce reliable dose response curves. The values are means ± SE; n = 8 for each group. Responses in the large and intermediate sized vessels were significantly different, $p < 0.05$.

FIGURE 4 - Scatter plot of vessel size versus maximal response to 10^{-4} M norepinephrine for all vessels (n = 36).

FIGURE 5 - Control and maximal dilator responses to 10^{-4} M acetylcholine expressed as a percentage of maximal Ca^{++} free diameter. Vessels were first preconstricted to 46±3.7% of maximal Ca^{++} diameter with KCl. Values are means ± SE; * indicates responses are significantly different from control, $p < 0.05$. Control n = 24, large n = 8, intermediate n = 8, and small n = 8; # indicates responses are significantly different from other sized vessels.

FIGURE 6 - Dose responses to acetylcholine for large, intermediate, and small sized canine coronary arteries. The values are means ± SE; n = 8 for each group. Responses in the large, intermediate, and small sized vessels were significantly different ($p < 0.05$) from each other.

FIGURE 7 - Scatter plot of vessel size versus maximal response to 10^{-4} M acetylcholine for all vessels. All vessels were precontracted with KCl (dashed line) prior to the addition of acetylcholine (n = 33).

FIGURE 8 - Control and maximal dilator responses to 10^{-4} M adenosine expressed as a percentage of maximal Ca^{++} free diameter. Vessels were first preconstricted to 44±4.1% of maximal Ca^{++} diameter with KCl. Values are means ± SE, * indicates responses are significantly different from control, $p < 0.05$. Control n = 24, large n = 8, intermediate n = 8, and small n = 8. # indicates responses are significantly different from other vessel sizes.

FIGURE 9 - Dose responses to adenosine for large, intermediate, and small sized canine coronary arteries. The values are means ± SE; n = 8 for each group. Responses in the large, intermediate, and small sized vessels were significantly different ($p < 0.05$) from each other.

FIGURE 10 - Scatter plot of vessel size versus maximal response to 10^{-4} M adenosine for all vessels. Vessels were precontracted with KCl (dashed line) prior to the addition of adenosine (n = 36).

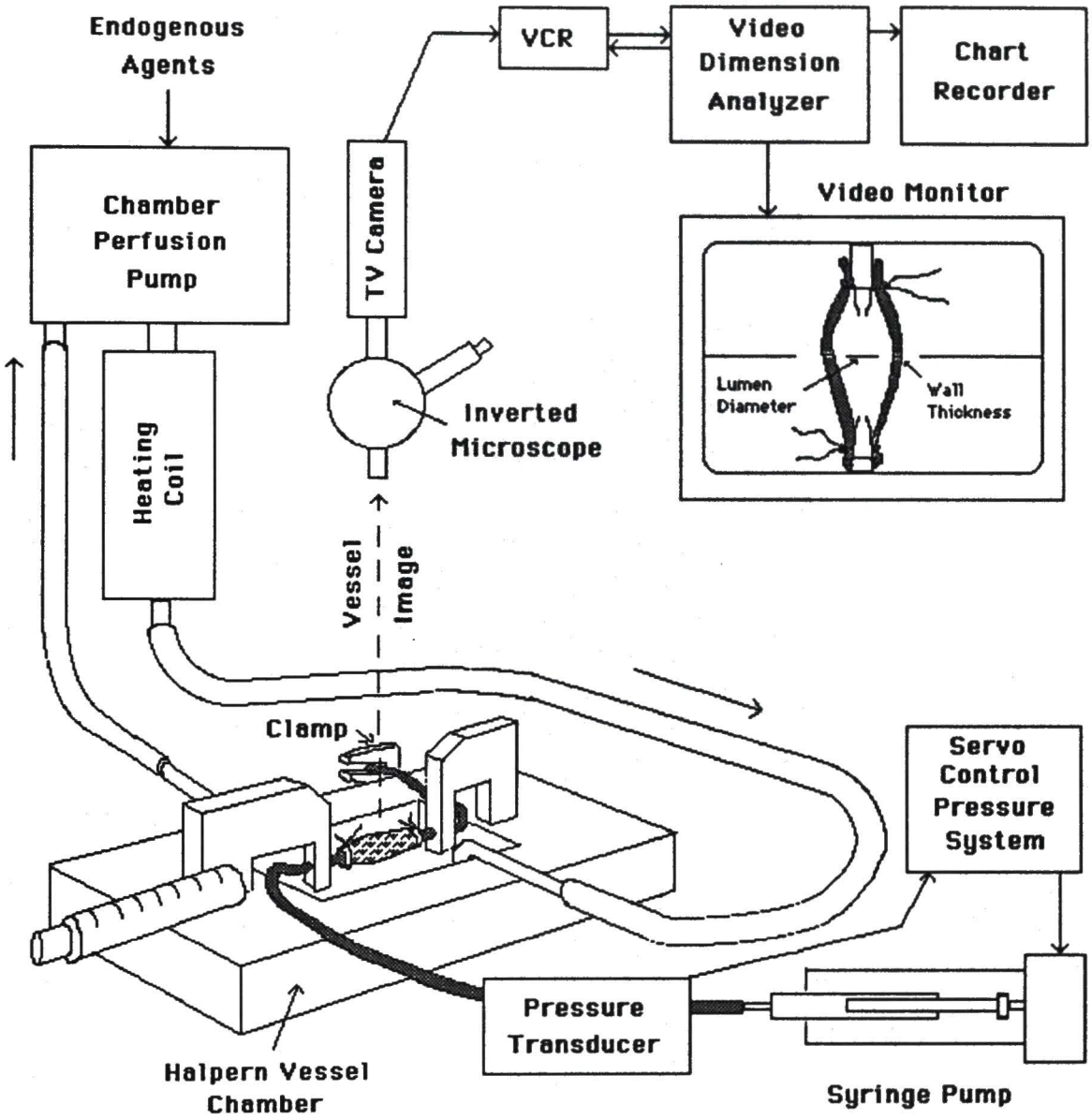
Endogenous Agents
Chamber Perfusion Pump
Heating Coil
TV Camera
VCR
Video Dimension Analyzer
Chart Recorder
Video Monitor
Lumen Diameter
Wall Thickness
Inverted Microscope
Vessel Image
Clamp
Servo Control Pressure System
Pressure Transducer
Halpern Vessel Chamber
Syringe Pump

FIGURE 1

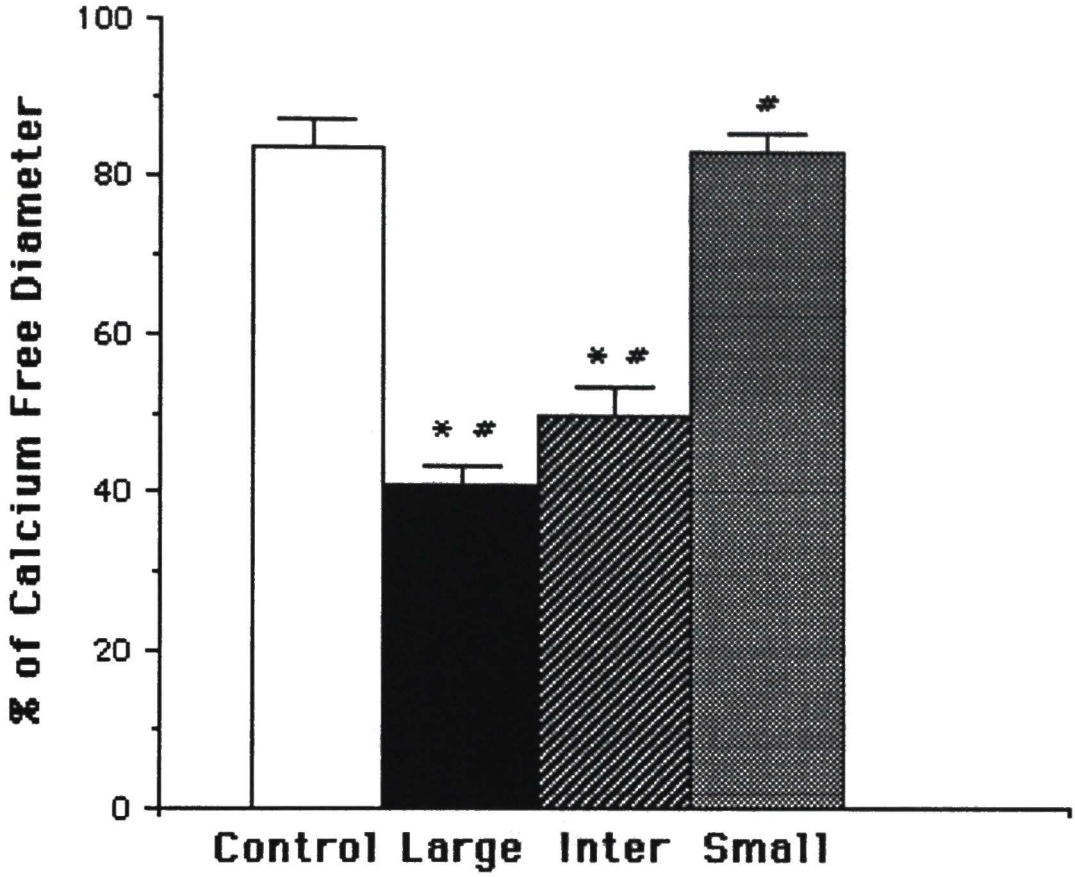
100
80
60
40
20
0
% of Calcium Free Diameter
* #
* #
#
Control Large Inter Small

FIGURE 2

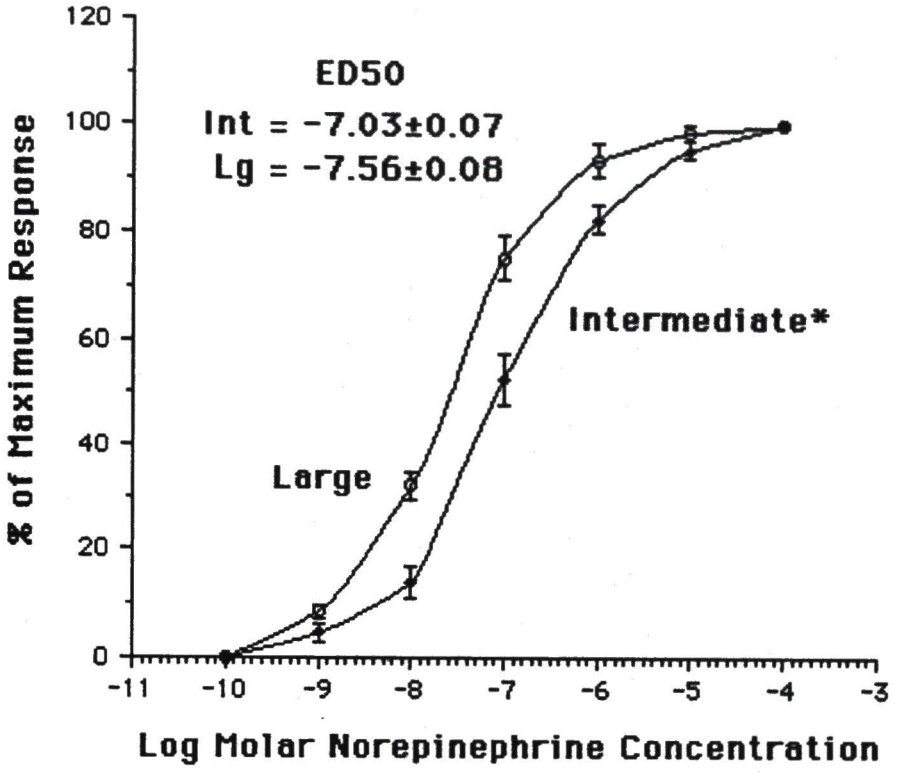
ED50
Int = -7.03±0.07
Lg = -7.56±0.08
Intermediate*
Large
% of Maximum Response
Log Molar Norepinephrine Concentration
120
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
-4
-3

FIGURE 3

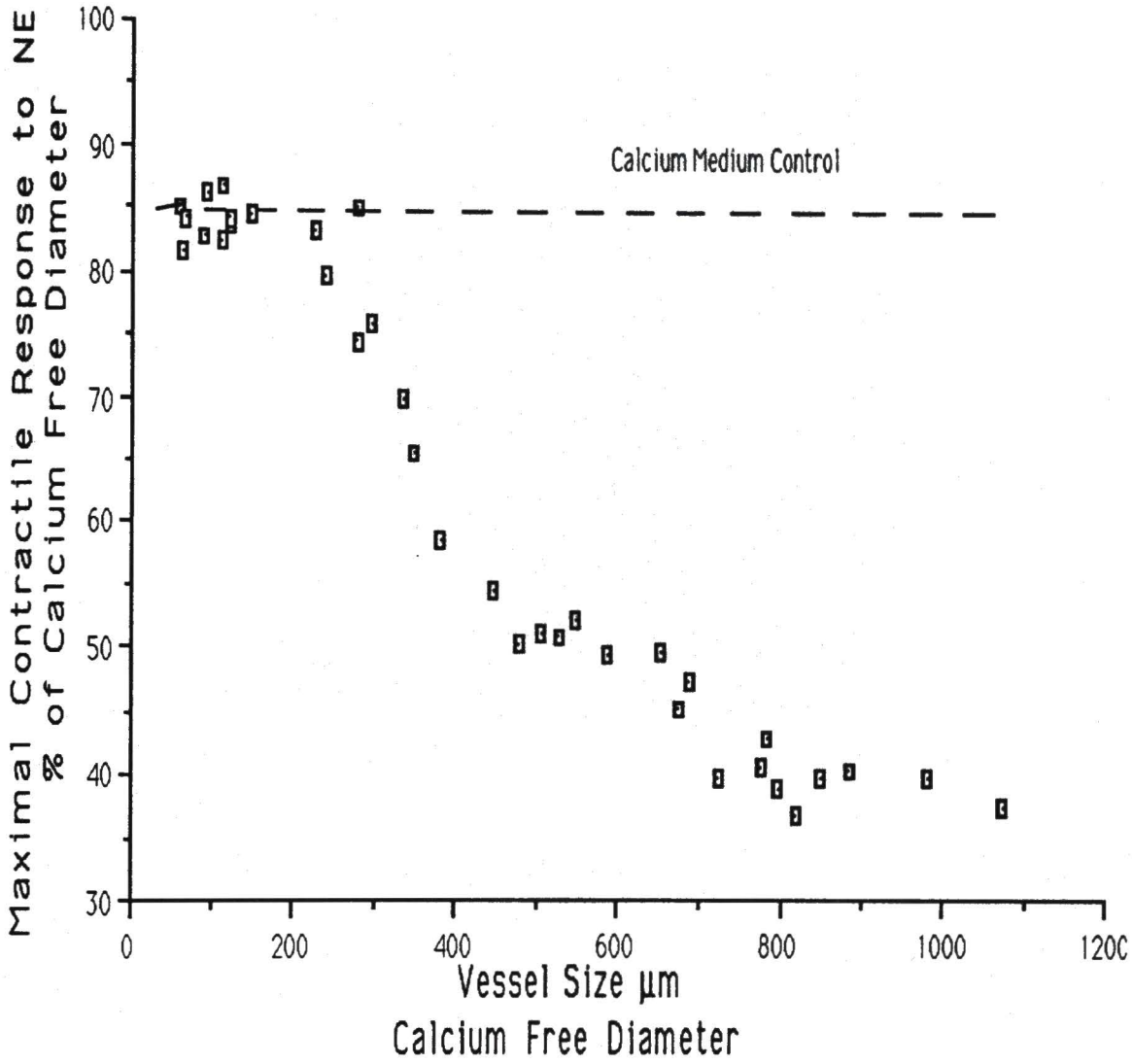
Maximal Contractile Response to NE
% of Calcium Free Diameter
100
90
80
70
60
50
40
30
Calcium Medium Control
0
200
400
600
800
1000
1200
Vessel Size µm
Calcium Free Diameter

FIGURE 4

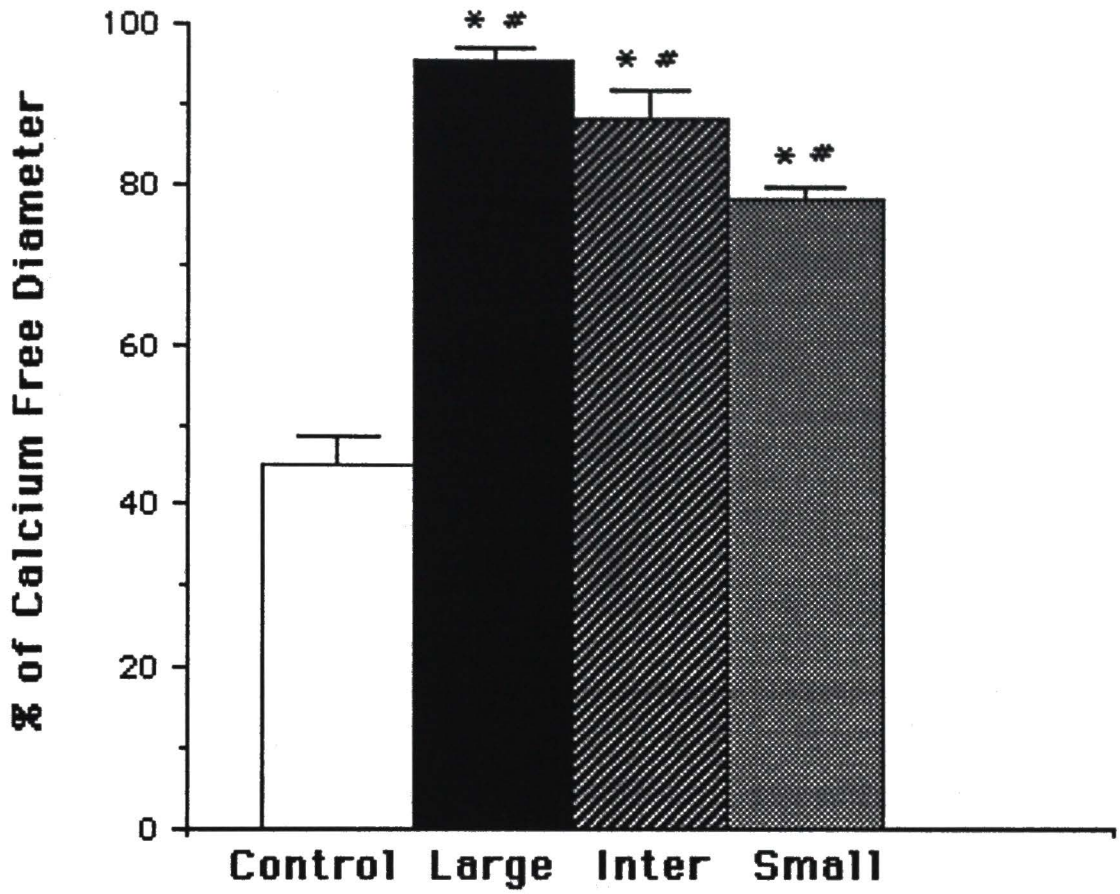
% of Calcium Free Diameter
100
80
60
40
20
0
* #
* #
* #
Control
Large
Inter
Small

FIGURE 5

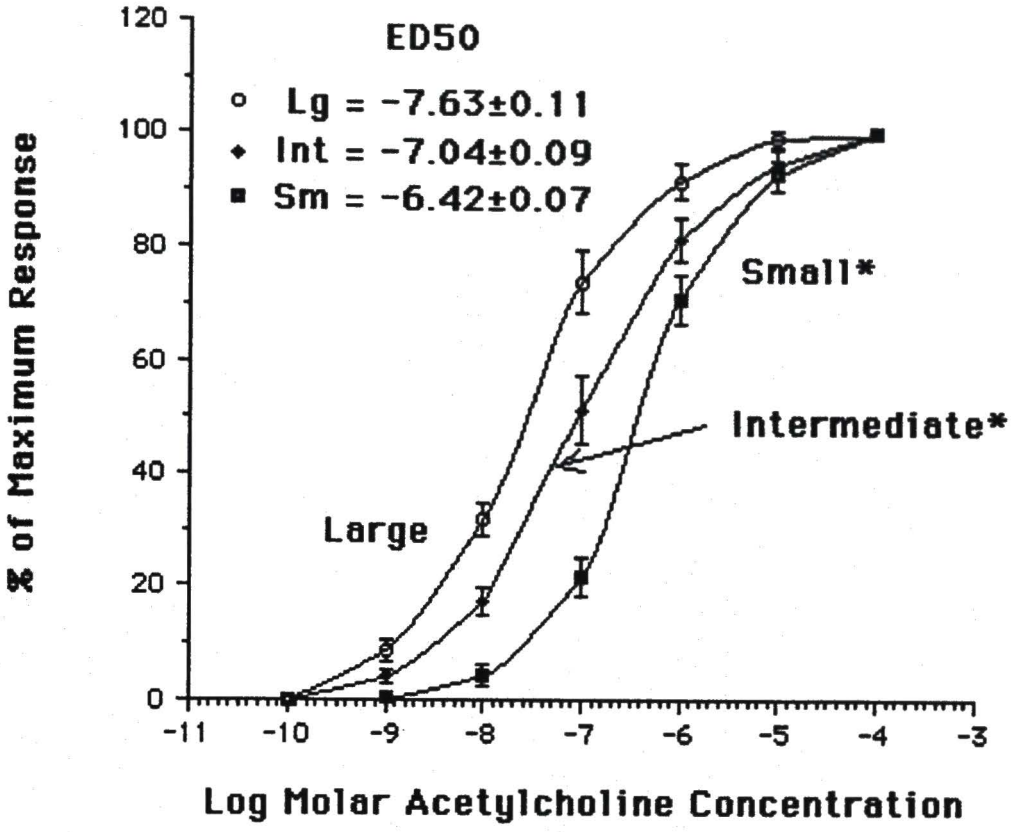
ED50
Lg = -7.63±0.11
Int = -7.04±0.09
Sm = -6.42±0.07
Small*
Intermediate*
Large
% of Maximum Response
Log Molar Acetylcholine Concentration
120
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
-4
-3

FIGURE 6

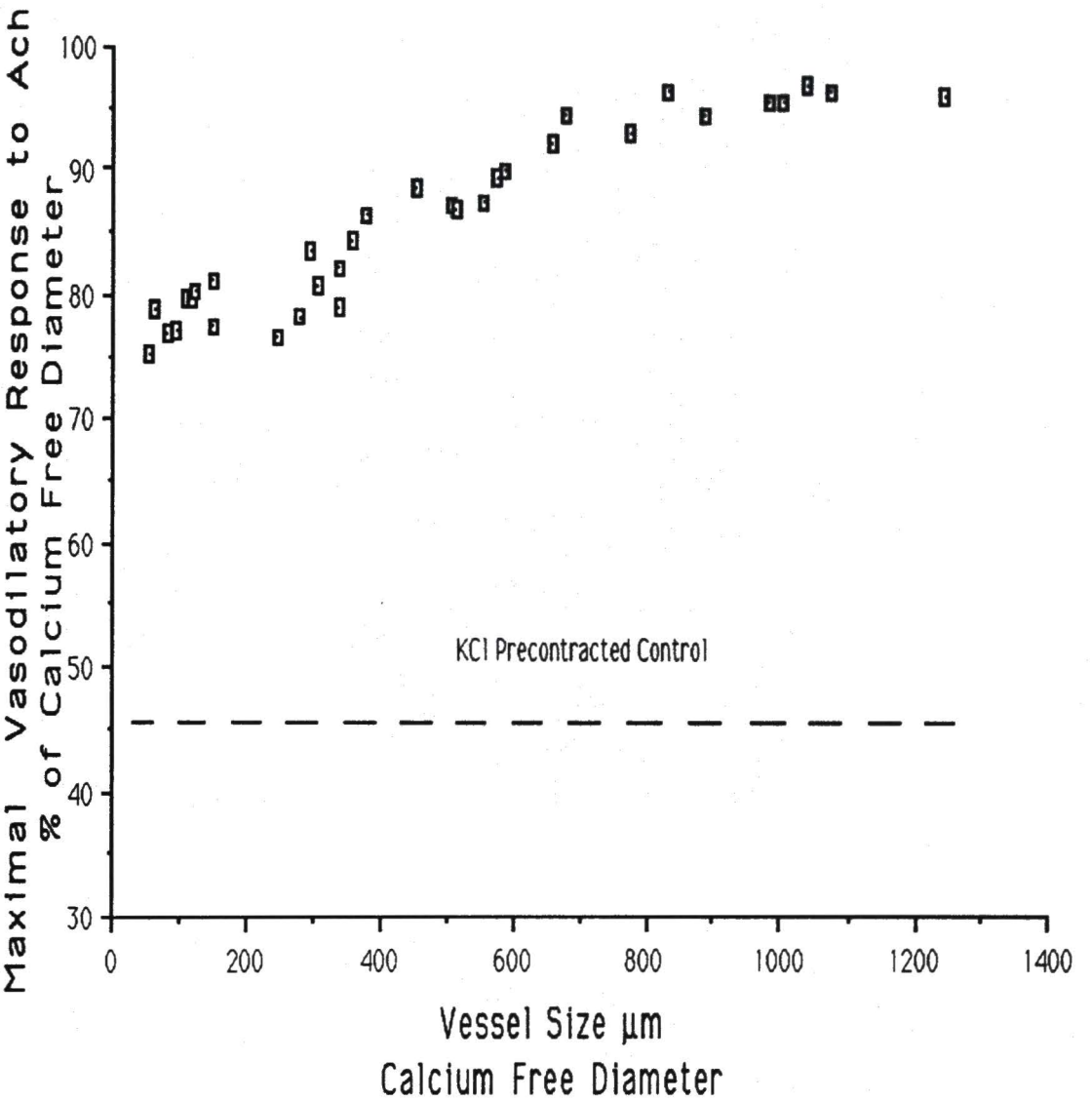
Maximal Vasodilatory Response to Ach
% of Calcium Free Diameter
100
90
80
70
60
50
40
30
KCl Precontracted Control
0
200
400
600
800
1000
1200
1400
Vessel Size µm
Calcium Free Diameter

FIGURE 7

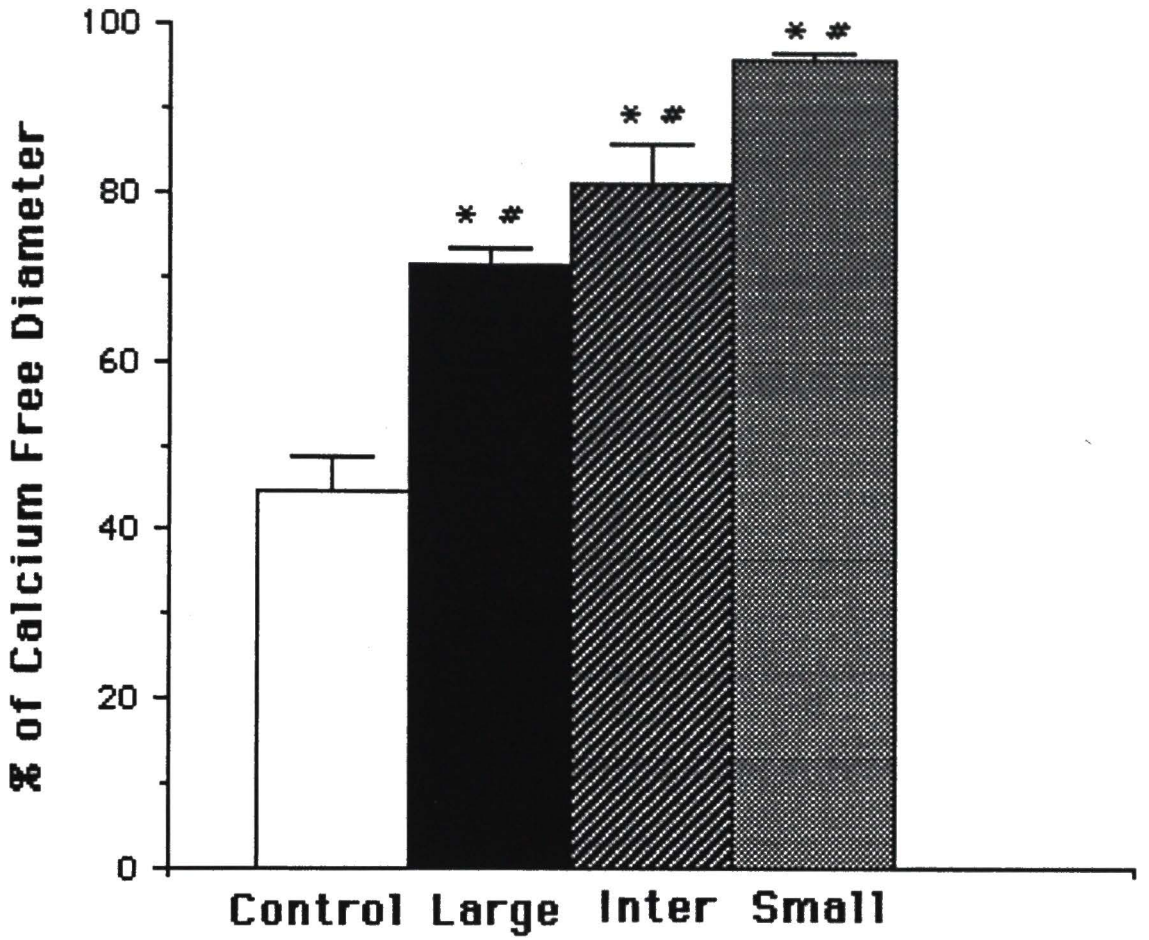
100
80
60
40
20
0
% of Calcium Free Diameter
* #
* #
* #
Control
Large
Inter
Small

FIGURE 8

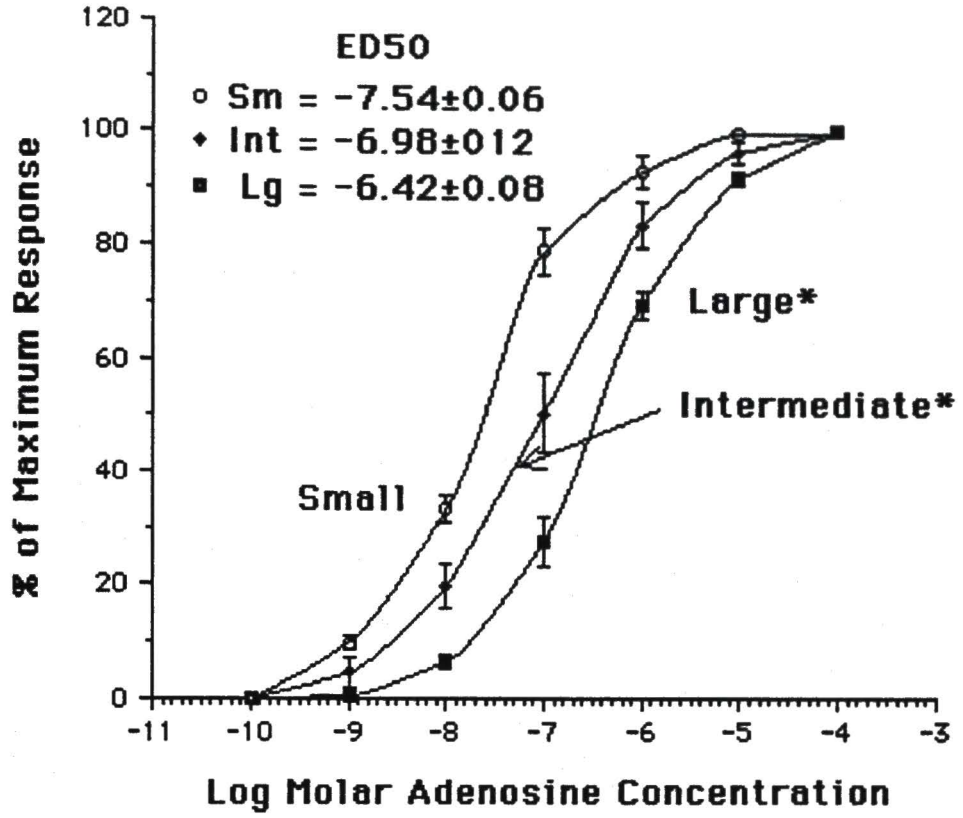
ED50
Sm = -7.54±0.06
Int = -6.98±012
Lg = -6.42±0.08
Large*
Intermediate*
Small
% of Maximum Response
Log Molar Adenosine Concentration
120
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
-4
-3

FIGURE 9

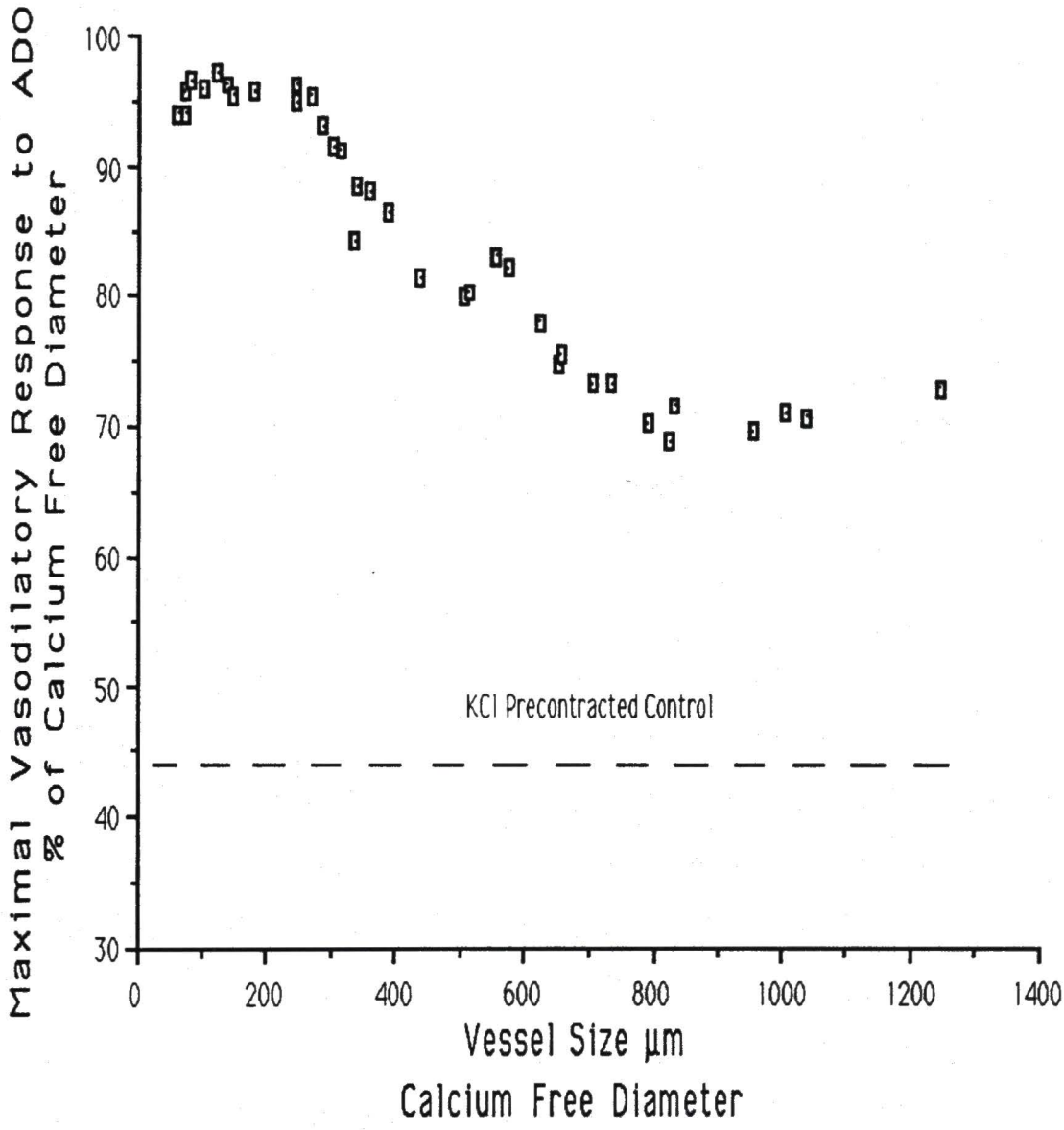
Maximal Vasodilatory Response to ADO
% of Calcium Free Diameter
100
90
80
70
60
50
40
30
KCl Precontracted Control
0
200
400
600
800
1000
1200
1400
Vessel Size µm
Calcium Free Diameter

FIGURE 10

CHAPTER 3

ENDOTHELIAL DERIVED NITRIC OXIDE PRODUCTION DETERMINES THE RESPONSES OF CANINE CORONARY RESISTANCE ARTERIES TO NOREPINEPHRINE.

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Abstract:

Responses of small and intermediate isolated canine coronary resistance arteries (lumen diameter 147±42μm, and 531±37 μm respectively) to norepinephrine were evaluated before and after mechanical or pharmacological interruption of endothelial derived relaxing activity. Blockade of relaxing activity was verified by the absence of acetylcholine-mediated relaxation. Isolated arteries were mounted in a vessel chamber (Halpern), the lumens were pressurized to 40 mmHg at zero flow and the vessel diameters were determined with a video dimension analyzer (Living Systems). Maximal vessel lumen diameter (D_{max}) was determined in a Ca^{++} free medium and a reference vessel diameter (84±5.3% of D_{max}) was established by equilibration of vessels in a medium containing 2.0 mM Ca^{++}. Increasing norepinephrine concentrations added to the chamber, in small arteries, produced a slight decrease in diameter (82±2.1% of D_{max}) in seven vessels and a slight increase (86±4.4% of D_{max}) in

eleven others. The overall changes were not significantly different from control in the small arteries (n = 18). In the intermediate arteries increasing norepinephrine concentrations produced a significant constriction to 51±4.7% of D_{max} in all arteries compared to control ($p<0.05$): ED_{50} = 0.081±0.018 μM (n=4). Following blockade of endothelial derived nitric oxide production with the nitric oxide synthase inhibitor N-Nitro-L-Arginine Methylester (L-NAME) 10^{-5} M the small vessels spontaneously constricted to 73±4.1% of D_{max} , and the intermediate to 72±2.7% of D_{max} indicating a significant basal release of nitric oxide. Graded additions of norepinephrine in small arteries reduced the vessel diameter to 56.6±2.3% of D_{max} ($p<0.05$) with an ED_{50} of 0.249±0.023 μM, and 43±3.4% of D_{max} ($p<0.05$) with an ED_{50} of 0.033±0.016 μM for the intermediate arteries. After mechanical removal of the endothelial cell layer, norepinephrine produced a reduction in vessel diameter (to 61.4±2.3% of D_{max} for small, and 52±3.8% of D_{max} for intermediate arteries) nearly identical to that observed under nitric oxide blockade and with nearly indistinguishable ED_{50}'s (0.245±0.021 μM for small, and 0.035±0.017 μM for the intermediate vessels). These data suggest that the weak and equivocal response of coronary resistance arteries to norepinephrine results from the competitive dilatory influence of endothelial derived nitric oxide production and not to the absence of norepinephrine receptors.

Key Words: Isolated vessel, Norepinephrine, Acetylcholine, Nitric oxide, Coronary arteries, Endothelium

Introduction:

The interaction of the sympathetic nervous system with the coronary vasculature has been studied extensively [3, 8, 15-17, 23, 26, 30, 35, 37, 46, 48, 49, 58, 65, 70]. Stimulating sympathetic innervation or administering norepinephrine generally constricts large canine coronary arteries and reduces coronary blood flow through the direct activation of postjunctional α-adrenergic receptors [26, 25, 37-42, 46, 77, 80, 81]. However, recent studies of the coronary microcirculation in pigs and cats have demonstrated that the small resistance arteries (<300μm) do not respond well to norepinephrine [16, 63, 82]. Most of these studies were conducted in intact animals with secondary metabolic, neural, hormonal, and endothelial influences still intact and functioning. Therefore, opposing secondary effects may have moderated the direct action of norepinephrine on the coronary resistance arteries. Quillen et. al. [70] recently employed the isolated microvessel methodology developed by Osol and Halpern [68] and Duling et. al. [24] in pigs. They demonstrated that this method preserves endothelial and smooth muscle function while removing the indirect effects of norepinephrine, which are secondary to the adrenergic activities on the local myocardium. They observed a norepinephrine mediated vasodilation in small isolated resistance arteries and suggested that norepinephrine acts through both smooth muscle β-receptors and the endothelial release of nitric oxide [70]. Similarly, we reported that small canine coronary arteries in that they seldom contracted when norepinephrine was added to the perfusate [69].

These results in isolated vessels suggest that sympathetic stimulation should dilate the coronary vasculature and increase coronary blood flow [70]. Paradoxically, norepinephrine infusion and / or sympathetic stimulation leads to coronary vasoconstriction and reduced blood flow [26, 25, 46, 48, 73, 77, 80, 81].

Since the majority of coronary vascular resistance resides with the smallest coronary arteries [17], understanding the mechanisms regulating their diameter becomes important. The absence of constrictor responses to added norepinephrine has lead to suggestions that these vessels lacked functional α-adrenergic receptors [16, 70]. Alternatively, we suggest that the role and relative influence of endothelially mediated relaxation increases as vessel size and wall thickness are reduced. This study was conducted to evaluate the hypothesis that the weak norepinephrine mediated constrictor responses in the small coronary resistance arteries are the result of opposition from significant endothelial mediated vasodilation.

Materials and Methods:

Twenty seven mongrel dogs of either sex were anesthetized with sodium pentobarbital (34 mg/Kg i.v.) and the hearts removed through a left throacotamy. The hearts were immediately placed in cold, calcium free physiological salt solution (PSS) containing 1 gm of albumin per 100 ml of PSS. The left anterior descending coronary artery (LAD) was exposed and cannulated. The LAD was perfused with a mixture of calcium free PSS, gelatin, and india ink at 30 to 35°C until all the surface arteries were clearly visible. The heart was replaced in iced PSS-albumin for approximately 5 minutes to allow the perfusate to gel. The LAD region was cut free and immersed in iced calcium free PSS-albumin solution. Constituents of the calcium free PSS solution were as follows (all quantities in mM) 145 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 1.2 NaH_2PO_4, 5.0 glucose, 2.0 pyruvate, 0.02 ethylenediaminetetraacetic acid, 3.0 3-(N-morpholino) propanesulfonic acid [54]. The pH was adjusted to 7.4 at the required temperature [53]. The PSS-gelatin-india ink suspension was prepared by mixing 10 ml. of warm PSS, 0.2 ml

of non-dialyzed and non-waterproof india ink (Koh-I-Noor), and 0.36 gm porcine skin gelatin [53]. The PSS, gelatin, india ink mixture forms a solid at temperatures below 20°C which facilitates microvessel dissection. The mixture liquefies again when physiological temperatures are reestablished and is easily flushed from the vessel.

Epicardial arteries with lumenal diameters between 60 and 200 μm and between 400 and 600 μm were selected from the LAD perfusion territory and dissected free. The vessels were carefully transferred to a lucite tissue chamber (Halpern) and mounted between two glass cannulas (O.D. 40 - 120 μm), utilizing the methods described by Osol, Halpern, and Duling et. al. [24, 44, 68]. The tissue chamber was transferred to a microscope stage attached to a video dimension analyzer (Living Systems Inc.), a video tape recorder, and a strip chart recorder (see Figure 1). The arteries were distended with an intraluminal pressure of 40 mmHg which approximates the normal intraluminal pressures for coronary resistance arteries [17]. All studies were conducted at zero flow to prevent flow induced endothelial release of nitric oxide [9, 30, 44, 53]. The extravascular chamber was perfused (12 to 16 ml/min.) with calcium free PSS-albumin and the temperature allowed to rise to 37°C where it was maintained with an external circulating heat exchanger. After equilibration of the isolated artery for one hour in calcium free PSS the maximal vasodilation (D_{max}) was recorded. Calcium (2.0 mM) was added to the chamber perfusate and the vessel was again allowed to equilibrate for approximately one hour.

Vessels selected for experimentation met viability criteria introduced by Duling et. al. [24]; all vessels developed spontaneous tone at 37°C, developed myogenic responses when intraluminal pressure was altered, and responded

appropriately to vasoactive agents. Any vessels which failed to meet these criteria were rejected (n = 3).

The vessels were subjected to increasing concentrations of L-norepinephrine from 10^{-10} to 10^{-4} M. After the initial norepinephrine dose responses vessels were returned to calcium containing PSS-albumin and allowed to equilibrate until a steady state was observed (about 20 min.). To insure that the endothelial cell layer was intact and functioning normally, vessels were preconstricted to approximately 50% of D_{max} with KCl and graded doses of acetylcholine were added 10^{-10} to 10^{-4} M. Vessels which failed to dilate following acetylcholine were removed from the study (n=2). The vessels were returned to calcium containing PSS-albumin and again allowed to equilibrate. After a steady state had been reached, the vessel was treated with the nitric oxide synthetase inhibitor N-Nitro-L-Argine Methylester, (L-NAME) 10^{-5} M to block endothelial production of nitric oxide (NO). The efficacy of the NO blockade was evaluated in each vessel by the absence of dilation in vessels precontracted with KCl following the addition of acetylcholine 10^{-4} M. The acetylcholine and KCl were then flushed from the extravascular perfusate with fresh calcium medium and the vessel allowed to returned to starting diameter. Under NO blockade, a second norepinephrine dose response was conducted. The vessel was thoroughly washed and reequilibrated in calcium containing PSS-albumin without L-NAME. Once the vessel had reached a steady state the endothelial cell layer was mechanically removed by sliding the vessel back and forth over a glass cannula [53]. The effectiveness of the mechanical disruption of the endothelial cell layer was verified by the absence of an acetylcholine (10^{-4} M) mediated dilation following the same procedure used for L-NAME. A final norepinephrine dose

response was conducted to independently verify the endothelial participation in the resistance vessel responses to norepinephrine.

Data Analysis:

Changes in vessel diameter were normalized as a percentage of the initial calcium free maximal diameter (D_{max}). The mean maximal responses, and their respective ED_{50}'s were compared by analysis of variance. A $p < 0.05$ was accepted as significantly different, and post-Hoc comparisons were made with the Sheffe's test. Values were expressed as means ± SE.

Results:

The average lumen diameter for small vessels in this study was 147±23 μm with a range of 57 to 243 μm, and 531±37 with a range of 417 to 594 for the intermediate sized vessels in calcium free PSS (D_{max}) (n=18 and n=4 respectively). When calcium was reintroduced into the perfusate, the arteries constricted to a new steady state at 84±5.3% of D_{max} which served, thereafter, as the reference or control. Increasing abluminal norepinephrine concentrations added to the vessel chamber containing the small arteries produced a slight decrease in lumen diameter (82±2.1% of D_{max}) in seven vessels and a slight increase (86±4.4% of D_{max}) in eleven others. The overall changes were not significantly different from control, and as such were not sufficient to permit the determination of an ED_{50}. In the intermediate arteries increasing norepinephrine concentrations produced a significant constriction to 51±4.7% of D_{max} in all arteries compared to control ($p<0.05$); ED_{50} = 0.081±0.018 μM (n=4) (see Figure 2).

A dose response to acetylcholine was conducted to evaluate the integrity of the endothelial cell layer and determine the maximal dose. After precontraction to 46±5.3% of D_{max} with KCl, acetylcholine produced a vasodilation to 82±2.5% of D_{max} which was not different from the control prior to KCl. When endothelial derived nitric oxide production was blocked by the administration of L-NAME all dilatory responses to acetylcholine were abolished (see Figures 2, and 4). However, in the absence of KCl precontractions vessels treated with L-NAME consistently constricted to 73±4.1% of D_{max} ($p<0.05$) for small vessels, and 72±2.7% of D_{max} for the intermediate arteries, suggesting a significant basal NO production in the absence of agonist (see Figures 3, and 4). Acetylcholine (10^{-4} M) had no effect on the increase in resting tone following the addition of L-NAME. The acetylcholine was washed out and a second norepinephrine dose response was conducted in the presence of L-NAME. In all cases, norepinephrine which was ineffective earlier in the same small vessels now produced a graded vasoconstriction to a maximum response of 56±2.3% of D_{max} with an ED_{50} of 0.249±0.023 μM (Figures 3 and 5). Norepinephrine produced a significantly increased constriction in the intermediate arteries in the presence of L-NAME to 43±3.4% of D_{max} ($p<0.05$) with an ED_{50} of 0.033±0.016 μM. The isolated vessel was then returned to calcium containing PSS and allowed to equilibrate.

Next, the endothelium was mechanically removed by abrading the inside of the vessel with the glass cannula [53]. To verify removal of the endothelium, vessels were preconstricted with KCl and a single dose of acetylcholine 10^{-4} M was added to the chamber. The addition of acetylcholine to the chamber perfusate resulted in a significant vasoconstriction in the small, and intermediate arteries to 39±2.7% of D_{max} , and 37±3.2% of D_{max} respectively, from the KCl

preconstricted value of 46±5.3% of D_{max} (see Figures 2 and 4). After acetylcholine washout, the addition of increasing doses of norepinephrine to the chamber produced significant constriction for the small arteries to 61±2.3% of D_{max} with an ED_{50} of 0.245±0.021 μM (see Figure 3 and 5). Norepinephrine produced a significantly increased constriction in the intermediate endothelial denuded arteries to 52±3.8% of D_{max} ($p<0.05$) with an ED_{50} of 0.035±0.017 μM. Neither the maximal constriction nor the ED_{50} were significantly different from that observed following pharmacological blockade with L-NAME.

Discussion:

The functional importance of small resistance vessels derives from reports that 60 to 70% of coronary resistance to blood flow resided in arteries below 200 μm in cat and rabbit hearts respectively [17, 64]. Arteries of this size have also been shown to differ from the larger arteries in their responses to a variety of endogenous vasoactive substances [16, 50]

The poor responses of small isolated coronary resistance vessels to norepinephrine have been attributed to both the absence of α_1-adrenergic receptors in this portion of the coronary vasculature and to the competitive dilatory effects of adrenergic β-receptors and / or the vascular endothelium. This study demonstrated that the equivocal response of the small canine coronary resistance arteries to norepinephrine results from the competing influence of endothelial nitric oxide. Whether the nitric oxide production in small and intermediate arteries is a constitutive activity of the endothelium or the result of adrenergic stimulation is unclear. The active constriction in the absence of agonist after L-NAME suggests that the basal release of nitric oxide is significant. This is supported further by the failure of maximal acetylcholine concentrations to

dilate untreated vessels beyond the resting reference diameter (84±5.3% of D_{max}). The consistent appearance of norepinephrine mediated vasoconstriction after inhibition of nitric oxide synthesis with L-NAME or removal of the endothelium provides clear support for the persistence of functioning α-adrenergic receptors in canine coronary arteries with lumen diameters between 60 and 200 μm.

The restoration of constrictor activity after removal of the endothelial influence in canine coronary vessels is in contrast to findings in isolated porcine vessels [70]. When hemoglobin, a nitric oxide scavenger, was added to the medium only minimal vasoconstriction could be elicited to norepinephrine infusions suggesting that the failure of these vessels to constrict was not the result of nitric oxide production. The completeness of hemoglobin suppression of endothelial derived nitric oxide was not tested by the addition of an endothelial dependent vasoactive agent nor was the endothelium mechanically disrupted [70]. Therefore, sufficient nitric oxide may have still reached the vascular smooth muscle and prevented significant vasoconstriction. The differences between this study and those in the pig may also be due to differences in the distribution of α-adrenoceptors in the coronary circulation between species [19].

The ratio of endothelial to smooth muscle cells is greatest in small resistance arteries and least in larger arteries. This could account for the near balance between endothelial mediated vasodilation and smooth muscle mediated vasoconstriction. Greater diffusion distances in larger coronary vessels, presumably results in more opportunity for nitric oxide degradation and smaller concentrations at smooth muscle cells further form the endothelium. A net vasoconstriction may result in larger arteries when the relaxing influence of the endothelium on the proximal inner rings of smooth muscle is overcome by the

constriction of multiple outer rings further removed from the endothelial influence. The properties of the vascular endothelium in the smallest coronary resistance arteries may also differ from those observed in larger transport vessels. For example, the endothelial capacity for nitric oxide production in small coronary arteries may be greater than that in larger arteries.

A norepinephrine-mediated release of endothelial derived nitric oxide in large epicardial coronary arteries from both dogs and pigs has been attributed to endothelial α_2-receptors [18]. Our study indicates that norepinephrine may also release nitric oxide from the endothelium of canine coronary resistance arteries, as suggested by Quillen et. al. [70] in the pig.

Acetylcholine vasodilates isolated vessels with intact endothelial cell layers and constricts those without the endothelial cell layers [30-32]. Jaiswal et. al. [45] demonstrated that M_3 muscarinic receptors were responsible for endothelium-dependent vasodilation, whereas M_2 muscarinic receptors mediated the constriction following removal of the endothelium [45]. Since we observed a similar endothelial dependent relaxation in endothelium intact and contraction in endothelium denuded vessels after acetylcholine, both muscarinic subtypes may also be present in the canine coronary microcirculation.

In summary, our study has demonstrated that the canine coronary vascular endothelium significantly modifies the response of the canine coronary resistance arteries to norepinephrine. The data further show that adrenergic receptors exists and function normally in the smallest canine coronary resistance arteries. Furthermore, this study indicates that the muscarinic receptor subtypes M_2 and M_3 may also be present in canine coronary resistance arteries.

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the degree Doctor of Philosophy. Supported in part by NIH Grants 5T32HL07652-05, HL29232, and HL34172.

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FIGURE LEGENDS

FIGURE 1 - Illustrates the systems employed to measure vessel lumen diameter and wall thickness. The chamber perfusion pump and heating coil maintains the perfusate at 37^{o}C and allows for rapid exchange of perfusate and the introduction of agents. The servo pressure system maintains the intraluminal pressure at 40 mmHg. The video dimension analyzer and microscope measure the vascular lumen and wall thickness from recorded TV images.

FIGURE 2 A- The effects of acetylcholine in small canine coronary resistance arteries following precontraction of the vessel with KCl (KCl). In control vessels acetylcholine produced a significant endothelium dependent relaxation, $p < 0.05$ (Control). After inhibition of nitric oxide synthetase with L-NAME acetylcholine had no significant effect (L-NAME). After mechanical removal of the endothelium, acetylcholine produced a significant vasoconstriction, $p < 0.05$ (ENDX).

FIGURE 2 B - The effects of acetylcholine in intermediate canine coronary resistance arteries following precontraction of the vessel with KCl (KCl). In control vessels acetylcholine produced a significant endothelium dependent relaxation, $p < 0.05$ (Control). After inhibition of nitric oxide synthetase with L-NAME acetylcholine had no significant effect (L-NAME). After mechanical removal of the endothelium, acetylcholine produced a significant vasoconstriction, $p < 0.05$ (ENDX).

FIGURE 3 A - Mean diameters for small coronary arteries under control conditions (Con), after norepinephrine (Con NE), after inhibition of nitric oxide synthase with L-NAME (L-NAME), after L-NAME plus norepinephrine (L-NAME NE), after mechanical removal of the endothelium (ENDX), and after mechanical removal of the endothelium plus norepinephrine (ENDX NE).

* Significantly different from the respective control, $p < 0.05$.

** Significantly different from control, $p < 0.05$.

FIGURE 3 B - Mean diameters for intermediate coronary arteries under control conditions (Con) , after norepinephrine (Con NE), after inhibition of nitric oxide synthase with L-NAME (L-NAME), after L-NAME plus norepinephrine (L-NAME NE), after mechanical removal of the endothelium (ENDX), and after mechanical removal of the endothelium plus norepinephrine (ENDX NE).

* Significantly different from the respective control, $p < 0.05$.

** Significantly different from control, $p < 0.05$.

FIGURE 4 - Strip chart recording of vessel diameter in response to (A) increasing doses of norepinephrine under control conditions, (B) in the presence of L-NAME after first testing the vessel for blockade of nitric oxide release with acetylcholine, and (C) after removal of the endothelial cell layer after testing the vessel for the completeness of the removal with acetylcholine.

FIGURE 5 A - Mean ± SE norepinephrine dose response curves, for small arteries, in the presence of L-NAME (squares) and after removal of the endothelium (diamonds). The two curves illustrate that no significant difference exists between the two conditions, $p < 0.05$.

FIGURE 5 B - Mean ± SE norepinephrine dose response curves, for intermediate arteries, under control conditions (squares), in the presence of L-NAME (diamonds) and after removal of the endothelium (solid squares). The L-NAME and the endothelium removed curves are not significantly different from each other but both are significantly different from the control norepinephrine dose response, $p < 0.05$.

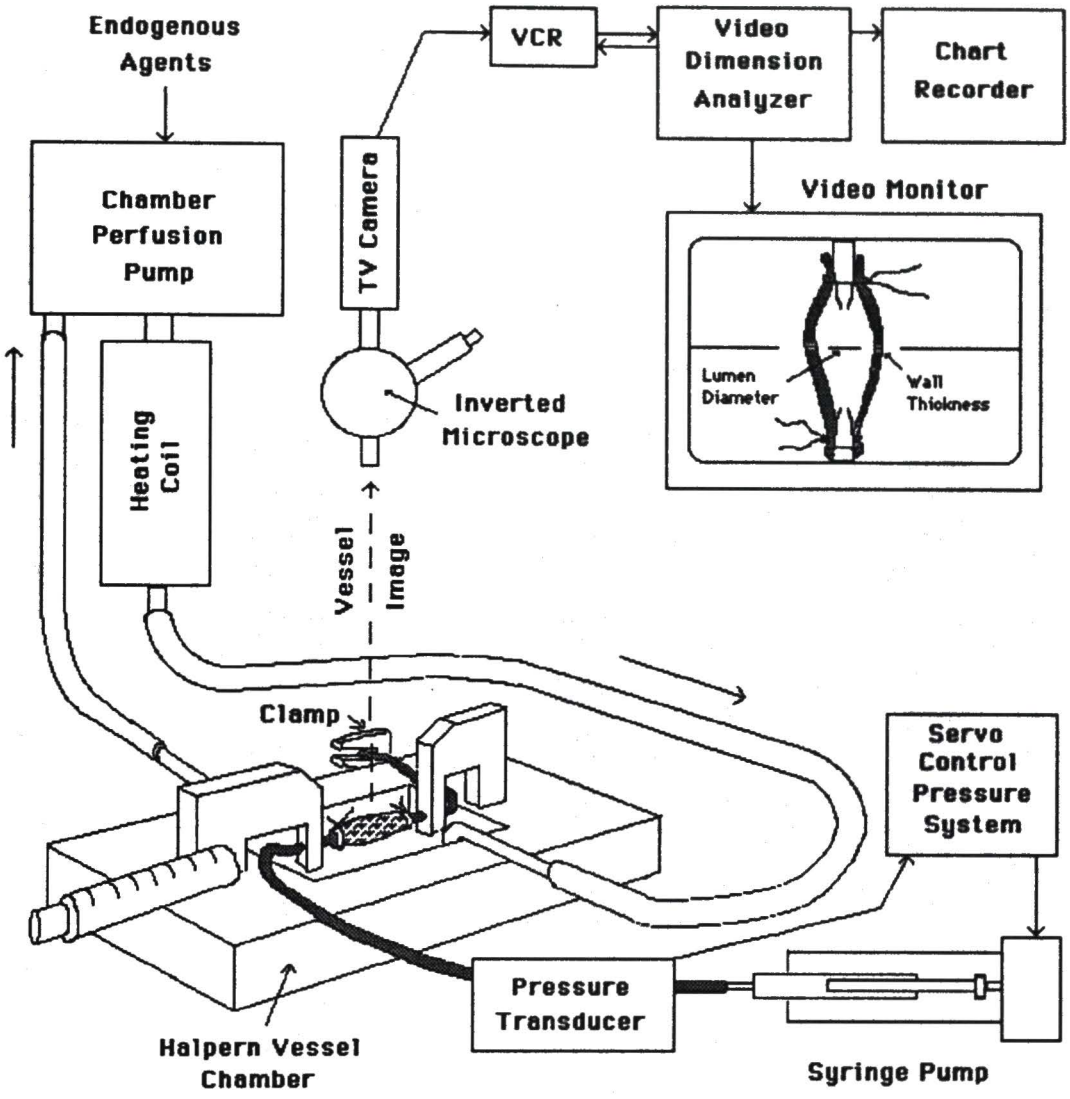
Endogenous Agents
Chamber Perfusion Pump
Heating Coil
TV Camera
VCR
Video Dimension Analyzer
Chart Recorder
Video Monitor
Lumen Diameter
Wall Thickness
Inverted Microscope
Vessel Image
Clamp
Servo Control Pressure System
Pressure Transducer
Halpern Vessel Chamber
Syringe Pump

FIGURE 1

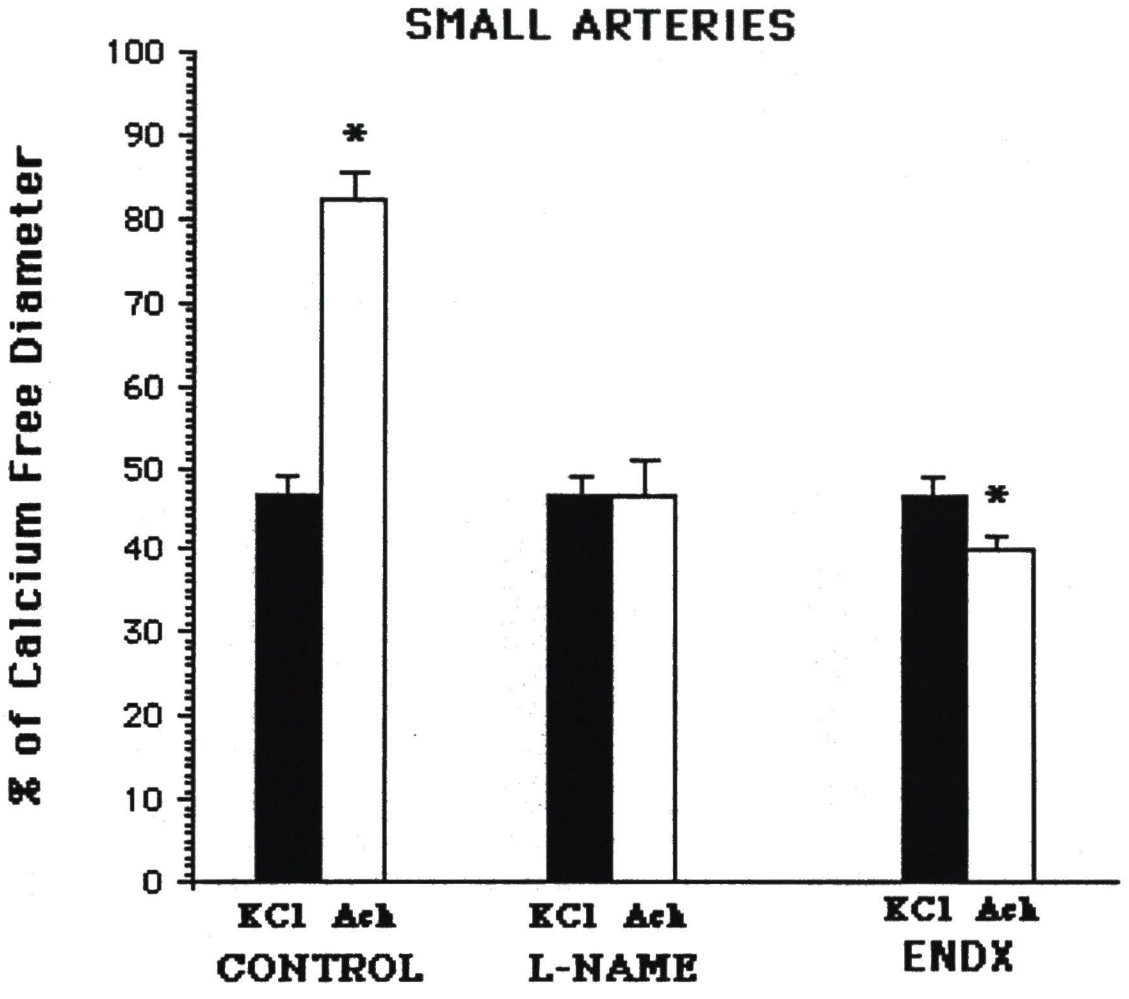
SMALL ARTERIES
% of Calcium Free Diameter
100
90
80
70
60
50
40
30
20
10
0
*
*
KCl Ach
CONTROL
KCl Ach
L-NAME
KCl Ach
ENDX

FIGURE 2 A

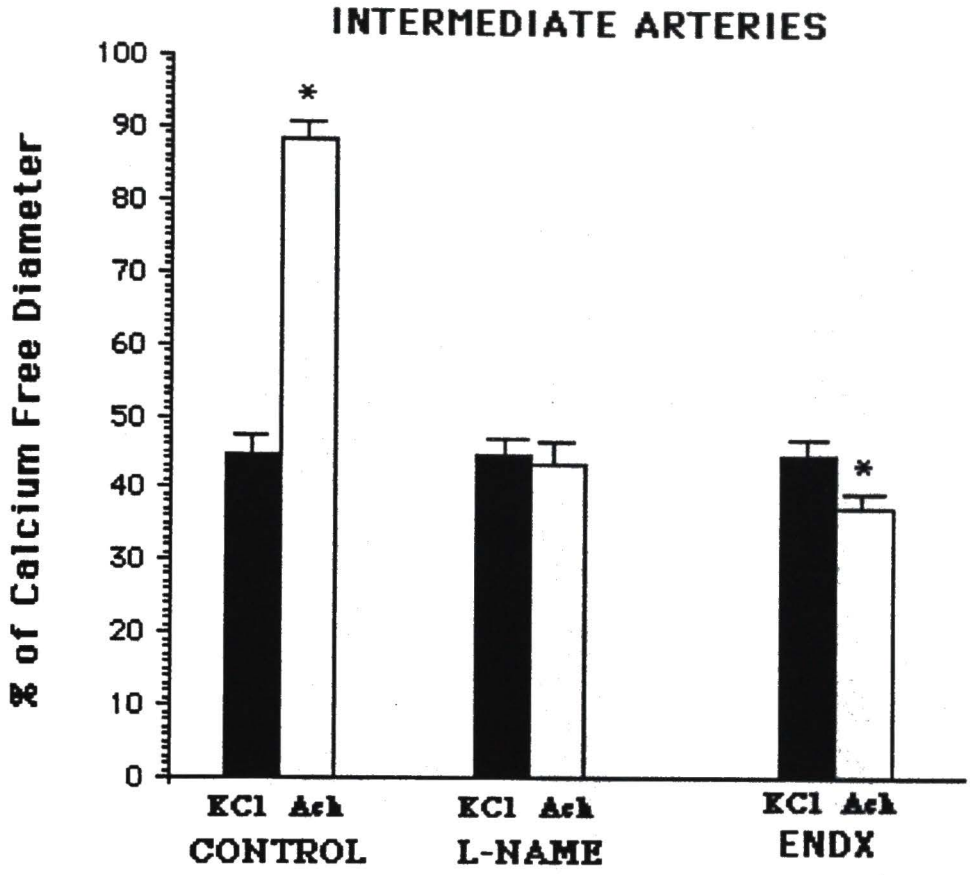
INTERMEDIATE ARTERIES
% of Calcium Free Diameter
100
90
80
70
60
50
40
30
20
10
0
*
*
KCl Ach
CONTROL
KCl Ach
L-NAME
KCl Ach
ENDX

FIGURE 2 B

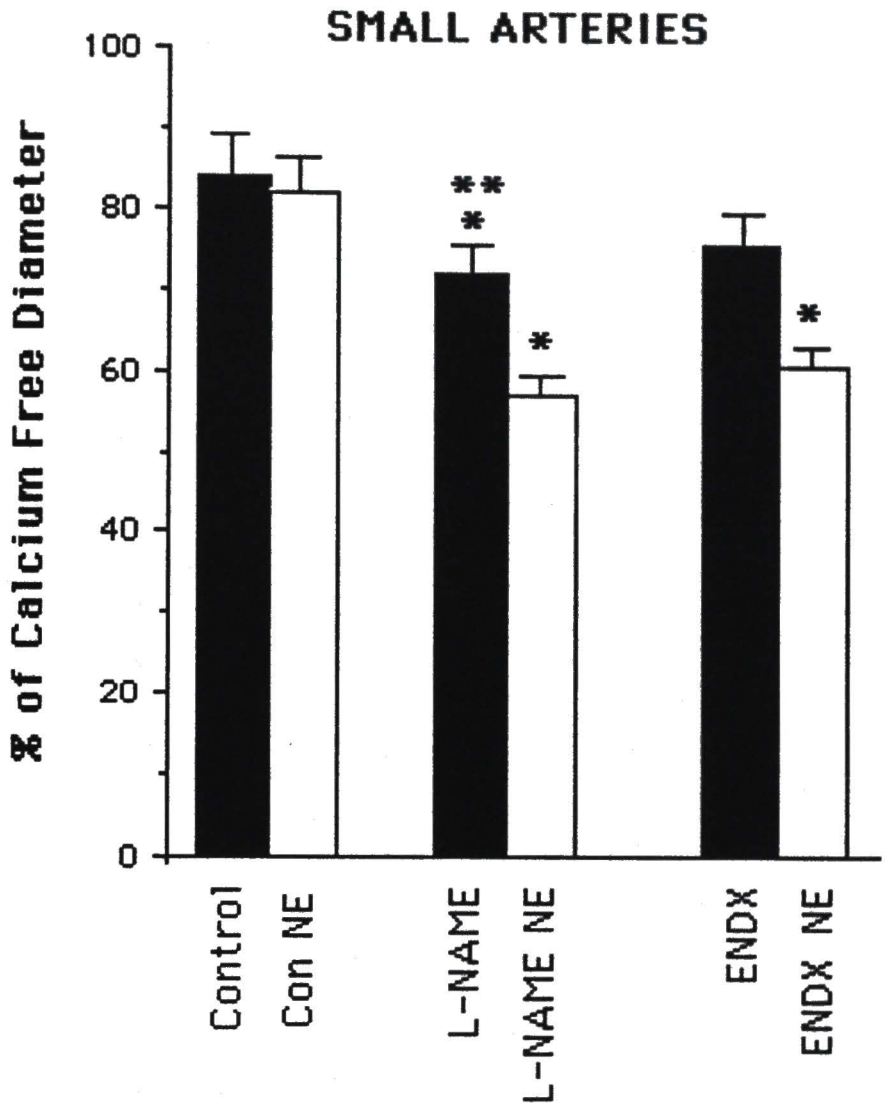
SMALL ARTERIES
% of Calcium Free Diameter
100
80
60
40
20
0
**
*
*
*
Control
Con NE
L-NAME
L-NAME NE
ENDX
ENDX NE

FIGURE 3 A

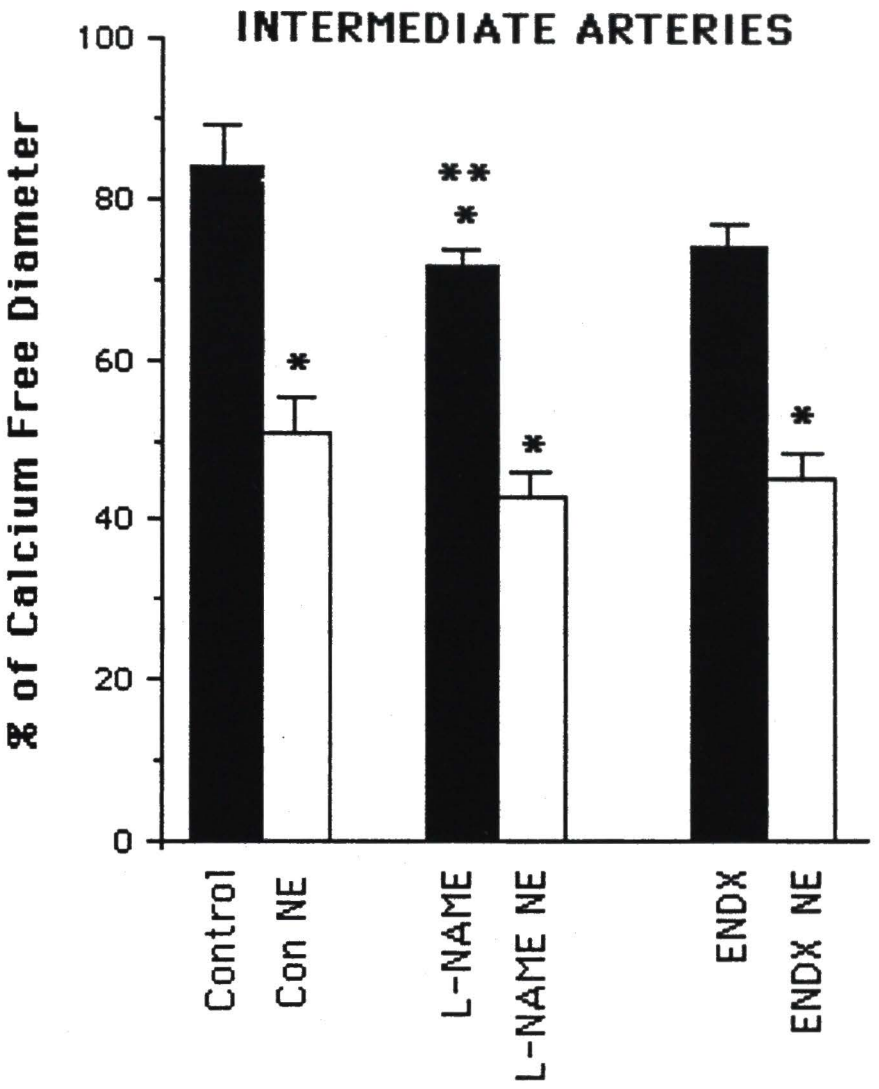
INTERMEDIATE ARTERIES
% of Calcium Free Diameter
100
80
60
40
20
0
**
*
*
*
*
Control
Con NE
L-NAME
L-NAME NE
ENDX
ENDX NE

FIGURE 3 B

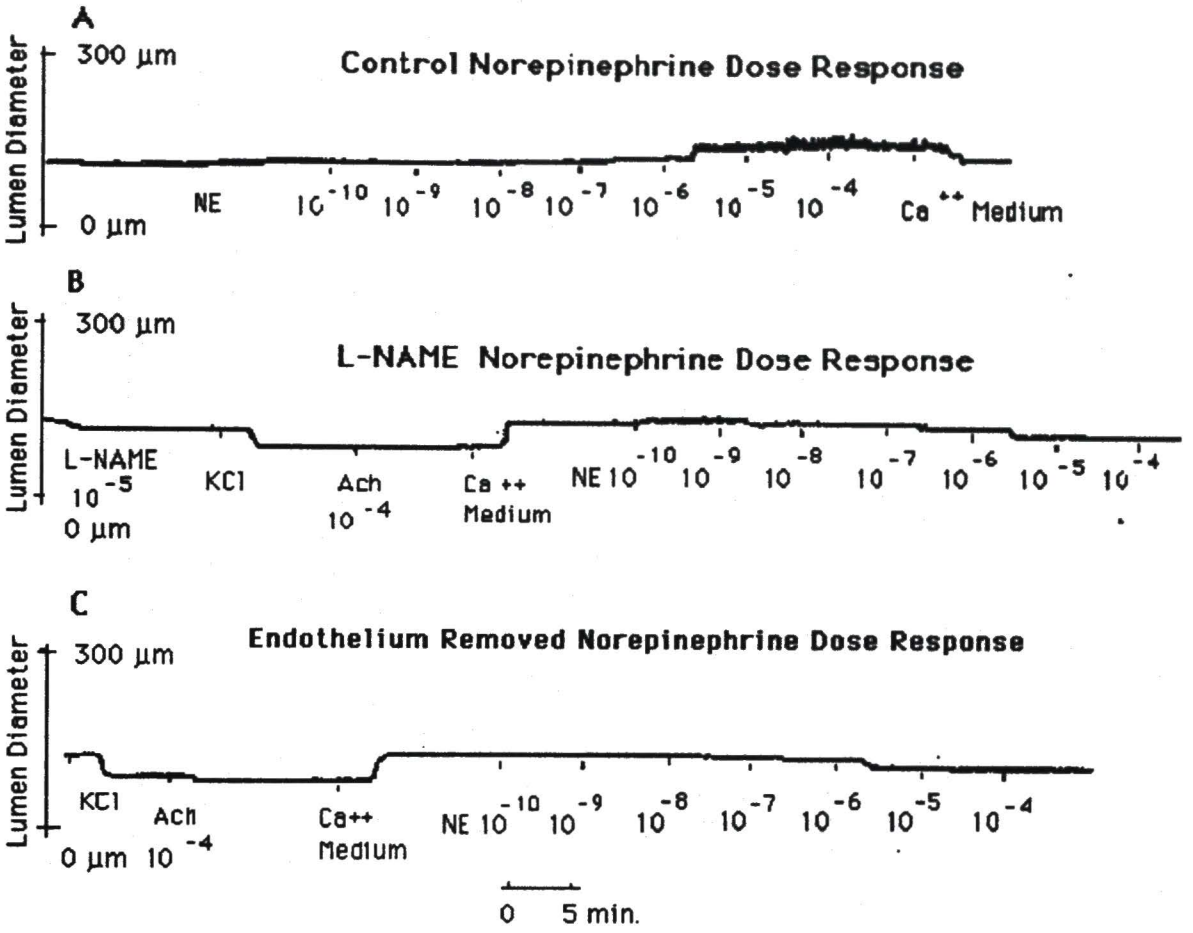
A
Control Norepinephrine Dose Response
Lumen Diameter
300 µm
0 µm
NE
10 -10 10 -9 10 -8 10 -7 10 -6 10 -5 10 -4
Ca ++ Medium
B
L-NAME Norepinephrine Dose Response
Lumen Diameter
300 µm
L-NAME 10 -5
0 µm
KCl
Ach 10 -4
Ca ++ Medium
NE 10 -10 10 -9 10 -8 10 -7 10 -6 10 -5 10 -4
C
Endothelium Removed Norepinephrine Dose Response
Lumen Diameter
300 µm
KCl
Ach
0 µm 10 -4
Ca++ Medium
NE 10 -10 10 -9 10 -8 10 -7 10 -6 10 -5 10 -4
0 5 min.

FIGURE 4

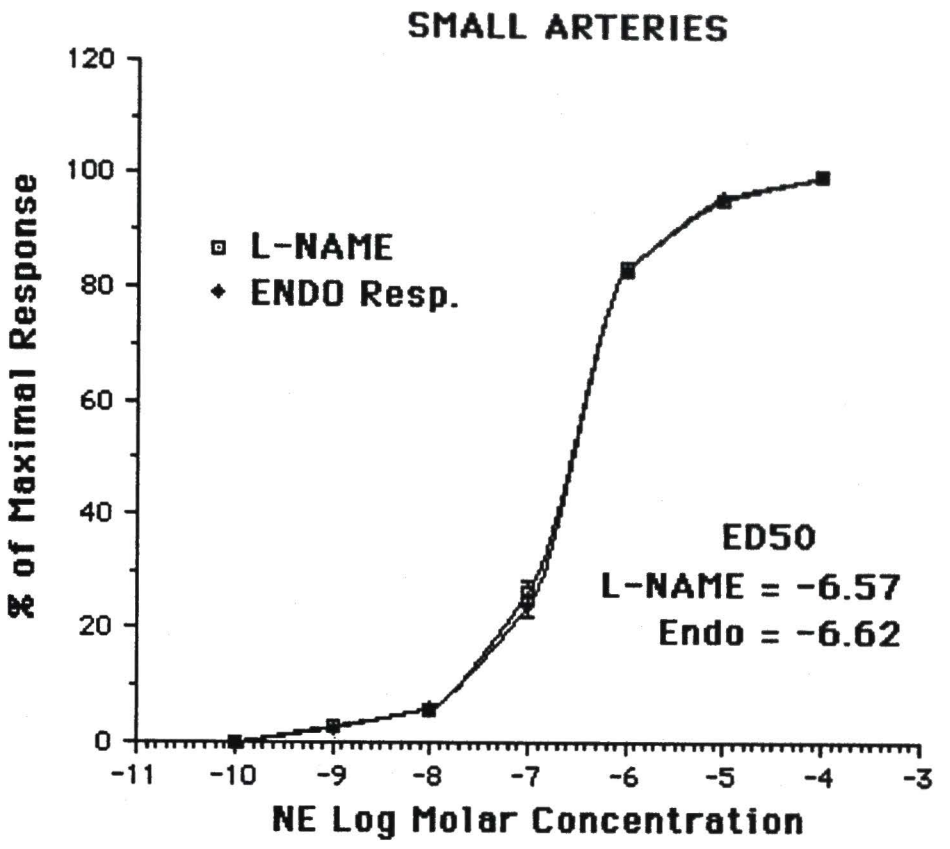
SMALL ARTERIES
% of Maximal Response
120
100
80
60
40
20
0
L-NAME
ENDO Resp.
ED50
L-NAME = -6.57
Endo = -6.62
-11
-10
-9
-8
-7
-6
-5
-4
-3
NE Log Molar Concentration

FIGURE 5 A

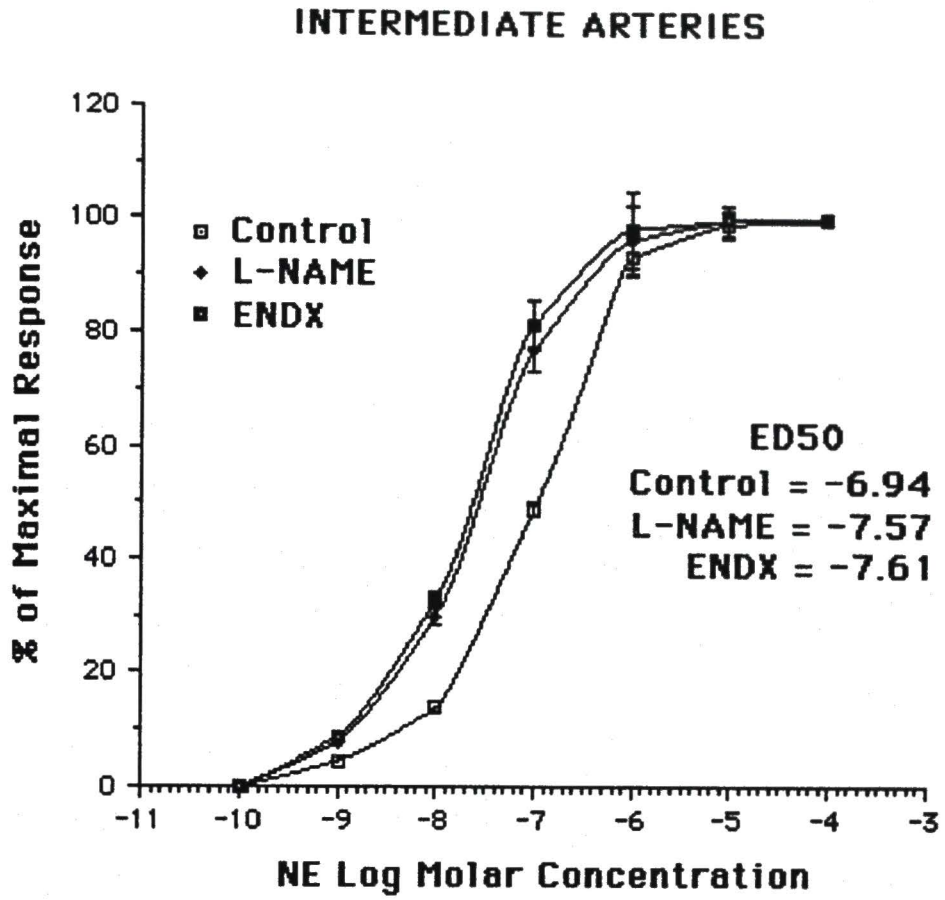
INTERMEDIATE ARTERIES
% of Maximal Response
120
100
80
60
40
20
0
Control
L-NAME
ENDX
ED50
Control = -6.94
L-NAME = -7.57
ENDX = -7.61
-11
-10
-9
-8
-7
-6
-5
-4
-3
NE Log Molar Concentration

FIGURE 5 B

Chapter 4

CONCLUSIONS

This study has demonstrated that large canine coronary arteries are more responsive to norepinephrine and acetylcholine than are intermediate coronary arteries, which are more responsive than the small coronary arteries. This finding is in agreement with the hypothesis that large coronary arteries are more concerned with responding to global changes in the coronary circulation mediated by the autonomic nervous system.

The greater effects of norepinephrine and acetylcholine on the large transport arteries are differentiated from the intermediate coronary arteries by a clear transition zone occurring between 600 to 700 μm in lumen diameters, and again between intermediate and small coronary arteries at 300 to 400 μm in lumen diameters. This finding suggests that functional differences exists between large, intermediate, and small canine coronary arteries.

Adenosine has a greater effect on the small resistance arteries than on the large transport arteries with transition zones occurring in approximately the same size ranges. Similar to the response observed with norepinephrine. This finding indicates that the small canine coronary arteries tend to be more sensitive to local metabolic stimulation than are the large or intermediate coronary arteries.

This study demonstrates that the response of the small canine coronary arteries to norepinephrine was equivocal, and that the canine coronary vascular

endothelium significantly modifies the response through the release of nitric oxide. It further shows that functional adrenergic receptors exists in canine coronary resistance arteries. Furthermore, this study indicates that both endothelial (M_2) and smooth muscle (M_3) muscarinic receptor subtypes may be present in canine coronary resistance arteries.

These studies support the following three hypothesis:

1. Canine coronary artery responses to the neurotransmitters norepinephrine and acetylcholine are greater in large conduit arteries and less in small distributive arteries.

2. The response to the local metabolite adenosine is greater in the small distributive arteries and less in the large conduit arteries.

3. Endothelial mediated responses are present in all vessels but the relative influence of the endothelium is greatest in the smallest vessels.