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ENDOTHELIUM-DEPENDENT RELAXATION OF BLOOD VESSELS

by

Michael Ray Hynes

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF PHARMACOLOGY

In Partial Fulfillment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY
WITH A MAJOR IN PHARMACOLOGY AND TOXICOLOGY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Michael Ray Hynes

entitled Endothelium-Dependent Relaxation of Blood Vessels

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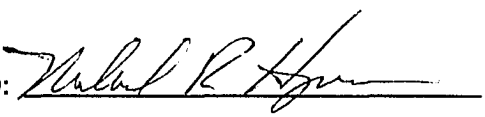
SIGNED: 

DEDICATION

This dissertation is dedicated to my mother, Mariane Hynes, my sister, Julie Brown, and all of my aunts, uncles and cousins who gave me nothing but support and encouragement. Knowing they were behind me made it possible

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ABSTRACT

Dilation of blood vessels in response to a large number of agents has been shown to be dependent on an intact vascular endothelium. The present studies examine some aspects of endothelium-dependent vasodilation in blood vessels of the rabbit and rat. Using the rabbit ear artery and the subtype-selective muscarinic antagonist pirenzepine, muscarinic receptors of the endothelium and smooth muscle cells were shown to be of the low affinity M_2 subtype. Inhibition of [^{3}H](-)quinuclidinyl benzilate was used to determine affinity for the smooth muscle receptors while antagonism of methacholine induced vasodilation yielded the endothelial cell receptor affinity.

The effect of increasing age (1-27 months) on endothelium-dependent relaxation was studied in aortic rings, perfused tail artery and perfused mesenteric bed of the Fisher 344 rat. Both aortic ring segments and perfused caudal arteries showed an age-related increase in sensitivity of endothelium-mediated relaxation to the cholinergic agonist methacholine. This increased sensitivity occurs between the ages of 6 and 12 months, with no further significant increase up to 27 months of age, suggesting this is a consequence of growth and development rather than old age. No difference with age in cholinergic relaxation was observed in the perfused mesenteric bed indicating either no change of sensitivity in smaller resistance vessels or an effect which is hidden in this more complex perfused system. In contrast to findings with cholinergic stimulation, responses of the perfused caudal artery to the calcium ionophore A23187 were not altered with age. This suggests

that the alteration with age in response to methacholine involves the muscarinic receptor or receptor coupling mechanism rather than the generation of, or response to, endothelium-derived relaxing factor (EDRF).

The influence of endothelium on contractile responses was examined using the perfused caudal artery. Endothelium removal significantly increased contraction to the α-adrenergic agonists methoxamine and BH-T 920 as well as to transmural nerve stimulation. Inhibition of contraction to agents which must first cross the smooth muscle layer before reaching the endothelium suggests that a continuous or basal level of EDRF release is responsible for decreased contraction rather than an receptor stimulated release of EDRF.

CHAPTER 1

ENDOTHELIUM-DEPENDENT VASODILATION A REVIEW

The vascular endothelium, once known primarily for its passive roles as a selective barrier and thromboresistant surface, has in recent years been recognized as an active site of uptake, metabolism or synthesis of vasoactive substances such as prostaglandins, serotonin, adenine nucleotides and angiotensin (for review see Thorgeirsson and Robertson, 1978). This active role that endothelium plays became even more apparent when Furchgott and Zawadzki (1980) reported that the vasodilation produced by acetylcholine in rings of rabbit aorta was absolutely dependent on the presence of an intact endothelium. Their observations led them to hypothesize the existence of a non-prostaglandin vasodilator substance synthesized by the endothelium in response to cholinergic stimulation. Since then a large number of agents have been identified which have vascular activity that is partially or totally dependent on the presence of an intact endothelium (Table 1). This chapter will explore the phenomenon of endothelium-dependent responses beginning with the most widely studied of these agents, acetylcholine.

What is the Nature of the Cholinergic,Endothelium-Dependent Response?

Acetylcholine is active as an endothelium-dependent vasodilator in a wide variety of tissues from different species of animals (Table 2). Its vasodilatory activity has been shown to be totally endothelium-dependent in all tissues examined except the posterior auricular artery of the cat, which appears to have both

endothelium-dependent and independent components (Brayden and Bevan, 1985). Removal of the endothelium not only eliminates the relaxation response to acetylcholine, but often reveals a direct contractile effect on the smooth muscle (Angus et al., 1983; Weinheimer and Osswald, 1986; Ignarro et al., 1986). Both the endothelium-dependent relaxation and the endothelium-independent contraction are mediated by muscarinic receptors as indicated by competitive blockade with the muscarinic antagonist atropine (De Mey and Vanhoutte, 1981; Chand and Altura, 1981a; Lee, 1982; Gruetter and Lemke, 1986). Investigations into the question of whether the muscarinic receptors mediating relaxation are the same subtype as those which produce contraction are detailed in chapter 2 of this dissertation.

Endothelium-Derived Relaxing Factor

Having shown that relaxation to acetylcholine was abolished in aortic strips when the endothelium was removed (by rubbing of the intimal surface), Furchgott and Zawadzki (1980) also demonstrated the return of the response when the stripped vessel (endothelium denuded) was placed in a "sandwich" with another vessel having an intact endothelium. The two strips of aorta were placed together in a bath with their intimal surfaces in contact. Tension was measured only in the initially unresponsive stripped segment. Addition of acetylcholine to this "sandwich" preparation now produced a relaxation response in the stripped segment. From these experiments they concluded that stimulation of the endothelium generates a substance which diffuses to the smooth muscle producing relaxation. This substance was later named endothelium-derived relaxing factor or EDRF (Cherry et al., 1982). Support for the existence of EDRF came from additional "sandwiches" of vessels from rat and dog vessels (Van de Voorde and Leusen, 1983b; Furchgott,

Table 1

Substances that produce endothelium-dependent relaxation in certain blood vessels.

Acetylcholine	Norepinephrine
A23187	Epinephrine
Histamine	Vasopressin
Substance P	Fenolodopam
Neurokinins A and B	Leukotriene D4
octa-cholecystokinin	Melittin
Adenosine	Calcitonin Gene Related Peptide
ATP	Vasoactive Intestinal Polypeptide
ADP	Neurotensin
Bradykinin	Bombesin
Arachidonic Acid	Hydralazine
Serotonin	Ergonovine
Thrombin	Platelet Activating Factor

Specific references for each agent are included within this review

Table 2. Species and tissues which demonstrate cholinergic, endothelium-dependent relaxation.

Artery	Reference
Bovine	
Intrapulmonary	Ignarro et al., 1986
	Gruetter and Lemke, 1986
Canine	
Coronary	Furchgott and Zawadzki, 1980
Femoral	De Mey and Vanhoutte, 1981
	De Mey et al., 1982
	Angus et al., 1983
Intrapulmonary	Chand and Altura, 1981b
Mesenteric	Toda, 1984
Pulmonary	De Mey and Vanhoutte, 1982
Renal	Chand and Altura, 1981b
	Toda, 1984
Saphenous	De Mey and Vanhoutte, 1982
Splenic	De Mey and Vanhoutte, 1982
Feline	
Aorta	Van de Vroode and Leusen, 1982
mesenteric	Furchgott and Zawadzki, 1980
Pulmonary	Furchgott and Zawadzki, 1980
Iliac	Furchgott and Zawadzki, 1980
Cerebral	Lee, 1982
Rat	
Aorta	Rapoport and Murad, 1983
	Ignarro et al., 1986
Tail	Busse et al., 1983
Carotid	Secrest et al., 1985

Artery	Reference
Guinea-pig	
Aorta	Furchgott and Zawadzki, 1980
Pulmonary	Bolton and Clapp, 1986
	Van de Vroode and Leusen, 1982
Mesenteric	Bolton and Clapp, 1986
	Bolton et al., 1984
Human	
Cerebral	Thom et al., 1986
Coronary	Bossaller et al., 1985
Mesenteric	Furchgott, 1983
Ovarian	Furchgott, 1983
Pulmonary	Thom et al., 1986
Renal	Vanhoutte et al., 1986
Rabbit	
Aorta	Singer and Peach, 1983b
Ear	Furchgott and Zawadzki, 1980
Mesenteric	Bolton and Clapp, 1986
	Forstermann et al., 1986b
Mesenteric bed	Carvalho and Furchgott
Pulmonary	Furchgott and Zawadzki, 1980

1983) as well as modified versions using an intact ring of bovine intrapulmonary artery surrounding an inverted stripped ring (Gruetter and Lemke, 1986). Further confirmation has come from a number of cascade experiments using vessels of rabbit (Griffith et al., 1984a; Forstermann et al., 1985), dog (Forstermann et al., 1985; Rubanyi et al., 1985a), guinea-pig and rat (Bolton et al., 1984) in which an intact vessel segment was perfused in series with a stripped segment. No response to endothelium-dependent vasodilators was observed when the intact segment was bypassed; however, vasodilation could be produced in this segment when the perfusing medium was first passed through the intact, stimulated segment.

While the existence of EDRF is now well established, the identity of this substance (or substances) is still unknown. However, in later sections the proposed mechanism of action of EDRF will be discussed, which, combined with the properties of inhibitors of the endothelium-dependent response gives clues to the nature and possible identity of EDRF.

Influence of Contractile Agent and Level of Tone

The nature of the contractile agent generally has little influence on the relaxation response to acetylcholine. Rabbit aorta relaxed equally well when contracted by norepinephrine, histamine, serotonin, angiotensin II or $PGF_{2\alpha}$ (Furchgott and Zawadzki, 1980; Furchgott et al., 1981) as did dog pulmonary when contracted with norepinephrine, phenylephrine or serotonin (Chand and Altura, 1981a). However, equivalent contractions produced by replacing Na^+ ions in the buffer with K^+ ions were less sensitive to the relaxing effects of acetylcholine in vessels of the rabbit (Furchgott and Zawadzki, 1980; Ibengwe and Suzuki, 1986; Bolton and Clapp, 1986), dog (Chand and Altura, 1981a; Furchgott, 1983) and guinea-pig (Bolton et al., 1984; Bolton and Clapp, 1986).

The level of tone induced by the contractile agent also has some influence on the ability of acetylcholine to relax vessels. At levels of norepinephrine which induced contraction below 60% of maximum, acetylcholine produced 80-100% relaxation in the rabbit aorta (Furchgott et al., 1981; Diamond and Chu, 1983; Ibengwe and Suzuki, 1986), but as the contractile tone approaches 100% of maximum the relaxation response can decrease to, in many cases, less than 25%. Furthermore, if the contraction is produced by increasing K^+ ion in the bathing solution then the decreased ability of acetylcholine to relax vessels in the face of increasing tone is even more dramatic (Ibengwe and Suzuki, 1986). This inverse relationship between the level of induced tone and the magnitude of the vasodilatory response is not, however, specific only for endothelium-dependent agents. Diamond and Chu (1983) observed a similar relationship in the rabbit aorta between the level of tone induced by phenylephrine and the magnitude of endothelium-independent vasolilation produced by either nitroglycerin or nitroprusside.

Effect of Oxygen Tension and Temperature

Even before the relaxation response to acetylcholine was shown to depend on an intact endothelium, De Mey and Vanhoutte (1980) had demonstrated the dependence of this response on the level of dissolved oxygen. Using canine femoral arteries these authors found that anoxia (PO_2 < 1 mmHg) abolished the response to acetylcholine. Optimal response was obtained at a PO_2 level of 145 mmHg while fully oxygenated Krebs-Ringer's solution (PO_2 = 650 mmHg) as well as hypoxia (PO_2 = 35 mmHg) had slight inhibitory effects. However, under these same conditions there was no difference in relaxation produced by the endothelium-independent agent, adenosine. Similarly, in the rabbit aorta Furchgott and Zawadzki (1980) found inhibition of relaxation to acetylcholine by anoxia, but no effect on relaxations by

sodium nitrite, glyceryl trinitrite or isoproterenol. This apparent sensitivity to hypoxia may be tissue dependent, since De Mey and Vanhoutte (1980) found inhibition of relaxation to acetylcholine in the canine femoral artery at an oxygen tension of 35 mmHg, but Chand and Altura (1981) found that same level had no effect in the canine pulmonary artery. The exact role that hypoxia may play in the endothelium-dependent response to acetylcholine is further confused by the finding that in both dog femoral and rat tail arteries hypoxic (PO_2< 40 mmHg) perfusion itself produced an endothelium-dependent relaxation (Busse et al., 1983). This response differed from the acetylcholine-mediated response in that it was blocked by the cyclooxygenase inhibitor indomethacin. Thus, while the cholinergic endothelium-dependent response is clearly absent under anoxic conditions, the effect of other levels of oxygen tension is not fully understood and will be discussed further when the nature and stability of EDRF are considered.

Temperature has also been shown to affect acetylcholine-mediated relaxations (De Mey and Vanhoutte, 1980). Dog femoral arteries were shown to respond normally to acetylcholine when tested in the temperature range of 32-37°C; however, temperatures of 27°C and 42°C tended to decrease the response, while at 22°C the relaxation to acetylcholine was practically eliminated.

The Role of Cyclic Guanylate Monophosphate (cGMP)

The relaxations induced by the endothelium-independent nitrovasodilators such as nitroglycerin, nitroprusside and $NaNO_2$ have been closely tied to their ability to activate guanylate cyclase and elevate intracellular levels of cGMP in bovine (Axelsson et al., 1979; Kukovetz et al., 1979; Ignarro et al., 1981) and canine (Janis and Diamond, 1979; Diamond and Blisard, 1979) arteries. Similarly, acetylcholine has been shown to both relax tension and increase concentrations of cGMP in a dose-

and endothelium-dependent manner in rings from rat and rabbit aorta (Rapoport and Murad, 1983; Diamond and Chu, 1983) and bovine intrapulmonary artery (Gruetter and Lemke, 1986). The observed rate of relaxation to acetylcholine was very similar to the rate of cGMP increase. Indeed, cGMP elevation slightly preceded relaxation, as would be necessary if cGMP was a causal agent (Diamond and Chu, 1983). Both relaxation to acetylcholine and cGMP increase were blocked by atropine (Rapoport and Murad, 1983; Gruetter and Lemke, 1986). Furthermore, Fiscus et al. (1984) have demonstrated that both acetylcholine and sodium nitroprusside increase the activity of cGMP-dependent protein kinase in rat aorta and that the acetylcholine-induced increase is dependent on the presence of an intact endothelium. Neither vasodilator affected the level of cAMP or the activity of cAMP-dependent protein kinase in the presence or absence of endothelium (Rapoport and Murad, 1983; Diamond and Chu, 1983; Fiscus et al., 1984).

These results indicate that the mechanisms of endothelium-dependent relaxation via EDRF and endothelium-independent relaxation of the nitrovasodilators converge at guanylate cyclase. Whether guanylate cyclase is activated similarly by both mechanisms is unclear. However, the proposed pathway for the nitrovasodilators involves eventual biotransformation to the relatively stable, free radical nitric oxide (•NO) which binds to a hemoprotein associated with guanylate cyclase, activating that enzyme (Ignarro et al., 1981; Gruetter et al., 1981a). The oxidizing agent methylene blue inhibits this process (Gruetter et al., 1981a; 1981b), possibly by converting the heme from the ferrous to ferric form. Since methylene blue also inhibits the relaxation and cGMP increase produced by acetylcholine (Holtzman, 1982; Martin et al., 1985), it has been proposed that EDRF may also act at the heme site on guanylate cyclase, suggesting either a similarity of structure or chemical

properties for EDRF and nitric oxide (R.F. Furchgott, L.J. Ignarro, personal communication). This possibility will be explored further in the discussion of the nature of EDRF.

The Role of Calcium Ion

A possible role for translocation of extracellular calcium in endothelium-dependent vasodilation is suggested by the requirement of intact entothelium for the calcium channel activators, BAY K 8644 and (+)202,791 to produce relaxation in canine femoral arteries (Rubanyi et al., 1985b) and, more importantly, by the endothelium-dependent relaxations produced by the calcium ionophore A23187. A23187 is a potent vasodilator in bovine (Gruetter and Lemke, 1986), feline (Brayden and Bevan, 1985), canine (Cocks et al., 1985), rabbit (Furchgott and Zawadzki, 1980; Carvalho and Furchgott, 1981; Singer and Peach, 1983b; Weinheimer and Osswald, 1986), rat (Thomas et al., 1986), guinea-pig (Bolton et al., 1984; Bolton and Clapp, 1986; Weinheimer and Osswald, 1986) as well as human (Forstermann et al., 1986c) blood vessels, and in all tissues tested its actions are totally endothelium-dependent. Identical responses of A23187 and acetylcholine in the "sandwich" experiments and cGMP measurements discussed above, as well as parallel response to most inhibitors discussed below, implicate EDRF as the mediator of A23187-induced relaxation and further suggest calcium ion influx as a requirement for acetylcholine-induced relaxation.

The use of calcium free solutions (Singer and Peach, 1982; Winquist, et al., 1985) to decrease or totally block the cholinergic, endothelium-dependent relaxation of rat and rabbit aorta more directly indicates a role for calcium influx in this response. Cascade experiments have been done in which a perfused rat aorta was stimulated to produce EDRF, and a de-endothelized strip of aorta acted as a bioassay

for the presence and level of EDRF (Schoeffter and Miller, 1986). By independently controlling calcium concentrations in buffers of the perfused segment and the test strip, the effect of calcium on EDRF production was clearly separated from its effect on the contractile state of the smooth muscle. These experiments demonstrate an absolute dependence on external calcium for acetylcholine stimulated EDRF release.

Several possible mechanisms for the action of calcium ion in the production or release of EDRF are proposed by Weinheimer and Osswald (1986) in their report that the cholinergic relaxations of rabbit aorta were blocked by the calmodulin antagonists calmidazolin and W-7. One possibility is activation of phospholipase A_2, thought to be dependent on calmodulin (Wong and Cheung, 1979), leading to the release of arachidonic acid. The ability of the phospholipase A_2 inhibitors quinacrine and p-bromophenacyl-bromide to block cholinergic relaxations in rabbit (Singer and Peach, 1983b; Furchgott et al., 1982) and dog (Regoli et al., 1982; De Mey et al., 1982; Forstermann et al., 1985; Furchgott et al., 1982) add support to this idea. Calmodulin has also been implicated in the activation of lipoxygenase (Fiedler-Nagy, et al., 1983). This enzyme and the lipoxygenase pathway have also been suggested by Furchgott (1981) and others (Chand and Altura, 1981b; De Mey et al., 1982) as a possible pathway for EDRF production. Because of the broad range of activity of calmodulin, it is not inconceivable that it could be involved in the activation of both of these enzymes as well as others in the signal transduction from muscarinic receptor to EDRF release.

The mechanism by which calcium ions enter the endothelial cell is also unclear. Calcium channel blockers have shown mixed results as inhibitors of the cholinergic response. In rabbit aortic rings nifedipine and verapamil have decreased

the maximum relaxation to acetylcholine by 40-50 % leaving the ED_{50} values unchanged (Singer and Peach, 1982). Others report that these calcium channel blockers produced a 2-3 fold rightward shift in the dose-response curve to acetylcholine with only a small decrease in the maximum relaxation (Winquist et al., 1985). Nicardipine in this same tissue had no significant effect on the endothelium-dependent relaxation to acetylcholine (Jakakody et al., 1986).

While calcium channel blockers had only limited effect, inhibition of Na^+-Ca^{++} exchange by dichlorobenzamil completely abolished acetylcholine stimulated relaxation in rat and rabbit aorta without affecting the endothelium-independent relaxation of sodium nitroprusside or atrial naturetic factor (Winquist et al., 1985). Further support for Na^+-Ca^{++} exchange as the mechanism for calcium influx comes from experiments using cGMP elevation rather than relaxation as a measure of EDRF release. In rat aorta cyclic GMP elevation to maximum acetylcholine stimulation (1 μM) was unaffected by the calcium channel blockers, verapamil, nifedipine, diltiazem and bepridil, while replacement of Na^+ with choline or the presence of the Na^+-Ca^{++} exchange antagonist amiloride inhibited cGMP production totally or by 80%, respectively (Schoeffter and Miller, 1986). Similar treatment had no effect on nitroprusside-induced elevation of cGMP.

Thus, although evidence for the role of calcium channels is questionable and that for Na^+-Ca^{++} exchange appears more promising, the question is far from resolved. As already noted the ability of acetylcholine to relax vessels precontracted by elevated K^+ is significantly less than when contraction is produced by other agents. While this observation could be interpreted in terms of a change in membrane voltage with an effect on voltage-sensitive channels, it could also be due to an effect on Na^+-K^+ exchange via Na^+-K^+-ATPase. Inhibition of acetylcholine

relaxation in dog femoral rings by ouabain (De Mey and Vanhoutte, 1980) does seem to indicate some role for Na^+-K^+ exchange in this process, but whether this involves calcium ion transport has yet to be determined.

What is the Nature of EDRF?

Half-life

In 1984 Griffith et al. developed a cascade perfusion system composed of a segment of rabbit aorta with intact endothelium to generate EDRF and a stripped segment of rabbit coronary artery as a bioassay for the EDRF. As the distance between the vessels was extended the response of the test segment decreased. By assuming that the degree of relaxation was a measure of EDRF remaining active in the system they were able to calculate a half-life of approximately 6 seconds for EDRF. Comparable values have since been determined in similar systems of perfused rabbit and dog vessels (Rubanyi et al., 1985a; Khan and Furchgott, 1986), as well as a variation employing cultured endothelial cells to generate EDRF (Cocks et al., 1985; Gryglewski et al.,1986a). In this case bovine or porcine endothelial cells were grown on microcarrier beads, placed in a column and perfused. EDRF production was again determined by use of a stripped ring segment (rabbit, bovine or dog) as a bioassay. Such cascade systems which separate generation of EDRF from its site of action have proven to be useful tools in examining the mechanisms for production and action of EDRF as well as its chemical properties.

A Possible Role for Arachidonic Acid Metabolism

As discussed earlier quinacrine and p-bromophenacylbromide, inhibitors of phospholipase A_2, also inhibit acetylcholine-induced relaxations in rabbit and dog vessels suggesting a role for this enzyme in EDRF production. Furthermore, direct

stimulation of phospholipase A_2 by melitten, a polypeptide toxin from bee venom, produced relaxations in rabbit aorta that closely parallel those of acetylcholine (Forstermann and Neufang, 1985). Since phospholipase A_2 is known to release arachidonic acid from cellular membranes (Flower and Blackwell, 1976) and direct application of arachidonic acid to ring segments from rabbit (Singer and Peach, 1983a) and dog (De Mey and Vanhoutte, 1982; Vanhoutte and Rubanyi, 1985) produce relaxations that are at least partially endothelium-dependent, the arachidonic acid cascade leading to either prostaglandins or leukotrienes may be involved in the cholinergic relaxation response.

The established ability of endothelium to synthesize prostaglandins as well as the well known prostaglandin vasoactive properties and short half-life, all suggest the possibility that EDRF may be a prostaglandin. Prostaglandins have been shown to be produced in response to cholinergic stimulation by endothelial cells of intact rings (Forstermann and Neufang, 1985; Huang and Lee, 1985) and in cultures (Gryglewski et al.,1986a). However, the cyclooxygenase inhibitor, indomethacin, did not effect the acetyl-choline generated relaxation or increases in cGMP levels, even though prostaglandin production was completely blocked (Van de Voorde and Leusen, 1983b; Forstermann and Neufang, 1985). Thus, while arachidonic acid may be involved in endothelium-dependent relaxations, production of prostaglandins is apparently not.

In the other branch of the arachidonic acid cascade, oxidation by lipoxygenase leads to the production of the leuko-trienes. The vasoactive, labile leukotrienes again fit the general description of EDRF, and, unlike the case with prostaglandins, inhibitors of lipoxygenase also inhibit acetylcholine-induced relaxation. 5,8,11,14-eicosatetraynoic acid, a triple bond analogue of arachidonic

acid which inhibits both lipoxygenase and cyclooxygenase (Flower, 1974), has also been shown to block the cholinergic endothelium-dependent relaxation in rabbit (Furchgott and Zawadzki, 1980; Forstermann et al., 1985; Singer and Peach, 1983b; Regoli et al., 1982), rat (Van de Voorde and Leusen, 1982, 1983b; Davies and Williams, 1983) and dog (Forstermann et al., 1985; De Mey et al., 1982) vessels, while having no effect on responses to endothelium-independent vasodilators such as isoproterenol or the nitrates (Furchgott et al., 1981). Similarly, nordihydro-guiaretic acid, also known to inhibit lipoxygenase, has proven effective in blocking or reversing endothelium-dependent responses in rabbit aorta (Furchgott et al., 1982; Singer and Peach, 1983b; Forstermann and Neufang, 1985) and femoral artery (Forstermann et al., 1985) and dog renal artery (Chand and Altura, 1981b). A third lipoxygenase inhibitor, BW 755C (0.1 mM), was initially reported ineffective against cholinergic relaxation in rabbit aorta (Furchgott et al., 1981). However, when higher concentrations (0.15 mM) of BW 755C were used relaxations in the rat aorta were blocked (Davies and Williams, 1983), and at a concentration of 0.3 mM, relaxations were converted into small contractions in the rabbit aorta (Forstermann and Neufang, 1985).

If EDRF is a leukotriene (LT) it is not LTC_4, D_4 or E_4 all of which were ineffective in producing relaxation of rabbit aorta (Forstermann and Neufang, 1984a). While LTD_4 did produce relaxation in dog renal and mesenteric arteries, LTD_4 could not be EDRF since this response to LTD_4 was dependent on the presence of an intact endothelium. Furthermore, the response was blocked by the putative leukotriene antagonist FPL 55712 which had no effect on acetylcholine-induced relaxation in these tissues or in the rabbit aorta (Forstermann and Neufang, 1984a).

As an alternative to cyclooxygenase or lipoxygenase metabolism of arachidonic acid, Singer et al. (1984) have suggested a role for cytochrome P-450 in both arachidonic acid metabolism and endothelium-dependent relaxation. They found that the endothelium-dependent relaxations to arachidonic acid, A23187 and methacholine were inhibited by the cytochrome P-450 blockers, SKF-525A and metyrapone, while endothelium-independent relaxations to nitroprusside were undiminished. Combined with evidence for cytochrome P-450 metabolism of arachidonic acid as well as inhibition of cytochrome P-450 by 5,8,11,14-eicosatetraynoic acid and nordihydroguiaretic acid, this pathway is seen as a viable alternative in production of EDRF from arachidonic acid. Unfortunately the lack of specificity shown by SKF-525A, for instance its ability to inhibit calcium channels (Kalsner et al., 1978), has cast some doubt on this mechanism.

A report by Griffith et al. (1984a) dramatically increased the level of confusion generated by a lack of specificity of inhibitory agents. After first confirming the inhibition of relaxation of intact rabbit aorta by nordihydroguiaretic acid and phenidone, another lipoxygenase inhibitor, these agents were tested in a cascade system (rabbit aorta to coronary artery), where they were added between the intact vessel generating EDRF and the stripped test segment. Both agents were found to be equally effective as inhibitors when contact with the endothelial cells, lipoxygenase or even cytochrome P-450 was not possible. By altering the injection site and, therefore, the contact time with EDRF in the perfusate, it was concluded that direct chemical inactivation of EDRF blocked relaxation in this system rather than an inhibition of either synthesis or release of EDRF. While it is still possible that the arachidonic acid metabolites play an important role in EDRF generation, it is also possible that the inhibitory effects described above are partially or totally

due to other chemical properties of these substances such as antioxidant or free radical scavenging activity rather than their inhibition of arachidonic acid metabolism.

Inhibition by Antioxidants and Free Radical Scavengers

Using both rabbit aortic strips and the perfusion cascade system described above an exhaustive study of inhibition of endothelium-dependent relaxation was carried out with substances having different known combinations of chemical properties including antioxidants, specific and non-specific free radical scavengers, lipoxygenase inhibitors and alkylating agents (specific for carbonyl groups)(Griffith et al., 1984a). By comparing chemical activities with the ability to inhibit endothelium-dependent relaxation, it was concluded that specific lipoxygenase inhibition or oxygen centered free radicals were not involved. Most inhibitors were found to exhibit both antioxidant and non-specific free radical scavenging ability, leaving open the possibility that EDRF is a non-oxygen centered free radical. However, inhibition by phenylhydrazene and potassium borohydride (KBH_4), which specifically react with carbonyl groups, led to the conclusion that a carbonyl was at or near the active site, and that EDRF was probably an aldehyde, ketone or lactone.

Other investigators have also eliminated oxygen centered free radicals as potential EDRF candidates, and, further, found that inhibition of these radicals or more specifically, superoxide anions ($\cdot O_2^-$), by superoxide dismutase had a protective effect on EDRF in ring preparations (Rubanyi and Vanhoutte, 1986a), perfused vessel cascades (Rubanyi and Vanhoutte, 1986c) and cell culture cascades (Gryglewski et al., 1986b). These authors suggest that the superoxide anion may be acting as a reducing agent (antioxidant) to inactivate EDRF. Forstermann et al. (1985) have shown in perfusion cascades of rabbit and dog vessels that decreasing

the oxygen tension in the buffer solution to physiological levels (120-140 mmHg), which should decrease the level of oxygen free radicals, also has a protective effect on EDRF. In their lower oxygen system EDRF generated by rabbit vessels had a half-life of 24 sec, and that from dog vessels was increased to 49 sec. Rubanyi and Vanhoutte (1986c) found that a combination of low oxygen and superoxide dismutase would prolong the half-life of EDRF from dog femoral artery to 81 sec.

Other Inhibitors of EDRF.

Other agents that inhibit endothelium-dependent relaxation include the guanylate cyclase inhibitor, methylene blue, discussed earlier and the specific receptor antagonists of endothelium-dependent vasoactive agents which will be covered individually with these agents. However, there are several other agents that apparently block endothelium-dependent relaxation by inactivating EDRF which deserve individual attention.

Rubanyi et al. (1985a) using a cascade composed of a perfused canine femoral artery with a coronary artery test ring have shown that in the presence of phentolamine and propranolol the catecholamines norepinephrine and epinephrine reverse the relaxing effect of acetylcholine. This inhibition of relaxation was not a direct contrac-tile effect, and was still present when the catecholamines were added between vessel segments. Furthermore, the greater the length of contact time with EDRF the greater the inhibitory effect. Based on the previously discussed susceptibility of EDRF to reducing agents it seems likely that the catecholamines are behaving as antioxidants to inactivate EDRF. These observations are of particular interest since it is very often contractions induced by norepinephrine which must be reversed by endothelium-dependent agents generating EDRF. It is also interesting to note that norepinephrine has itself been shown to relax canine

and porcine arteries (Cocks and Angus, 1983) by an endothelium-dependent mechanism which appears to be EDRF-mediated. Endothelium-dependent catecholamine-induced relaxations will be discussed in greater detail below.

Forstermann et al. (1985) have reported that the addition of albumin (5 mg/ml) to the perfusion medium in rabbit or dog vessel cascades abolished relaxations produced by acetylcholine, suggesting extensive binding of EDRF to albumin. They also found that the presence of diluted serum (1:10) not only abolished the relaxation of the test ring but also attenuated relaxation of the generating segment. Conflicting results with albumin were observed by Thomas et al. (1986) in rings of rat aorta. These authors found buffers containing 5% albumin actually enhanced responses to acetylcholine suggesting a protective effect on EDRF. However, they confirmed the inhibitory effect of serum or plasma (human and rat) on relaxation, and suggest the possibility that the earlier report of albumin inhibition of EDRF was due to contami-nation of the albumin with serum. In either case the apparent inactivation of EDRF by components of blood suggest that the *in vivo* response to EDRF must be extremely localized.

Hemoglobin has also been found to inhibit cholinergic, endothelium-dependent relaxation as well as the endothelium-independent relaxation by glyceryl trinitrate in rabbit aortic rings (Martin et al., 1985; Edwards et al., 1986). As discussed earlier methylene blue inhibits endothelium-dependent and independent relaxation apparently by inactivating guanylate cyclase, and, therefore, it is possible that hemoglobin may also work through this mechanism. However, hemoglobin is believed to inhibit relax-ation by the nitrovasodilators by binding to their active metabolite, nitric oxide (•NO), rather than a direct effect on guanylate cyclase (Mittal et al., 1978; Gruetter et al., 1981a). It seems likely, therefore, that

hemoglobin inhibits endothelium-dependent relaxation through inactivation of EDRF. Further evidence for this mechanism has come from Cocks and Angus (1985) who reported that EDRF generated in a cell culture perfusion cascade was removed by passage through a column of hemoglobin (oxidized form) covalently bound to agarose, while agarose alone had no effect.

In similar experiments Cocks and Angus (1985) replaced the hemoglobin-agarose column with either a lipophilic or ion exchange column. EDRF removal by the anion but not cation exchange column indicates a negatively charged structure, and the lack of effect of the lipophilic column suggests that EDRF is not a lipid or fatty acid derivative and, therefore, not a metabolite of arachidonic acid.

Summary.

EDRF is a labile compound having a biological half-life of 5 to 10 seconds under most *in vitro* conditions, which can be extended to as long as 80 seconds by altering those conditions. Evidence exists that EDRF is an oxidative metabolite of arachidonic acid generated via either the lipoxygenase or cytochrome P-450 pathway or both. Inhibition by specific alkylating agents suggests the presence of a carbonyl group near the active site making it an aldehyde, ketone or lactone. Inactivation by other agents indicates the possibility of a particularly stable non-oxygen centered free radical. Similarity to the nitrovasodilators in ability to activate guanylate cyclase and susceptibility to methylene blue and hemoglobin also suggests a free radical. Finally, inactivation by anionic exchange, but not cationic or lipophilic columns, connotes a hydrophilic, negatively charged molecule.

What is the Role of Endothelium with Other Vasodilators?

While acetylcholine is the most widely known and examined endothelium-dependent vasodilator, there is an ever increasing list of agents found to produce relaxation in blood vessels via the endothelium and EDRF (Table 1). This section will discuss some of the more widely studied of these agents including the tissues in which they have been shown to be active, nature of the receptors mediating the response and comparisons to acetylcholine.

Calcium Ionophore, A23187

Next to acetylcholine the most widely studied endothelium-dependent vasodilator is the calcium ionophore, A23187. Unlike acetylcholine or most of the other endothelium-dependent vasodilators, the action of A23187 is not dependent on the presence of specific receptors to produce relaxation, thus making it the most versatile of the group. A23187 has produced endothelium-dependent relaxation in vessels from all species studied including rat (Folco et al., 1982; Rapoport and Murad, 1983), rabbit (Singer and Peach, 1982, 1983b; Weinheimer and Osswald, 1986; Martin et al., 1985), cat (Brayden and Bevan, 1985), dog (Cocks et al., 1985), pig (Gordon and Martin, 1983), cow (Gruetter and Lemke, 1986) and human (Forstermann et al., 1986c; Thom et al., 1986). A23187 parallels acetylcholine in ability to stimulate an increase in cGMP (Rapoport and Murad, 1983; Furchgott and Jothianandan, 1983; Gruetter and Lemke, 1986) and susceptibility to blockade by inhibitors, including calcium channel blockers (Singer and Peach, 1982), the lipoxygenase inhibitors 5,8,11,14-eicosatetraynoic acid and nordihydroguiaretic acid (Singer and Peach, 1983b), cytochrome P-450 inhibitors (Izzo et al., 1983; Singer et al., 1984), antioxidants and free radical scavengers (Griffith et al.,

1984a), methylene blue (Gruetter and Lemke, 1986; Martin et al., 1985), blood serum (Thomas et al., 1986) and hemoglobin (Forstermann et al., 1986c; Thom et al., 1986). Relaxation to A23187 is unaffected by indomethacin (Singer et al., 1984; Gruetter and Lemke, 1986), and, unlike acetylcholine, it is also unaffected by the phospholipase A_2 inhibitor,quinacrine (Singer and Peach, 1983b; Rapoport and Murad, 1983; Forstermann et al., 1986c).

Generally, A23187 is more potent than acetylcholine and, as described by Furchgott (1983), a more powerful vasodilator. A 6 min exposure of rabbit aortic rings to a maximally effective concentration of A23187 makes the vessel segment unresponsive to acetylcholine for at least 44 min after washout, suggesting that A23187 had fully activated the mechanism for EDRF production or release making any further activation by acetylcholine impossible.

Histamine

Histamine has produced endothelium-dependent relaxations in isolated preparation of the aorta(Van de Voorde and Leusen, 1982, 1983b; Davies and Williams, 1984; Carrier et al., 1984), carotid (Leusen and Van de Voorde, 1985) and mesenteric artery (White and Carrier, 1986) of the rat , the guinea-pig pulmonary artery (Weinheimer and Osswald, 1986) and monkey coronary artery (Toda, 1986). In the dog mesenteric artery histamine-induced vasodilation is only partially endothelium-dependent, and, unlike the responses above, was partially inhibited by indomethacin. Relaxations of the rat aorta were inhibited by 5,8,11,14-eicosatetraynoic acid (Van de Voorde and Leusen, 1982, 1983b; Rapoport and Murad, 1983; Davies and Williams, 1983), hydroquinone (Van de Voorde and Leusen, 1982, 1983b), BW 755C (Davies and Williams, 1983) and anoxia (Van de Voorde and Leusen, 1983b). Quinacrine (10 µM) was found to have either no effect (Van de Voorde and Leusen,

1982) or to produce partial (30%) inhibition (Rapoport and Murad, 1983). This pattern of inhibition suggests that histamine produces endothelium-dependent vasodilation, similar to acetylcholine, via EDRF; however, limited inhibition by quinacrine further suggests that the activity of histamine, like that of A23187, is not dependent on phospholipase A_2 activation.

Inhibition of responses in both rat aorta and mesentery by the H_1 antagonists diphenhydramine and mepyramine but not the H_2 antagonist cimetidine indicate that the relaxation was mediated by H_1 endothelial receptors in these vessels (Van de Voorde and Leusen, 1982; Carrier et al., 1984; White and Carrier, 1986). In a perfusion cascade with intact or stripped human umbilical arteries or veins with a stripped rat aortic ring as the bioassay for EDRF production, histamine produced an endothelium-dependent relaxation of the test ring that could be inhibited by nordihydroguiaretic acid, methylene blue and mepyramine, but was unaffected by indomethacin or cimetidine suggesting that these vessels were also producing EDRF via H_1 endothelial receptors (Leusen and Van de Voorde, 1986).

A direct contractile response to histamine has also been observed in some tissues. Rabbit and guinea-pig aorta (Leusen and Van de Voorde, 1985) and rabbit middle cerebral artery (Secombe et al., 1986) contracted to histamine, and the relaxations of rat aorta and guinea-pig pulmonary artery were converted to contractions with endothelial removal (Van de Voorde and Leusen, 1983b; Weinheimer and Osswald, 1986). Toda (1986) examined histamine responses in human and monkey coronary arteries and found a relaxation response with intact monkey vessels, contraction with intact human arteries and contraction in both vessels when stripped of endothelium. Using selective inhibitors on both intact and stripped vessels, it was concluded that in each vessel both dilator and constrictor effects

of histamine were present. Vasodilation was produced via H_1 receptors on endothelium and via H_2 receptors on smooth muscle, while constriction was due to H_1 receptor activation on smooth muscle cells. The net effect was constriction in human and dilation in monkeys. Mixed responses were also observed in the rabbit middle cerebral artery where blockade by the H_1 antagonist chlorpheniramine reversed constriction into dilation that was partially dependent on intact endothelium and inhibited by cimetidine (Secombe et al., 1986). This again suggests endothelium-mediated relaxation to histamine, however in this case mediated by H_2 receptors.

Histamine is also an effective vasodilator in the perfused rat hindquarters (Van de Voorde and Leusen, 1983a). While endothelium removal is not possible in this system, inhibition by 5,8,11,14-eicosatetraynoic acid and quinacrine suggest endothelial involvement. Both H_1 and H_2 antagonists inhibited this response, but by using combinations of the histamine antagonists, 5,8,11,14-eicosatetraynoic acid and quinacrine these authors again concluded that the H_1 receptor mediates the endothelium-dependent dilations.

While histamine has been shown to have mixed dilator and constrictor effects in isolated vessels or vascular beds, it generally produces a dose-dependent decrease in blood pressure that is mediated by both H_1 and H_2 receptor subtypes (Owen, 1977). The observations described above make it clear that the endothelium and EDRF play a major role in the vascular activity of histamine and must be considered when the physiological and pathological significance of histamine is discussed.

Substance P

Substance P is a potent vasodilator with total dependence on an intact endothelium for its activity on a variety of isolated vessels from rabbits (Zawadzki

et al., 1981; D'Orleans-Just et al., 1985b), dog (Zawadzki et al., 1981; Angus et al., 1983; D'Orleans-Just et al., 1985a), cat (Zawadzki et al., 1981) and guinea-pig (Bolton and Clapp, 1986). Human (Forstermann et al., 1986c) and pig coronary arteries (Beny et al., 1986a) also relaxed in response to substance P. Substance P relaxation was inhibited by 5,8,11,14-eicosatetraynoic acid and quinacrine in rabbit, dog and cat vessels while indomethacin had no effect (Zawadzki et al., 1981; D'Orleans-Just et al., 1985b). Hemoglobin was found to inhibit substance P response in the guinea-pig vessels (Bolton and Clapp, 1986). Peptides related to substance P such as neurokinins A and B, kassinin, and octa-substance P (SP 1-8) had similar patterns of relaxation and inhibition in rabbit and dog vessels (D'Orleans-Just et al., 1985b; Zawadzki et al., 1983).

Substance P differs from other endothelium-dependent vasodilators by its extreme potency and susceptibility to desensitization. The threshold concentrations for substance P-mediated response were 10 and 1 pM in isolated rabbit and dog vessels respectively (Zawadzki et al., 1981) and as low as 0.1 nM in dog femoral artery *in situ* (Angus et al., 1983). Exposure to concentrations of 3 to 30 nM substance P desensitizes the test vessel to any further relaxation response to substance P or the substance P like peptides described above (Zawadzki et al., 1981; Furchgott, 1983). Desensitization to substance P had no effect on the response to other endothelium-dependent vasodilators and disappeared after washout of the desensitizing dose. Desensitization therefore seems to be at the level of the substance P receptor rather than in the mechanism of EDRF production.

The Purines (ATP, ADP, Adenosine)

The purines, in a manner similar to histamine, have produced mixed results (both contraction and relaxation) in isolated blood vessel preparations. A variety of

precontracted canine (De Mey and Vanhoutte, 1981, 1982; De Mey et al., 1982; Angus et al., 1983; Vanhoutte and Rubanyi, 1985), rabbit (Furchgott et al., 1981), rat (Kennedy et al., 1985; Thomas et al., 1986), guinea-pig (Bolton and Clapp, 1986) and human (Vanhoutte et al., 1986) vessels relax in an endothelium-dependent fashion to either or both ATP and ADP. The intact rabbit ear artery when precontracted also relaxed to ATP; however, at resting tone ATP produced a contraction that was enhanced by endothelial removal (Kennedy and Burnstock, 1985). Also in ear artery, adenosine, which normally produces endothelium-independent relaxation (Furchgott et al., 1981; De Mey and Vanhoutte, 1982), generated a relaxation that was at least partially endothelium-dependent (Kennedy and Burnstock, 1985). Removal of endothelium from canine femoral artery converted a relaxation to ATP into contraction (Angus et al., 1983), and in the guinea-pig mesenteric artery it increased the size of the contraction to ATP that had been previously observed in the presence of endothelium (Bolton et al., 1984).

As observed with histamine, the mixed responses seem to result from a mixture of receptor subtypes found on both the smooth muscle and endothelium. The smooth muscle cells of rabbit ear artery appear to have both the P_1 purinoceptor mediating relaxation (to both adenosine and ATP) and the P_2 purinoceptor mediating contraction (to ATP), while the endothelium has only P_1 receptors (adenosine and ATP) (Kennedy and Burnstock, 1985). The rat femoral artery has a similar distribution on its smooth muscle cells; however, the P_2 subtype mediates relaxation via the endothelium (Kennedy et al., 1985). The rat aorta also has only P_2 receptors on both endothelium and smooth muscle mediating relaxation and contraction, respectively (White et al., 1985). Differences in relative agonist potency in both of these vessels as well as differential response to blockade by arylazido amimopropionyl

ATP ($ANAPP_3$) in the femoral artery suggest that different subtypes of the P_2 purinoceptor mediate responses on the smooth muscle and endothelium.

It is unclear at this time whether the endothelium-dependent relaxation to the purines is mediated via EDRF since few of the above studies attempted to determine the nature of the vasodilatory agent. Cyclooxygenase inhibitors were unable to inhibit relaxation in either rabbit aorta (Furchgott et al., 1981) or dog femoral artery (De Mey et al., 1982). Responses in rabbit aorta were blocked by 5,8,11,14-eicosatetraynoic acid and quinacrine (Furchgott, 1981); however, those of the dog femoral artery were unaffected (De Mey et al., 1982). Thus, even in these vessels a role for EDRF is uncertain.

It is certain, however, that part of the vascular response to the purines is mediated by the endothelium. Therefore, any attempt to determine the role that purines may play in the control of vascular tone must consider not only the complex interaction of contraction and dilatation mediated by the smooth muscle purinoceptor subtypes, but also the endothelium and its ability to produce a vasodilator agent.

Bradykinin.

The response of isolated vessels to bradykinin is as complicated as the responses observed to histamine and the purines. Bradykinin may produce endothelium-dependent or independent relaxation or contraction depending on the species and tissue. Bradykinin produced endothelium-dependent relaxation in a number of canine arteries (Altura and Chand, 1981; Cherry et al., 1982; Regoli et al., 1982; Cocks et al., 1985), bovine intrapulmonary artery and vein (Ignarro et al., 1986), porcine aorta (Gordon and Martin, 1983) and human mesenteric artery (Cherry et al., 1982). Relaxation to bradykinin in these vessels is unaffected by

indomethacin and thus appears to be mediated by EDRF. However, cat and rabbit superior mesenteric artery (Cherry et al., 1982) as well as rabbit celiac and extrapulmonary arteries (Forstermann et al., 1986b) relax to bradykinin in both the presence and absence of endothelium. These responses, unlike the direct relaxation observed with histamine and the purines, are an indirect effect mediated by prostaglandins. Bradykinin induced prostaglandin release has been observed in rabbit vessels, and endothelium-independent relaxations to bradykinin have all been inhibited by indomethacin (Cherry et al., 1982; Forstermann et al., 1986b).

Some rabbit vessels, including aorta and renal artery (Cherry et al., 1982) and jugular and mesenteric veins (Regoli et al., 1982) either with or without endothelium, only contract to bradykinin, while dog renal and pulmonary arteries relax with intact endothelium, but contract when stripped (Chand and Altura, 1981b). Thus the observed response of a given tissue to bradykinin may be a combination of the indirect effects of endothelium-dependent (EDRF) and independent (prostaglandins) relaxation and an apparently direct contractile effect.

Bradykinin has been found to be particularly effective in the generation of EDRF from endothelial cells in culture. In multipassage endothelial cell cultures grown from bovine aorta (Cocks et al., 1985) the agonist order of potency for EDRF generation was bradykinin $\geq$ A23187 >> acetylcholine $\geq$ substance P, and in those from pig aorta (Gryglewski et al.,1986a) the order was bradykinin $\geq$ A23187 and acetylcholine was found ineffective. Whether these results are due to the original responsiveness of these endothelial cells or a result of the culturing process is currently under investigation.

The physiological or pathological role that bradykinin plays in the vasculature in not well understood. From the above observations, it clearly has potential as

both a vasoconstrictor and vasodilator, and, as discussed, its final net effect may depend on the endothelium-mediated component. Chand and Altura (1981b) discussed a number of disease states, particularly in the pulmonary vasculature, where a change in the ability of the endothelium to produce a vasodilator in response to bradykinin may play a role in the pathology. While no definite links have yet been established, the potential physiological and pathological effects of endothelium-dependent responses to bradykinin, like those of the other endothelium-dependent vasodilators, deserve further consideration.

Arachidonic Acid

As discussed above arachidonic acid is suspected of being a key precursor molecule in the production of EDRF. However, the results of experiments attempting to clarify the role of arachidonic acid by examining its direct effect on isolated blood vessels have been, thus far, inconclusive. Arachidonic acid produced a contraction in intact rabbit aorta that was enhanced by endothelium removal (Singer and Peach, 1983a; Forstermann and Neufang, 1985). Indomethacin inhibited the contractile response in intact aorta, and at concentrations of arachidonic acid greater than 30 μM converted the response into relaxation. Contraction of stripped vessels to arachidonic acid was also partially inhibited by indomethacin; however, no relaxations were observed. Thus in rabbit aorta arachidonic acid appears to have two contractile effects with one sensitive to indomethacin and both independent of the endothelium. There also seems to be an endothelium-dependent relaxation response to arachidonic acid which is enhanced by indomethacin either directly or by inhibition of contraction or both.

Precontracted canine femoral, saphenous, pulmonary and splenic arteries were all found to relax in a concentration-dependent manner to arachidonic acid, and

in all vessels endothelium removal shifted the concentration-response curve to the right (De Mey and Vanhoutte, 1982). In the only canine vessel tested with indomethacin, the femoral artery, the relaxation response to arachidonic acid was totally blocked indicating EDRF had no role in this response (De Mey et al., 1982).

While arachidonic acid does seem able to generate at least partially endothelium-dependent relaxation in rabbit and dog blood vessels, inhibition by indomethacin suggests a greater role for prostaglandins and only a small possible role for EDRF in the rabbit aorta. These results do not, however, rule out arachidonic acid as the precursor molecule for EDRF for two reasons: 1) EDRF formation from arachidonic acid may be in specific compartments not reached by exogenous arachidonic acid, and 2) in the compartments that are reached by exogenous arachidonic acid other metabolic products formed may have inhibitory effects on EDRF production or release. Thus the role of arachidonic acid in endothelium-dependent relaxation responses via EDRF is still unclear.

Aggregating Platelets, Serotonin, Thrombin and Vasopressin

When exposed to aggregating platelets dog coronary arteries were found to contract when at basal tone, but they would relax if already precontracted (Cohen et al., 1983a, 1983b). Endothelium removal potentiated the contractile response and eliminated the relaxation (Cohen et al., 1983a, 1983b). It has been proposed that this response is at least partially mediated by serotonin since: 1) aggregating platelets were found to release serotonin; 2) the relaxation response to aggregating platelets was inhibited by the serotonin antagonists methiothepin and methysergide ($5HT_1$); 3) contractile responses were inhibited by ketanserin ($5HT_2$) and 4) exogenous serotonin produced similar responses in dog and pig coronary arteries (Cohen et al., 1983a; Cocks and Angus, 1983; Houston et al., 1985).

However, ADP, which also produced endothelium-dependent relaxation in dog coronary artery, was also shown to be released by aggregating platelets, and furthermore, apyrase, an enzyme that hydrolyzes ADP to AMP, attenuated both the endothelium-dependent relaxation responses to ADP and that to aggregating platelets without affecting the response to serotonin (Housten et al., 1985, 1986). These findings suggest a key role for ADP in the endothelium-dependent relaxation response to aggregating platelets.

Thrombin, a vasoactive compound whose production may be stimulated by aggregating platelets, has also been shown to have at least partially endothelium-dependent responses. Precontracted segments of rabbit and dog coronary (Garland and Bevan, 1985; Ku, 1986; De Mey et al., 1982) and dog basilar (Katusic et al., 1984) when exposed to thrombin relax in the presence and contract in the absence of endothelium. Neither type of response was affected by cyclooxgenase inhibitors, but both responses could be blocked by heparin (De Mey et al., 1982; Ku, 1986).

Vasopressin, which like thrombin is elevated during hemorrhage and is normally considered a vasoconstrictor, produced endothelium-dependent relaxation of dog basilar artery, relaxation of dog coronary that was partially endothelium-dependent but only contraction in dog femoral artery (Katusic et al., 1984)

Taken together the above observations suggest a protective role for the endothelium during injury or blood loss. The relaxation response to aggregating platelets specifically seen in coronary vessels, whether produced via serotonin or ADP, would be protective against infarction both during injury and under normal conditions. Relaxations of both coronary and cerebral arteries to thrombin and vasopressin would help maintain blood flow to the heart and brain during

hemorrhage while simultaneously causing contraction and a decrease in blood flow to less essential organs.

Flow Rate

Changes in flow rate have been found to produce vasodilation in blood vessels in an endothelium-dependent manner. In a perfusion cascade system (dog femoral to coronary) an increase in steady flow or the introduction of pulsatile flow triggered the endothelium-dependent release of a non-prostaglandin vasodilator with characteristics similar to EDRF (Rubanyi et al., 1986). However, in mesenteric vessels at constant pressure, Tesfamariam et al. (1985) found that during flow, contractile responses to nerve stimulation and exogenous norepinephrine were less that at no flow. They hypothesized that flow washed away an inhibitory substance that had been spontaneously released by the endothelium. While these results may initially appear contradictory, it is possible that the apparent EDRF release observed in the cascade experiment when flow was increased is a result of the change in flow rather than the actual level of flow. This would, therefore, be consistent with the flow/no flow experiments. Similar results have been observed in dog femoral arteries *in situ*, where an increase in blood flow at constant inflow pressure increased the artery diameter, but only in the presence of an intact endothelium (Smiesko et al., 1985). This again suggests endothelium-dependent release of a vasodilator substance induced by changes in flow. The mechanism leading to the release of this substance and whether it is EDRF has yet to be determined.

Chronic changes in blood flow also appear to alter the responses of blood vessels to endothelium-dependent vasodilators. When dog femoral arteries were exposed to a 3 fold increase in blood flow by use of an arteriovenous fistula, then removed after 6 weeks and the endothelium-dependent responses tested, the vessels

which had increased flow were found to have increased endothelium-dependent relaxations to acetylcholine (Miller et al., 1986). Both the acute and chronic effects of flow which are mediated by the endothelium suggest that the endothelium plays an important role in the mechanics of blood flow dynamics as well as pharmacological responses.

Catecholamines

Cocks and Angus (1983) have reported epinephrine- and norepinephrine-induced endothelium-dependent relaxations of precontracted canine and porcine coronary arteries in the presence of propranolol and prazosin. A lack of effect with the α_1 specific agonist methoxamine and blockade by specific α_2 antagonists indicate the relaxation is mediated by the α_2-adrenoceptor. Unfortunately, no attempt was made to determine the nature of the vasodilator substance which was apparently released by the endothelium. In a similar study of dog coronary responses where Rubanyi and Vanhoutte (1985) found the presence of endothelium to potentiate relaxations to ß-adrenergic agonists. In this case, the effect was inhibited by indomethacin suggesting involvement of prostaglandins rather than EDRF.

Fenoldopam (SK&F 82526), an apparent dopamine agonist, produced dose-dependent relaxations in the rabbit splenic artery that were partially dependent on the presence of intact endothelium (Ohlstein et al., 1984). These relaxations were potentiated in intact but not stripped tissues by a phosphodiesterase inhibitor thus suggesting a possible role for cGMP and EDRF in this response.

The above observations coupled with reports from a number of laboratories that endothelium removal leads to increased contractile response to norepinephrine as well as other vasocon-strictors have led to speculation that the endothelium might provide a form of physiological antagonism to high levels of circulating

vasoconstrictor substances by releasing a vasodilator substance when stimulated by various types of agents (Eglème et al., 1984). Alternatively, it has also been proposed that the endothelium continuously releases a vasodilator substance. The absence of this agent when endothelium is removed would enhance constriction (Martin et al., 1986). This controversy will be discussed further in chapter 4.

Other Agents

Recently a number of vasoactive peptides have been shown to produce endothelium-dependent relaxation in blood vessels. Calcitonin gene related peptide produced endothelium-dependent relaxation in a number of human arteries including the pulmonary, gastric, coronary, splanchnic and cerebral (Sever et al., 1986; Thom et al., 1986). Vasoactive intestinal polypeptide relaxations were endothelium-dependent in rat aorta (Davies and Williams, 1983; Katusic et al., 1984) and bovine intra-pulmonary artery (Ignarro et al., 1986) but not porcine coronary (Beny et al., 1986a). Neurotensin and bombesin have also been shown to produce weak endothelium-dependent relaxation in some segments of dog carotid artery (D'Orleans-Just et al., 1985b). However, another recently discovered vasodilator peptide, atrial natriuretic factor, has been found to act entirely independently of the endothelium (Winquist et al., 1985).

Relaxations to the antihypertensive drug hydralazine have also been found to be partially endothelium-dependent in rabbit aorta where endothelium removal produced a 10 fold shift in the dose-response curve (Folco et al., 1982). However, no other antihypertensive drugs including minoxidil, diazoxide, the nitrates or prazosin were found to be dependent on the endothelium (Folco et al., 1982).

The ergot alkaloid, ergonovine, generally considered to be a vasoconstrictor and used clinically to induce vasospasm in susceptible patients produced

endothelium-dependent relaxations in rabbit aorta precontracted with serotonin, phenylephrine or histamine (Griffith et al., 1984b). This relaxation was unaffected by serotonin or alpha-adrenergic antagonists, but was reversed by agents shown to inhibit EDRF. The sensitivity of some patients to ergonovine suggests a possible contributary role of altered endothelial reactivity in coronary vasospasm.

What is The Role of Endothelium in Pathological States?

While it is clear from the above discussion that the vascular endothelium is involved in a number of responses of blood vessels to vasoactive agents, the importance of this involvement in the normal functioning of the organism has yet to be determined. It has been observed, however, that in certain pathological conditions such as hypertension, atherosclerosis and diabetes mellitus there are significant changes in the endothelium-dependent responses of blood vessels.

Hypertension

An alteration in the endothelium-dependent relaxations of isolated blood vessels has been observed with hypertension, whether genetically or experimentally induced. Aortic ring segments from genetically hypertensive rats have shown decreased maximum response to acetylcholine and A23187, while the maximum response to nitroprusside was unaffected (Winquist et al., 1984). Small mesenteric arteries (200 μm) from genetically hypertensive rats showed similar decreases in maximum relaxation to acetylcholine, bradykinin and histamine (De Mey and Gray, 1985).

When hypertension was induced in rats using renal clip or DOCA-salt techniques similar decreases were again observed in the endothelium-dependent relaxations of aortic segments to acetylcholine, A23187 and histamine (Lockette et

al., 1986; Van de Voorde and Leusen, 1986). These changes were reversed, however, if blood pressure was allowed to return to normal. A bioassay for EDRF (perfused aorta to aortic ring cascade) found no decrease in the apparent release of EDRF from vessels of hypertensive animals suggesting the deficit in response was due to changes in the smooth muscle response to EDRF (Van de Voorde and Leusen, 1986).

Luscher and Vanhoutte (1986a) have found, however, that acetylcholine induces contractions in quiescent, intact aortic ring segments from genetically hypertensive rats but not from stripped vessels or vessels from control animals with or without endothelium. They have also shown that in the presence of ketanserin ($5HT_2$ antagonist) to minimize the direct contractile effects on vascular smooth muscle, precontracted, intact aortic rings from hypertensive animals contract while those from controls relax when exposed to serotonin (Luscher and Vanhoutte, 1986b). Both the acetylcholine- and serotonin-induced, endothelium-dependent contractions were blocked by indomethacin but not inhibitors of leukotriene, prostacyclin or thromboxane synthetases. From these observations the authors suggest that the hypertensive endothelium releases a contracting factor that is a cyclooxgenase product, but not prostacyclin or thromboxane. They further suggest that this contracting factor is responsible for the observed differences in endothelium-dependent relaxations to acetylcholine, rather than a change in EDRF production or its coupling, as proposed above.

Since the changes in blood pressure precede the changes in endothelium-dependent responses in both genetic (De Mey and Gray, 1985) and experimental (Van de Voorde and Leusen, 1986) hypertension, it seems likely that the altered endothelium is an effect rather than a cause of hypertension. However, the direction of the altered response, toward greater contractions, suggests that it may contribute to a

further increase in already elevated blood pressure, and thus may eventually be found to be a suitable site for clinical intervention.

Hypercholesterolemia and Atherosclerosis

Several recent studies have examined the effect of hypercholesterolemia and atherosclerosis on endothelium-dependent relaxations. Hypercholesterolemia in the absence of atherosclerosis was found to significantly decrease or change to small contractions the relaxation response of rabbit aortic rings to acetylcholine (Ibengwe and Suzuki, 1986; Habib et al., 1985). While hypercholesterolemia alone was ineffective on vessels from monkey and dog, when atherosclerotic vessels were examined, rabbit aorta, monkey femoral and human coronary all had decreased relaxations response to acetylcholine (Bossaller et al., 1985; Harrison et al., 1986). However, relaxations to neither the endothelium-dependent agent A23187 nor the endothelium-independent agent nitroglycerine were altered in atherosclerotic vessels suggesting a deficit at the cholinergic receptor or receptor coupling rather than in release of EDRF or responsiveness of smooth muscle to EDRF.

Diabetes Mellitus

While diabetes mellitus is known to produce alteration in vascular reactivity, only a limited number of studies have examined its effect on endothelium-dependent responses. Drug-induced diabetes does not appear to have any effect on endothelium-dependent relaxation responses to acetylcholine in rat aorta and mesenteric vessels and rabbit aorta or on the endothelium-dependent relaxations to histamine in rat aorta (Fortes et al., 1983; Stitzel et al., 1985; White and Carrier, 1986). However, isolated rat mesenteric artery rings *in vitro* (White and Carrier, 1986) and rat mesenteric microvessels *in situ* (Fortes et al., 1983) were found to

have increased sensitivity to both histamine and bradykinin, respectively. Whether these responses were endothelium-dependent was not determined in the microvessel experiments, but in the mesenteric rings the response was abolished with removal of the endothelium.

Although limited in species and vessels examined, these studies do indicate an effect of diabetes on some endothelium-dependent relaxation responses. However, the role, if any, for this alteration in endothelium-dependent responsiveness on the pathogenesis of diabetes, has yet to be uncovered.

What is the Rationale for the Current Studies?

Chapters 2 through 4 consist of three manuscripts which have been or soon will be submitted for publication. They are presented fundamentally as they were submitted with only a few minor revisions primarily to adhere to the current format. Each manuscript was written to stand alone, and while the studies they describe are different, they are unified by their common exploration of the phenomenon of endothelium-dependent relaxation of blood vessels.

It is the purpose of this section to explain in somewhat greater detail and candor than is found in each chapter, the rationale, objectives and hypotheses that preceded the work. The final chapter of this dissertation is a short summation which discusses how well the objectives were met and the validity of these initial hypotheses.

The Muscarinic Receptor Subtype on Vascular Endothelium

In the original report on endothelium-dependent vasodilation, Furchgott and Zawadzki (1980) observed that both the relaxation of intact rabbit aortic ring segments and the contractions of those stripped of endothelium were blocked by

atropine indicating mediation by muscarinic receptors. Concurrently, reports were appearing which suggested that the muscarinic antagonist pirenzepine was able to distinguish two subtypes of muscarinic receptor (Brown et al., 1980; Hammer et al., 1980; Hammer and Giachetti, 1982). Both functional (Brown et al., 1980) and radioligand binding studies (Hammer et al., 1980; Hammer and Giachetti, 1982; Watson et al., 1983) identified receptors with a high affinity for pirenzepine (M_1) in the sympathetic ganglia and cerebral cortex while more peripheral tissues such as the myocardium and ileum had the lower affinity (M_2) subtype. Shortly thereafter, in a review of the current state of knowledge of endothelium-dependent vascular responses, Furchgott (1983) asked whether the muscarinic receptors present on endothelial cells mediating relaxation were the same as those on smooth muscle cells mediating contraction. The objective of this current study was to determine the answer to this question.

Preliminary experiments using ring segments of rabbit ear artery had shown endothelium-dependent relaxation to cholinergic agonists, but no contractile response in either intact or stripped segments. Thus, ear artery unlike the aorta did not appear to have smooth muscle muscarinic receptors. This made ear artery ideal for examining the nature of endothelial muscarinic receptors while avoiding the complication of smooth muscle receptors.

The nature of the endothelial muscarinic receptor was to be determined in two series of experiments, both using the selective antagonist, pirenzepine. Inhibition of relaxation by different concentrations of pirenzepine would allow determination of antagonist affinity via Schild plot analysis (Arunlakshana and Schild, 1958). Parallel experiments were to be done using the well studied antagonist atropine to validate the procedures used with pirenzepine.

A second, somewhat more complex, approach was also to be used which involved inhibition of binding of the high affinity muscarinic antagonist quinuclidinyl benzilate by pirenzepine. First the binding characteristics of labeled quinuclidinyl benzilate ([^{3}H](-)QNB) had to be determined in both stripped and intact ear artery membrane homogenates. Binding could then be inhibited by both pirenzepine and atropine allowing the calculation of another set of affinity values for these agents (Cheng and Prusoff,1973).

This study began with a working hypothesis which was based on earlier observations of no functional activity for smooth muscle muscarinic receptors and a primarily peripheral distribution of M_2 subtype receptors. That hypothesis was:

Cholinergic, muscarinic receptors of the rabbit ear artery are primarily on the vascular endothelium and are of the low affinity, M_2 subtype.

Increasing Age and Endothelium-Dependent Vasodilation

It has been well established that advancing age produces a number of significant changes in circulatory hemodynamics in both man and animals. including increased blood pressure as well as altered responses to exercise and non-exercise stress and a variety of therapeutic drugs (Kalbfleisch et al., 1977; Miller, 1981; Palmer et al., 1978; Docherty, 1986). Considering the ever increasing number of agents which had been found to act through the endothelium it seemed possible that one mechanism for these age-related changes might be an alteration of the responsiveness of the vascular endothelium to vasoactive substances.

The objective of this study then was to determine if endothelium-dependent vasodilation was altered with advancing age. The initial approach was to be a comparison of the relaxation dose-response curves to a muscarinic agent such as methacholine in arteries from rats of four ages: 6, 12, 20 and 27 months.

Cholinergic responses were chosen for two reasons: 1) the endothelium-dependent response to acetylcholine had been demonstrated in most arteries tested making it an excellent marker for the endothelium-dependent mechanism, and 2) the previous study had established procedures for determining muscarinic receptor affinity which could be applied in these tissues as well. It was planned to later determine the responsiveness to other substances with endothelium-dependence (bradykinin and substance P) as well as those that act independently of the endothelium (isoproterenol and nitroprusside).

An earlier report on the vascular effects of acetylcholine described a decrease in vasodilation with age in human renal circulation (Hollenberg et al., 1974). A study using the perfused rat mesentery reported no change in response to acetylcholine with aging (Fleisch and Spaethe, 1981); however, only ages 3 weeks to 6 months were considered.

Thus, little information was available regarding endothelium-dependent responses and age other than reports that the relaxation response to acetylcholine is maintained in youth (rats), but declines with old age (humans). But faced with the observation that blood pressure increases with age and the increasingly obvious physiological significance of endothelium-dependent vasodilation, the aging study began with the following hypothesis: **Cholinergic, endothelium-dependent relaxation declines in the rat vasculature from maturity (6 months) to senescence (27 months).**

Enhanced Contraction with Removal of Endothelium

While the aging study was under way, several reports appeared which described enhanced contractile response in rat aorta to α-adrenoceptor agonists with removal of the endothelium (Eglème et al., 1984; Lues and Schüman, 1984;

Miller et al., 1984; Godfrained et al., 1985). While contractile responses to both α_1- and α_2-agonists were enhanced, the relative increase in response was much greater for α_2-selective agents. Since α_2-adrenoceptor mediated release of EDRF had already been reported in porcine and canine coronary arteries (Cocks and Angus, 1983), it was proposed that α_2-adrenoceptors on rat aortic endothelium were similarly stimulated to release EDRF which then acted to inhibit the α-stimulated contraction (Eglème et al., 1984). However, the inability of the α_2-adrenoceptor selective antagonist rauwolscine to mimic endothelial removal, and certain inconsistencies in the relative enhancement of different α_1- and α_2-adrenoceptor selective agonists, cast doubt on the validity of this proposal (Lues and Schümann, 1984).

Using a perfusion cascade system Griffith et al.(1984), observed increases in the contractile state of test segments when the perfused segments was stripped of endothelium, leading to the conclusion that an unstimulated, basal level of EDRF was released from the endothelium. This possibility was quickly presented as an alternative to α_2-adrenoceptor mediated release of EDRF (Miller et al., 1984; Martin et al., 1985). While this proposal was consistent with most of the observations, no explanation of the preferential enhancement of α_2-selective agonists had as yet been presented.

Cyclic GMP production was found to decrease in rat aorta with removal of endothelium suggesting basal release of EDRF, however, cGMP production was also shown to increase with exposure to both α_1- and α_2-adrenoceptor selective agonists, suggesting stimulated release of EDRF (Bigaud et al., 1984; Miller et al., 1984). It seemed possible, therefore, that both stimulated and basal release may be occurring.

The purpose of this third current study was to explore this controversy further, using the perfused rat caudal artery. Preliminary experiments with this preparation had shown enhancement of contraction by exogenous agonists with endothelium removal. These studies further showed previously unreported enhancement of transmural nerve stimulated responses. Uptake by both nerves and smooth muscle cells of norepinephrine released by nerve stimulation made it likely that enhancement with endothelial removal was, in this case, due to the elimination of the basal level of EDRF. Since in this perfusion system the bathing solution was separate from the perfusing solution, it appeared possible that addition of antagonists only to the perfusion to inhibit responses mediated by endothelium might not affect contractile responses. It was hoped, in this way, to determine if there is a stimulated as well as basal EDRF release.

This study, therefore, was begun under the following hypothesis: **Removal of endothelium enhances the contractile responses to both exogenous agonists and electrically stimulated nerve responses. This enhancement is a result of elimination of EDRF which is released from endothelium at a basal level as well as a result of α-adrenoceptor stimulation.**

CHAPTER 2

CHARACTERIZATION OF MUSCARINIC RECEPTORS OF THE RABBIT EAR ARTERY SMOOTH MUSCLE AND ENDOTHELIUM

Dilation of blood vessels in response to cholinergic agents has been shown to be dependent on an intact vascular endothelium both *in vivo* and *in vitro* (Furchgott and Zawadzki, 1980; Angus et al., 1983). In the absence of endothelium these agonists not only fail to relax but may even cause a contraction (Furchgott and Bhadrakom, 1953; Furchgott and Zawadzki, 1980; Angus et al., 1983). Both relaxation and contraction are blocked by the muscarinic antagonist atropine suggesting that muscarinic cholinergic receptors on the vascular endothelium and smooth muscle mediate relaxation and contraction, respectively.

Recent studies of muscarinic receptors using the muscarinic antagonist pirenzepine have provided evidence for the existence of receptor subtypes. Functional observations have shown pirenzepine to be a more potent antagonist of muscarinic receptors mediating depolarization of the rat superior cervical ganglion than of those producing contraction of ileal smooth muscle (Brown et al., 1980). In direct and indirect radioligand binding studies pirenzepine has also displayed tissue dependent receptor affinities. Pirenzepine was found to have a higher affinity for the muscarinic receptors in sympathetic ganglia and cerebral cortex than for muscarinic receptors in the myocardium and ileum (Hammer et al., 1980; Hammer and Giachetti, 1982; Watson et al., 1983). Thus pirenzepine provides a means of discriminating between the muscarinic receptor subtypes which have been given the

designations of M_1 and M_2 (Goyal and Rattan, 1978). The binding sites with high pirenzepine affinity in the sympathetic ganglia and cerebral cortex have been designated M_1, whereas the low affinity sites of the myocardium and ileum are classified as M_2.

The subclass of muscarinic receptors which mediates cholinergic responses of blood vessels has not previously been fully explored. Therefore, in the present study, pirenzepine was used to characterize muscarinic receptors of the rabbit ear artery. The ability of pirenzepine to block the functional relaxation response of ring segments *in vitro* to methacholine yielded the affinity of pirenzepine for the muscarinic receptors of the endothelium. The affinity of pirenzepine for vascular smooth muscle cell receptors was determined by the inhibition of [^{3}H](-)quinuclidinyl benzilate ([^{3}H](-)QNB) binding to membrane homogenates.

Methods

Adult New Zealand white rabbits (2-3 kg) of either sex were decapitated. Ear arteries were removed and placed in Krebs' solution equilibrated with 95% O_2-5% CO_2. The composition of the Krebs' solution was (millimolar):Na^+, 147.6; K^+, 6.4; Ca^{++}, 1.6; Mg^{++}, 1.2; Cl^-, 130; HCO_3^-, 26; SO_4^{2-}, 1.2; $H_2PO_4^-$, 1.2; glucose, 11; and disodium ethylenediamine tetracetate (EDTA), 0.027. Tissue for the radioligand binding assay was weighed and frozen at -20°C (1-3 weeks) until used.

Functional Studies.

Ring segments (2 mm length were either left intact of had the endothelium removed by running a 2 inch length of doubled 5-0 silk through the lumen. Endothelium removal was confirmed at the end of each experiment by silver staining and light microscopic examination of the intimal surface (Abrol et al., 1984). The

segments were placed in tissue baths with Krebs' solution at 37°C, equilibrated for 60 minutes, and stretched to a resting tension of 1 gram until stable.

Relaxation responses were obtained by cumulative addition of methacholine to ring segments contracted to a stable plateau by Norepinephrine (NE) (3×10^{-7} M). The comparison of the induced relaxation in vessels with endothelium intact or removed was made using a single methacholine concentration response curve per ring segment.

In repeated exposure to methacholine the response declined between the first and second exposures, but remained relatively constant between the second and third. Therefore, for determination of the antagonist dissociation constant (K_B) the following protocol was used to enable each ring segment to serve as its own control: 1) Initial response to NE and relaxation the methacholine (not used in the determination), followed by three washes and a twenty minute equilibration period. 2) Contraction to NE and second relaxation concentration response curve to methacholine (used as the tissue control), followed by three washes, addition of the antagonist (atropine or pirenzepine) and a thirty minute equilibration period. 3) Contraction to NE and the final relaxation response curve to methacholine. At the end of each experiment, maximum relaxation was determined by addition of 30 mM $NaNO_2$ and maximum contraction by addition of NE (10^{-5} M).

The relaxation induced by methacholine was plotted as a percentage of the maximum relaxation to $NaNO_2$. EC_{50} values for each response were determined at 50% of the maximum relaxation to methacholine on a least squares regression through the linear portion of the concentration-response curve. K_B's were

calculated as described by Tallarida and Jacob (1979) from the agonist dose-ratio (A'/A) in the absence and presence of an antagonist (B) using the equation:

$$K_B = \frac{(B)}{(A'/A - 1)} \qquad (1)$$

Since several concentrations of antagonist were used in each experiment the method of plotting described by Arunlakshana and Schild (1958) was used. Any shifts in the dose-ratio related to length of time in the baths or tissue fatigue were eliminated by dividing the observed dose-ratio in the presence of antagonist by the control dose-ratio to give the corrected dose-ratio (Furchgott, 1972; Duckles and Bevan, 1976).

[^{3}H](-)QNB Binding Assay.

Ear arteries used for the radioligand binding studies were either fresh of frozen at -20°C for up to 3 weeks. Endothelium was left intact or removed by either mechanical or collagenase stripping. The mechanically stripped vessels were cut longitudinally, and the internal surface was rubbed with the edge of forceps. The collagenase stripped vessels were flushed with Krebs' solution, filled with 1% Type I collagenases solution (Sigma) and incubated at 37°C for 15 minutes followed by additional flushing with Krebs' solution. Removal of the endothelium was confirmed by light microscopic examination of stained cross sections.

The tissue was minced with scissors into 10 ml of ice cold 50 mM Na/KPO_4 buffer (pH 7.4) (used also in resuspension and as final incubation medium) and homogenized using a polytron (Brinkman Instruments) at a setting of 5 for 15 seconds and 30 seconds followed by 15 seconds at a setting of 8. The homogenate was centrifuged for 15 minutes at 3000 x g, the pellet discarded, and the

supernatant centrifuged at 120,000 x g for 60 minutes at 5°C. The supernatant was discarded, and the pellet resuspended using short bursts on the polytron.

In all radioligand binding experiments, aliquots of homogenate, [^{3}H](-)QNB specific activity 30.2-33.1 Ci/mmol, New England Nuclear) and other drugs were incubated at 37°C in a final volume of 0.4 ml. Non-specific [^{3}H](-)QNB binding was determined in the presence of 10^{-6} M atropine sulfate. Incubation was terminated by rapid addition of 5 ml ice cold buffer, followed by filtration through GF/C glass fiber filters (Whatman) over vacuum and three 5 ml rinses with ice cold buffer. A Brandel M24 Cell Harvester machine (Brandel Biomedical Research and Development Laboratories) was used for filtration. [^{3}H](-)QNB retained on the filter was extracted for 16 hr with 8 ml of scintillation fluid [2 liters Toluene (MCB), 1 liter Triton X-100 (Westchem), and 16 grams Omniflour (New England Nuclear)]. Radioactivity was determined in a Searle Analytic 81 liquid scintillation counter. Proteins were assayed by the method of Lowry et al. (1951) using bovine serum albumin as a standard. All experimental points were determined in duplicate.

By increasing the length of the incubation time the rate of association (k_i) of [^{3}H](-)QNB could be determined. After steady-state was achieved (60 min) the addition of 10^{-6} M atropine blocked any further association, and the rate of dissociation (k_{-1}) was determined. Saturation of specific binding sites was determined by increasing the concentration of [^{3}H](-)QNB in the presence and absence of 10^{-6} M atropine in a 60 min incubation. Inhibition of [^{3}H](-)QNB binding by a number of drugs was observed by addition of the drugs to the [^{3}H](-)QNB membrane homogenate mixture prior to incubation.

Analysis of Binding Data

The analysis of binding data was based on equation 2 which derives from the law of mass action:

$$RL = \frac{B_{max} \cdot L}{K_d + L} \qquad (2)$$

where L is the free concentration of [^{3}H](-)QNB, RL is the bimolecular complex formed, whose concentration is measured in the filtration assay described above, Bmax is the total number of binding sites and K_d is the dissociation constant.

Values for the K_d and Bmax were determined from the equilibrium concentration of [^{3}H](-)QNB bound (RL) and free (L) by a nonlinear regression to equation 2 using the MLAB system distributed by the National Institutes of Health.

The rate of change in concentration of the components of the binding reaction is described by the differential equation:

$$\frac{d\,RL}{d\,t} = k_1 (L)(R) - k_{-1} (RL) \qquad (3)$$

where R is the concentration of unoccupied binding sites. As described previously the concentration of [^{3}H](-)QNB bound (RL) was measured during both association and dissociation and then fit using non-linear regression to equation 3 to give values for the rates of association (k_1) and dissociation (k_{-1}). The value for K_d was then calculated from the rate constants:

$$K_d = \frac{k_{-1}}{k_1} \qquad (4)$$

The competitive inhibition of specific [^{3}H](-)QNB binding by other drugs is described by the equation from Cheng and Prusoff (1973):

$$K_i = \frac{IC_{50}}{1 + (L / K_d)} \qquad (5)$$

where K_i is the equilibrium dissociation constant for the competing drug at the [^{3}H](-)QNB binding site and IC_{50} is the concentration of competing drug needed to inhibit 50% of the specific [^{3}H](-)QNB binding.

Statistical evaluation

Results are expressed as the mean ± SEM. Statistical differences between two means ($P < .05$) were determined by Student's t-test for unpaired observations. Differences between three groups were evaluated by analysis of variance and Newman-Keuls Multiple Range Test ($P < .05$)(Snedecor and Cochran, 1967).

Drugs

The following drugs were used: norepinephrine bitartrate, acetylcholine chloride, methacholine chloride, atropine sulfate, oxotremorine, hexamethonium bromide, [Sigma Chemical Co. (St. Louis, MO)], pirenzepine hydrochloride, [Boehringer Ingelheim Ltd.(Ridgefield CT)], nicotine hydrogen tartrate, [J.T. Baker (Phillipsburg NJ)]. All drugs were prepared fresh daily.

Results

Functional Studies

Ring segments of rabbit ear artery precontracted with norepinephrine relax in a concentration-dependent manner to methacholine (Fig 1). After the removal of the endothelium, there was a decline of norepinephrine contraction amounting to

21% ± 4 (n=4). This was judged to reflect a spontaneous fade, rather than a relaxation response to methacholine, as addition of methacholine to the bath did not consistently alter the rate of this fade. Thus after complete removal of the endothelium, there was no relaxation in response to concentrations of methacholine up to 10^{-4} M. This same concentration of methacholine was also unable to produce any contraction in vessels that were intact (n=4) or without endothelium (n=4) whether precontracted with NE or at basal tone.

The relaxation response to methacholine in intact vessels was inhibited by the muscarinic antagonists atropine and pirenzepine. An example of this concentration dependent inhibition by pirenzepine is shown in Figure 2. Pretreatment with concentrations of pirenzepine up to 10^{-5} M had no effect on maximum relaxation produced by methacholine and caused only a slight decrease in the slope of the concentration-response curve (Fig. 2). Values for pK_B (pA_2) and Schild plot slope were 9.0 ± 0.6 and 1.2 ± 0.4 (n=3) for the non-discriminating muscarinic antagonist atropine. For pirenzepine, the discriminating ($M_1 > M_2$) muscarinic antagonist, values were 6.5 ± 0.1 and 1.1 ± 0.4 (n=6), respectively (Table 3).

Radioligand binding studies

Specific [^{3}H](-)QNB binding to membranes of the rabbit ear artery was linear with protein concentration up to 0.5 mg/ml (Fig. 3). Steady-state binding of [^{3}H](-)QNB to these membranes was observed within 40 min at 37°C (Fig. 4). Non-specific binding in the presence of 10^{-6} M atropine was constant at all time points and comprised < 10% of total binding. Addition of 10^{-6} M atropine to homogenates after 1 hour of incubation resulted in a time-dependent decrease in bound [^{3}H](-)QNB (Fig. 4). Rate constants for association (k_1) (2.0 x 10^{-3} $pM^{-1}min^{-1}$) and dissociation (k_{-1}) (1.0 x $10^{-2}min^{-1}$) were derived, and the K_d of [^{3}H](-)QNB was then

provided by the quotient of these values ($K_d = k_{-1}/k_1$ = 5 pM) (n=2). A K_d of 37 ± 14 pM (n=4) was determined by computer analysis of saturation isotherms (Fig. 5). The density of binding sites, B_{max} , was 83 ± 11 fmol/mg protein (n=4) (Table 3).

Removal of the vascular endothelium by mechanical stripping or incubation with collagenase solution had no significant effect on the binding affinity of [^{3}H](-)QNB. However, the density of binding sites of the mechanically stripped tissue was significantly increased over controls and collagenase stripped tissue (Table 3).

The binding of [^{3}H](-)QNB was inhibited in a concentration dependent manner in the presence of a number of cholinergic agonist and antagonists (Fig. 6). The binding affinity as determined by inhibition of [^{3}H](-)QNB binding was greatest for the non-discriminating muscarinic antagonist atropine with a K_i of 1.3 ± 0.1 nM. Pirenzepine, the discriminating muscarinic antagonist, also inhibited [^{3}H](-)QNB binding but with a lower affinity (K_i = 550 ± 20 nM) as did the muscarinic agonists acetylcholine and oxotremorine. Inhibition by agents specific for nicotinic receptors (hexamethonium and nicotine) became significant only at concentrations greater than 10^{-4} M. The inhibition data were also analyzed by Hill plot. The muscarinic antagonists tested had Hill coefficients close to 1.0, whereas the cholinergic agonists tested had Hill coefficients less than 1.0 (Table 4).

The affinities of the muscarinic antagonists atropine and pirenzepine for inhibition of [^{3}H](-)QNB binding to ear artery membranes (K_i) were compared to their affinities for antagonism of the methacholine-induced relaxation of precontracted ear artery ring segments (K_B)(Table 5). While the two drugs were clearly different from each other neither showed a significant difference in affinity between binding (K_i) and functional (K_B) studies.

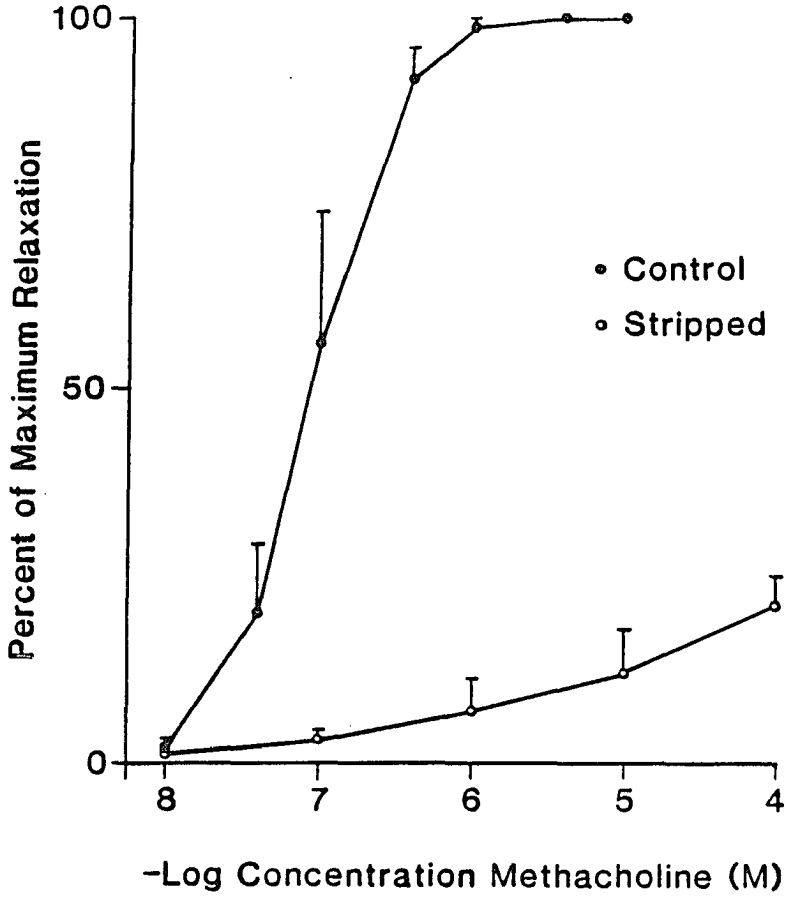

Figure 1. Effect of endothelium removal on methacholine induced relaxation of ear artery ring segments *in vitro* . After contraction to a stable plateau with 3 x 10^{-7} M norepinephrine, methacholine was added cumulatively (n=4).

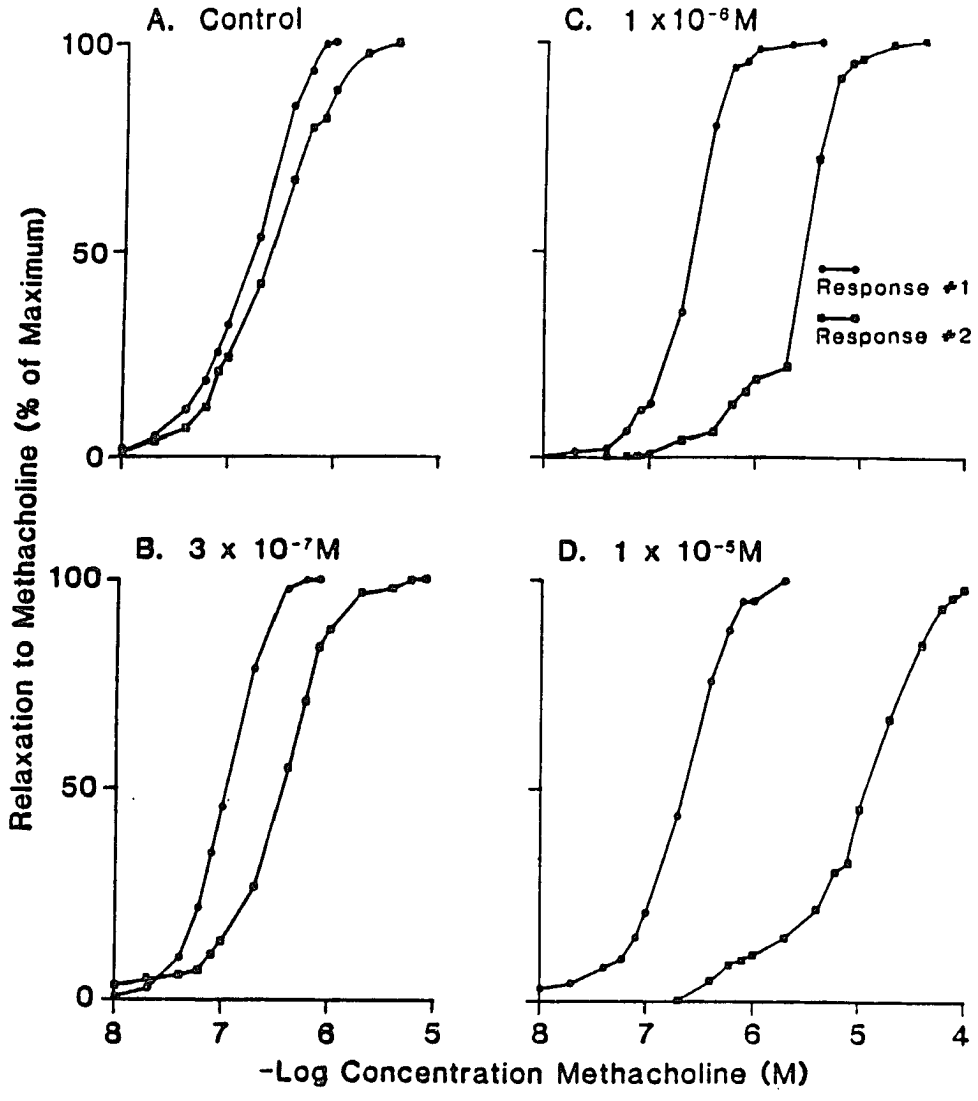

Figure 2. Effect of pirenzepine on methacholine induced relaxation of four consecutive ear artery ring segments. Each ring was precontracted to a stable plateau with 3 x 10^{-7} M norepinephrine. Two methacholine concentration-response curves were determined in each tissue, with pirenzepine added 30 min before the second curve. No antagonist was added to A, while effects of 3 concentrations of pirenzepine are shown in B,C, and D.

Table 3

Binding characteristics of [^{3}H](-)QNB in membrane preparations from intact and endothelium stripped rabbit ear arteries.

Treatment	n	K_d (pM)	B_{max} (fmol/mg protein)
Control	4	37 ± 14	83 ± 11
Mechanically stripped	3	33 ± 1	140 ± 18 *
Collagenase stripped	3	54 ± 27	86 ± 5

* $P < .05$; Different from both control and collagenase stripped .

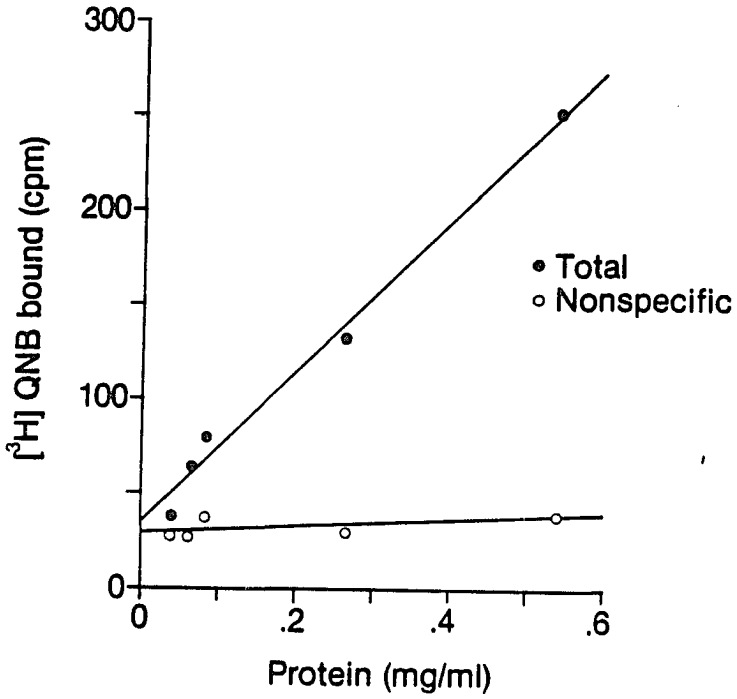

Figure 3. Effect of protein concentration on total and non-specific [^{3}H](-)QNB binding to rabbit ear artery homogenates. The indicated concentration were incubated with 69 pM [^{3}H](-)QNB as described under "Methods". Non-specific binding was determined in the presence of 10^{-6} M atropine. All points were determined in duplicate.

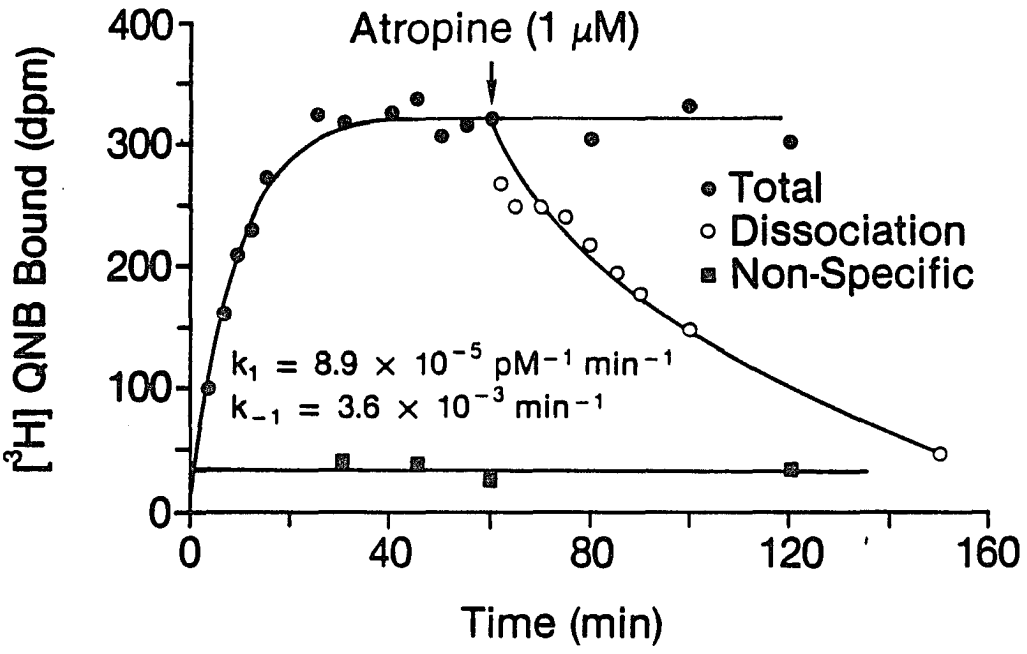

Figure 4. Rates of association and dissociation of [^{3}H](-)QNB binding as a function of time. Non-specific binding was determined in the presence of 10^{-6} M atropine. At the arrow atropine was added to samples indicated by the open circles , and dissociation of the [^{3}H](-)QNB-receptor complex was monitored. The assay contained 0.19 mg/ml protein and the concentration of [^{3}H](-)QNB was 48 pM. Each point is the mean of duplicate determinations from a single experiment.

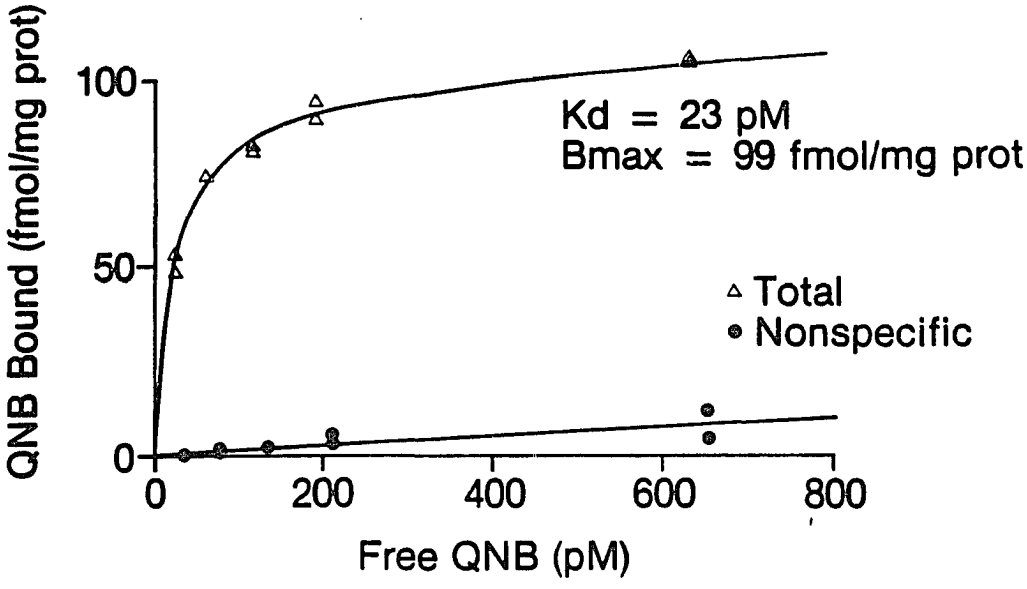

Figure 5. Total and non-specific [^{3}H](-)QNB binding to rabbit ear artery membrane homogenate. K_d and B_{max} were determined by non-linear regression of the data. Values shown are from a single experiment. Protein concentration was 0.144 mg/ml.

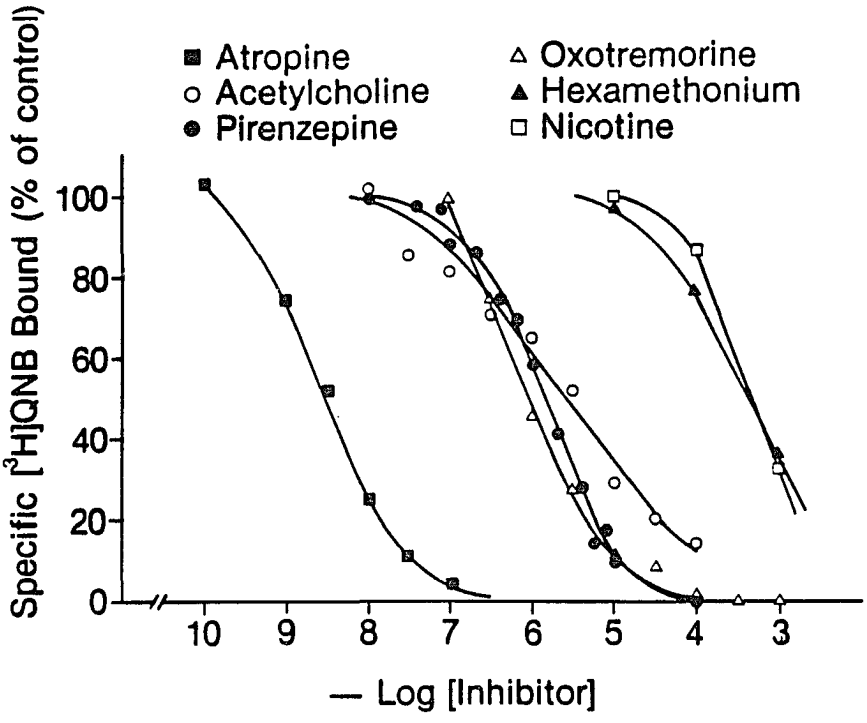

Figure 6. Inhibition by cholinergic agents of specific [^{3}H](-)QNB binding to rabbit ear artery membrane homogentates. Data are plotted as the percentage of specific binding of [^{3}H](-)QNB. [^{3}H](-)QNB concentrations ranged from 44 to 56 pM. Each point is the mean of duplicate determinations from a single experiment.

Table 4

Inhibition of [^{3}H](-)QNB binding.

Inhibitor	n	IC_{50} (μM)	n_H
Atropine	3	0.0028 ± .0003	0.8 ± .2
Pirenzepine	3	1.24 ± .08	0.9 ± .1
Acetylcholine	2	1.96	0.4
Oxotremorine	2	0.59	0.6
Nicotine	2	193	0.8

Values are means ± S.E.M. nH is the Hill coefficient.

Table 5

pK_i and pK_B values for atropine and pirenzepine in the rabbit ear artery.

Antagonist	pK_i	pK_B
Atropine	8.90 ± .05 (1.3 nM)	9.0 ± 0.6 (1.0 nM)
Pirenzepine	6.26 ± .02 (550 nM)	6.5 ± 0.1 (320 nM)

There is no significant difference between pKi and pK_B values for each drug ($P>.05$). Parentheses contain the corresponding mean K_i and K_B values.

Discussion

Functional studies.

The endothelium-dependent relaxation of precontracted rabbit ear artery ring segments (Fig. 1) was antagonized in a concentration-dependent manner by the muscarinic antagonists pirenzepine (Fig. 2) and atropine (not shown). In each case concentrations of the antagonists shifted the response curve to methacholine to the right in a parallel manner (as in Fig. 2).

The pK_B (pA_2) values for pirenzepine and atropine (6.5 ± 0.1 and 9.0 ± 0.6) compared favorably with those found by a number of investigators in tissues previously determined as containing the low affinity (M_2) subtype of muscarinic receptor. In the atria pK_B values for pirenzepine range from 6.2 to 6.7 while for atropine a pK_B of 8.7 has been reported. In the smooth muscle of the intestine the range of reported pK_B values for pirenzepine is 6.5 to 7.1 and for atropine 8.2 to 9.2 (Brown et al., 1980; Barlow et al., 1981; Fuder, 1982; Del Tacca et al., 1984). The pK_B value we obtained for pirenzepine in the endothelium is clearly distinct from that observed in a tissue identified by pirenzepine as M_1, such as the superior cervical ganglion, where the pK_B is 8.36 (Brown et al., 1980). We conclude from these observations that the muscarinic receptor of the rabbit ear artery endothelium which mediates relaxation is of the low affinity M_2 subtype.

Recently Eglen and Whiting (1985) have reported somewhat higher pA_2 values for pirenzepine on the endothelium of rabbit aorta (7.6 to 7.9) and dog femoral artery (7.6). However, since these affinities are not consistent with either an M_1 or M_2 subtype, these authors have suggested that the muscarinic receptor which mediates vasodilation differs from all previously identified subtypes. We are unable, at this time, to explain these results which differ from our

own. It is possible that tissue differences may play a role, but, perhaps a more likely explanation is the interference produced by muscarinic smooth muscle contraction which has been observed in both rabbit aorta (Furchgott and Zawadzki, 1980; Furchgott and Bhadrakom, 1953) and dog femoral artery (Angus et al., 1983), but which did not occur in our studies of the rabbit ear artery.

Radioligand Binding Studies

The direct binding of the muscarinic antagonist, [^{3}H](-)QNB, to ear artery membrane homogenates was characterized by protein linearity (Fig. 3), high affinity, saturability (Fig. 5) and inhibition by competitive cholinergic muscarinic drugs (Fig. 6). Affinity of [^{3}H](-)QNB for ear artery receptors was similar to that found in the rat brain (Yamamura and Snyder, 1974a), atria (Fields et al., 1978), guinea pig ileal smooth muscle (Yamamura and Snyder, 1974b), and the dog portal vein (Taniguchi et al., 1983). Scatchard and Hill plot analysis of [^{3}H](-)QNB binding (not shown) indicated that ear artery muscarinic receptors consist of a single population of binding sites. The B_{max} value we found was very similar to that found in dog and rabbit cerebral arteries (Ferron et al., 1984) and dog portal vein (Taniguchi et al., 1983), but considerably lower (693 fmol/mg protein compared to 83 in our study) than the B_{max} found in bovine cerebral vessels (Estrada and Krause, 1982).

Inhibition by pirenzepine of [^{3}H](-)QNB binding was used to determine the subtype of muscarinic receptor found in ear artery homogenates. Although the response of the ear artery to cholinergic stimulation is apparently mediated by receptors located on the endothelium, it became apparent that our radioligand binding technique primarily measures receptors on smooth muscle. If the binding density (B_{max}) reflects functional receptors mediating relaxation then removal of the endothelium prior to homogenization should produce a significant decrease in

B_{max}. In fact, mechanical stripping produced a significant increase in B_{max}. A similar increase has been observed with [^{3}H]prazosin binding in bovine aorta by Carmen-Krzan (1985) who suggested that endothelial removal may uncover new binding sites. This seems improbable in our experiments since collagenase stripping which also removed the endothelium left B_{max} unchanged. Our observed increase in B_{max} is most likely the result of a vigorous mechanical stripping technique which also removed some other tissue (such as adventitia or basal lamina) that yielded few binding sites but which contributed to the protein level of the tissue homogenate of unstripped vessels.

The lack of decrease in B_{max} with removal of the endothelium may indicate a similar distribution of receptors on both endothelium and smooth muscle. Alternatively, it is possible that differences in receptor density exist, but were not observed, since the amount of smooth muscle present in the ear artery is so much greater than the amount of endothelium. Similarly, the predominance of smooth muscle tissue would mask any contributions by prejunctional muscarinic receptors located on adrenergic nerves to the measured K_d or B_{max}. This illustrates on of the problems of radioligand binding in heterogeneous tissues. Significant binding may occur at sites that have less functional importance and, in some cases, these sites may predominate in number over those of greater functional significance.

It might be presumed that the muscarinic binding sites on vascular smooth muscle of the ear artery reflect the presence of receptors mediating contraction, as has been described in rabbit aorta (Furchgott and Zawadzki, 1980; Furchgott and Bhadrakom, 1953) and dog femoral artery (Angus et al., 1983). However, in the rabbit ear artery we never saw contraction to methacholine either in the presence or absence of tone or endothelium. Therefore, we do not know what function, if

any, might be ascribed to the muscarinic binding sites on the smooth muscle of this vessel.

Inhibition of binding by muscarinic agonists and antagonists gave IC_{50} and K_I values (Table 4) similar to those previously observed in the brain, intestine, heart and blood vessels (Yamamura and Snyder, 1974a, 1974b; Fields et al., 1978; Taniguchi et al., 1983). Of particular interest was the affinity of the M_1 selective antagonist pirenzepine (K_I = 550 ± 20 nM). This value is very similar to the low affinity receptors of the atria and intestine (200-800 nM) and clearly distinct from high affinity sites of the CNS (2-25 nM) (Hammer et al., 1980; Watson et al., 1983; Hammer and Giachetti, 1982). The Hill coefficient near 1.0 indicates a homogeneous population of receptors. Hill coefficients of less than 1.0 for acetylcholine and oxotremorine suggest a more complex interaction between these agonists and the muscarinic receptor as previously observed and discussed by others (Birdsall et al., 1978; Burgermeister et al., 1978; Wastek and Yamamura, 1978).

By blocking both functional responses and radioligand binding with the M_1 selective antagonist pirenzepine we have determined that all of the muscarinic receptors of the rabbit ear artery are of the low affinity M_2 subtype. While there is no statistical difference shown between the functional and binding values (Table 5) it might be noted that the value determined in the binding study is somewhat lower than that from the functional study. This, as discussed by Hammer (1982), has been consistently observed for pirenzepine with muscarinic receptors in several different tissues. However, when using either the binding or functional approach, pirenzepine still discriminates between receptor subtypes.

In summary, both functional responses to cholinergic stimulation, mediated predominantly by the endothelium, and muscarinic radioligand binding, which

is dominated by the functionally less important smooth muscle sites, clearly show that both cell types of the rabbit ear artery contain the low affinity M_2 subtype of muscarinic receptor.

CHAPTER 3

EFFECT OF INCREASING AGE ON THE ENDOTHELIUM-MEDIATED RELAXATION OF RAT BLOOD VESSELS IN VITRO

Advancing age produces a number of significant changes in circulatory hemodynamics in both man and animals. These include increased blood pressure as well as altered responses to exercise and non-exercise stress and a variety of therapeutic drugs (Kalbfleisch et al., 1977; Miller, 1981; Palmer et al., 1978; Docherty, 1986). One possible mechanism for these age-related changes is an alteration of the responsiveness of the vascular endothelium to vasoactive substances.

Furchgott and Zawadzki (1980) demonstrated that the vasodilatory action of acetylcholine was totally dependent on the presence of an intact endothelium. In response to cholinergic stimulation the endothelial cells produce an unidentified, labile "endothelium-derived relaxing factor" (EDRF) which causes the smooth muscle cells to relax. Since Furchgott's discovery, vasodilatation produced by a variety of substances, including bradykinin, histamine, serotonin, catecholamines and others, has been shown to be partially or totally dependent on the presence of the endothelium (Furchgott, 1983; Vanhoutte and Miller, 1985).

Because of the potential importance of the endothelial system for regulation of vascular tone and the possibility that the effectiveness of this system might change with age, blood vessels from an established model of aging, the Fischer 344 rat, were studied to determine the effect of increasing age on the

cholinergic, endothelium-mediated vasodilatory response. Both contractile responses to adrenergic agonists and relaxation responses to the cholinergic agonist methacholine were examined in aortic ring segments, perfused caudal arteries and the perfused mesenteric bed from rats aged 1 to 27 months. Relaxation to the calcium ionophore A23187, which produces endothelium-dependent relaxations by the influx of calcium ions into the endothelial cell (Singer and Peach, 1982), was also examined in the perfused caudal artery to test the activity of the endothelial system independently of the muscarinic receptor.

Methods

Animals

Male Fischer 344 rats aged 1 to 27 months were obtained from colonies maintained by the National Institute of Aging. Animals were divided into 5 groups by age: 1, 6, 12, 20 and 27 months. Average age, weight, and number of animals in each group were: 1.4 ± 0.2 months, 141 ± 14 g, 5; 7.1 ± 0.2 months, 331 ± 4 g, 29; 12 ± 0.2 months, 380 ± 7 g, 23; 20.5 ± 0.7 months, 358 ± 10 g, 24; 25.5 ± 0.3 months, 350 ± 9 g, 17.

Aortic Ring Segments

After decapitation the thoracic aorta was carefully removed and placed into room temperature Krebs' solution which was composed as follows (mM): Na^+, 147.6; K^+, 6.4; Ca^{++}, 1.6; Mg^{++}, 1.2; Cl^-, 130; HCO_3^-, 26; SO_4^{--}, 1.2; $H_2PO_4^-$, 1.2; glucose, 11; disodium ethylenediamine tetracetate, 0.027. During all procedures care was taken not to stretch the vessels or damage the endothelium. Unless stated otherwise the Krebs' solution was at a temperature of 37°C and aerated with a gas mixture of 95% O_2-5% CO_2.

With the aid of a dissecting microscope the vessel was divided into 4 to 6 segments (4 mm length) which were either left intact or had the endothelium removed by running a 3 inch length of doubled cotton string through the lumen. Vessel segments were equilibrated for 60 min, stretched to optimum resting tension and exposed repeatedly (3-7 times with 15 min intervals between exposures) to 0.1 μM norepinephrine (NE) until a stable response was obtained. A cumulative concentration response curve to NE was determined in some of the segments to determine the EC_{80} value for NE (concentration that produced 80% of the maximum response). Separate vessel segments were then contracted with NE using the previously determined EC_{80} value and relaxed by the cumulative addition of methacholine. Maximum relaxation was obtained by exposing the precontracted segment to 5×10^{-5} M papaverine. Contraction and relaxation responses were determined in separate ring segments to avoid tissue fatigue which often led to spontaneous relaxation.

At the end of each experiment the percentage of intimal surface with intact endothelium was determined by silver staining (Abrol et al, 1984). After staining, the fixed segments were permanently mounted and coded for later evaluation. Using a dissecting microscope with a grid eyepiece two independent observers subdivided the intimal surface into small sections and estimated the percentage of intact endothelium in each section. These estimates were then averaged to give the percentage of the entire surface which still retained endothelium.

Perfused Caudal Arteries

After decapitation a 3 cm segment of the proximal caudal artery was removed and cannulated at each end with PE 50 tubing. Vessels were either left intact or had the endothelium removed by running a 2 inch length of doubled 5-0

silk through the lumen. The vessel segment was then placed in a tissue bath with Krebs' solution, stretched to the *in situ* length and perfused with Krebs' solution at a constant rate of 0.5 ml/min using a Buchler polystaltic pump (Model 2-6100). Perfusion pressure was monitored with a Statham P23 Db pressure transducer and recorded on a Soltec 1220 chart recorder. After an equilibration period of at least 45 min the segment was repeatedly exposed (2-3 times with 15 min intervals between exposures) to the α_1-adrenergic agonist methoxamine (added to the bath solution) until a stable response was obtained. A cumulative concentration-response curve to methoxamine was determined. Following washout the vessel was again contracted by methoxamine in the bathing solution to produce a stable pressure increase of 100 mm Hg. The relaxation response was determined by the addition of methacholine or the calcium ionophore A23187 as a 10 µl bolus into the perfusing solution through an injection port proximal to the segment. Doses were given in increasing concentration at intervals which allowed the perfusion pressure to return to the 100 mm Hg plateau plus 5 to 10 min further recovery time. Dose-response curves were generated and ED_{50} values (dose which gave 50% of maximum relaxation response) determined by least squares regression through the linear portion of the curve. Maximum possible relaxation was determined at the end of each experiment by exposure of the contracted vessel to 5×10^{-5} M papaverine in the bathing solution.

Perfused Mesentery

Rats were anesthetized with pentobarbital sodium at a dose of 65 mg/kg given i.p. The mesenteric vascular bed was removed and perfused by the method of McGregor (1965) with some modifications. The abdomen was opened and the animal heparinized by intravenous injection of 200 units of heparin into the inferior vena

cava. The colon proximal to the rectum, the duodenum proximal to the stomach and the superior mesenteric artery were ligated and cut. The now free mesenteric bed and intestine were then lifted out and placed in room temperature Krebs' solution. The superior mesenteric artery was cannulated using PE 90 tubing. All major branches of the superior mesenteric artery were ligated except the final four leading to the terminal ileum. The vascular bed was then separated from the intestine by cutting along the intestinal wall.

The mesenteric vascular bed was placed in a 60 ml organ bath at 37°C and perfused with Krebs' solution at a constant rate of 5 ml/min using a Buchler Polystaltic pump and superfused by gravity feed at a rate of 0.5 to 1.0 ml/min. Perfusion pressure was monitored using a Statham P23Db pressure transducer and a Soltec 1220 chart recorder.

After a one hour equilibration period the mesenteric bed was repeatedly contracted (2-3 times with 15 min intervals between exposures) with methoxamine (2 μM) in the perfusion solution until a stable response was obtained. The mesenteric bed was then contracted to a stable plateau pressure change of 100 mm Hg by methoxamine (2-10 μM). Relaxation responses were determined by 10 μl bolus injections of methacholine into the perfusion buffer through an injection port proximal to the mesenteric bed. Dose-response curves and ED_{50} values were determined as in the caudal artery.

Drugs

The following drugs were used: norepinephrine bitartrate, methacholine chloride, A23187, papaverine hydrochloride (Sigma Chemical Co., St. Louis, MO); methoxamine hydrochloride (Burroughs Welcome Co., Research Triangle Park, NC);

sodium pentobarbital (Western Medical Supply, Inc., Arcadia CA); and heparin sodium (Elkins-Sinn, Inc., Cherry Hill, NJ).

Statistics

Results are expressed as the mean ± S.E.M. Statistical differences between two means ($P < .05$) were determined by Student's t-test for unpaired observations. Differences between three or more groups were evaluated by analysis of variance and Newman-Keuls Multiple Range Test ($P < .05$) (Snedecor and Cochran, 1967).

Results

Aortic Ring Segments

No significant difference with age in either sensitivity or maximum contractile response to NE was observed in aortic ring segments (Fig. 7). Maximum responses to NE were 1.2 ± 0.1 g (5) in segments from 1-month-old rats and 1.2 ± 0.1 (10), 1.5 ± 0.7 (3), 1.2 ± 0.1 (10) and 1.7 ± 0.7 g (3) from 6-, 12-, 20- and 27-month-old animals, respectively. Responses to NE in segments which had the endothelium intentionally removed were also not different from controls, having maximum contractions of 1.2 ± 0.1 g (4) and 1.4 ± 0.1 g (3) for segments from 6- and 20-month-old rats, respectively.

As the age of the rat increased the sensitivity of aortic ring segments to the relaxing effects of methacholine tended to increase (Fig. 8). This trend was statistically significant, however, only in vessels from the 20-month-old animals where a 2.5 and 5 fold shift in EC_{50} values were observed when compared to 6- and 1-month-old animals, respectively (Table 6). Maximum relaxations were not different in vessels from animals of various ages.

Endothelial Damage

Approximately 40% of aortic ring segments from 6 and 12-month-old animals were found to have significantly reduced responses to methacholine (Table 7). Therefore, ring segments were divided into two groups, responding and non-responding, defining non-responding as having a maximum relaxation to methacholine of less than 50%. Only responding segments were included in the data of Fig. 8 and Table 6. Non-responding segments (not shown) had a much higher threshold to methacholine relaxation, very flat concentration-response curves and significantly lower maximum relaxations. Maximum relaxations were 20 ± 6 % (n=4) and 11 ± 4 % (n=6) for segments defined as non-responding from 6- and 20-month-old rats, respectively. Evaluation of the endothelial integrity of silver stained segments from both responding and non-responding groups showed that both had sustained extensive damage; however, damage to the non-responding group was significantly greater (Table 7). There was no significant difference in the degree of endothelial damage when responding vessels of the two age groups were compared. No endothelium remained on vessels which were intentionally stripped.

Perfused Caudal Artery

In view of the possible problems resulting from a partially damaged endothelium in ring segments, contractile and relaxation responses were examined in another *in vitro* system, the perfused caudal artery. Similar to aortic ring segments the contractile responses to methoxamine in perfused caudal arteries from rats aged 6-20 months did not change with age (Fig. 9). However, vessels from 1-month-old rats had pressure changes in response to methoxamine which were significantly greater than all other ages (Fig. 9). In order to avoid tissue damage, pressure changes greater than 160 mm Hg were avoided; therefore Fig. 9 shows only

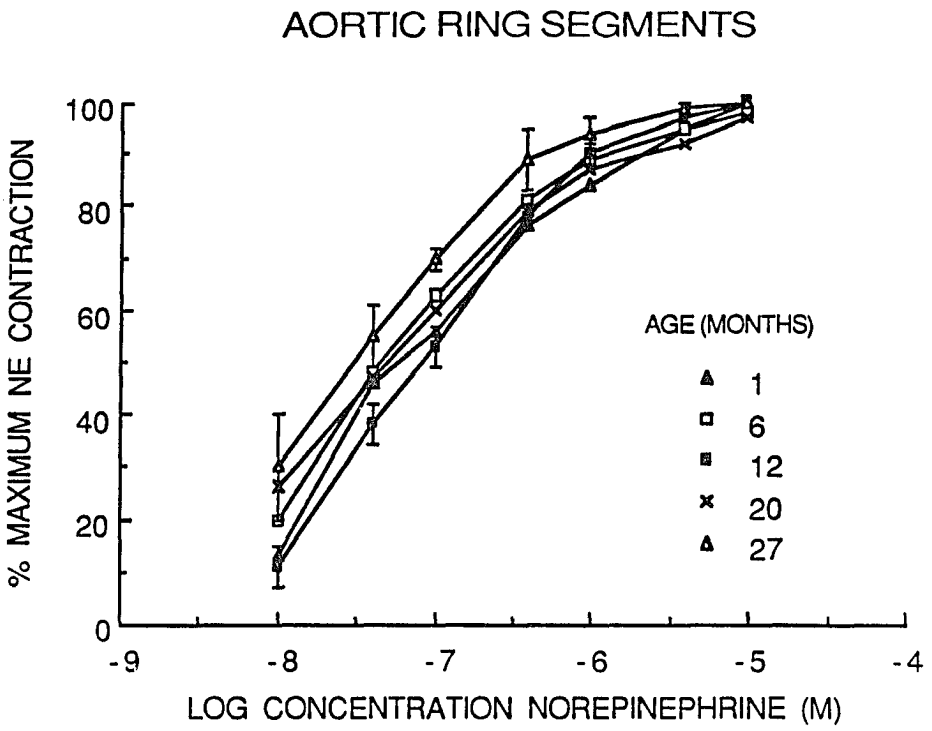

Figure 7. Concentration-response curves for the contractile response to norepinephrine of aortic ring segments from rats of different ages. Symbols represent the mean values ± S.E.M. (n = 3 to 10) and are expressed as the percentage of the maximum response to norepinephrine. Some standard errors were omitted for clarity.

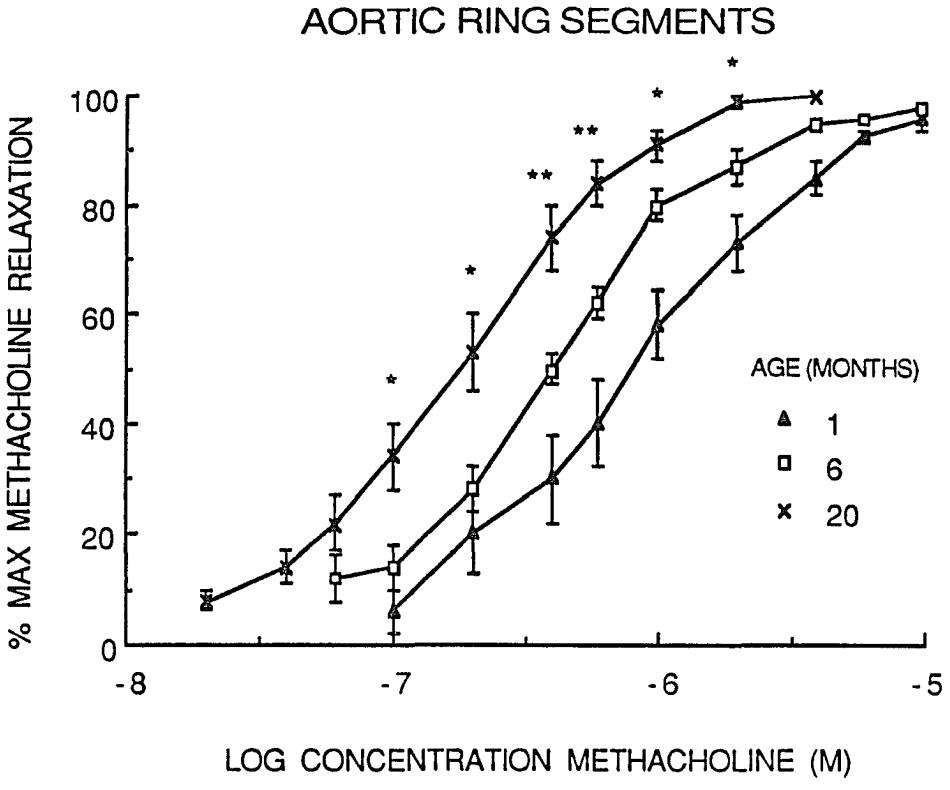

Figure 8. Concentration-response curves for the endothelium-dependent relaxation response to the cholinergic agonist methacholine of aortic ring segments from rats of different ages. Vessels were precontracted by NE (EC_{80}). Symbols represent the mean values ± S.E.M. (n = 5 to 9) and are expressed as the percentage of the maximum response to methacholine.

(* different from 1 and 6 month, ** all ages different, $P < .05$ by ANOVA)

Table 6

Influence of age on methacholine-induced relaxation in rat vascular preparations.

Vascular Preparation	Age (months)	Maximum (% papaverine)	-Log EC_{50} (M)	N
Aortic Ring Segment	1	62 ± 5	6.1 ± 0.1	5
	6	72 ± 5	6.4 ± 0.1	9
	20	70 ± 5	6.8 ± 0.1 **	5
			-Log ED_{50}(mol)	
Perfused Caudal Artery	6	73 ± 6	9.7 ± 0.1 *	11
	12	78 ± 5	10.6 ± 0.3	10
	20	73 ± 8	10.4 ± 0.2	8
	27	77 ± 8	10.5 ± 0.2	5
Perfused Mesentery	6	93 ± 1	9.8 ± 0.2	5
	12	89 ± 3	9.6 ± 0.2	5
	20	92 ± 2	9.7 ± 0.1	5

All values are mean ± S.E.M. Symbols indicate significant difference from all other ages (* $P < .05$, ** $P < .01$ by ANOVA)

Table 7

Percentage of intimal surface with intact endothelium in aortic ring segments.

	6 month	20 month
Responding	37 ± 4 (5)	32 ± 3 (6)
Non-responding	14 ± 6 (3) *	10 ± 6 (5) **

Mean ± S.E.M. (number of ring segments)(* $P < .05$, ** $P < .01$)

partial concentration-response curves and maximum responses could not be obtained. Because it was not possible to determine comparable levels of smooth muscle contraction in perfused vessels of one month old rats, and the major focus of this study is on effects of senescence, animals of this age were not further studied.

As in the aorta, increased sensitivity to methacholine with increased age was also observed in perfused caudal arteries (Fig. 10). Vessels from 12-, 20-, and 27-month-old animals were significantly more sensitive than 6month. ED_{50} values for 12-, 20- and 27-month animals showed 8, 5 and 6-fold differences when compared to 6-month values (Table 6). However, these values were not different from each other nor were there significant differences in the magnitude of maximum relaxation among any age group (Table 6). In both aortic segments and perfused caudal arteries removal of the endothelium eliminated any response to methacholine.

Perfused caudal arteries from 6- and 12-month-old rats precontracted with methoxamine gave a dose dependent relaxation to bolus injections of the calcium ionophore A23187 (Fig. 11). While the maximum relaxation responses to A23187 were similar to those of methacholine, no differences were observed in the sensitivity to A23187 or in maximum responses when vessels from 6- and 12-month-old rats were compared (Table 8).

Perfused Mesentery

Vascular relaxation responses to methacholine of the perfused mesenteric vasculature from 6-, 12- and 20-month-old rats are shown in Fig. 12. No differences were observed in either sensitivity or maximum relaxation response (Table 6).

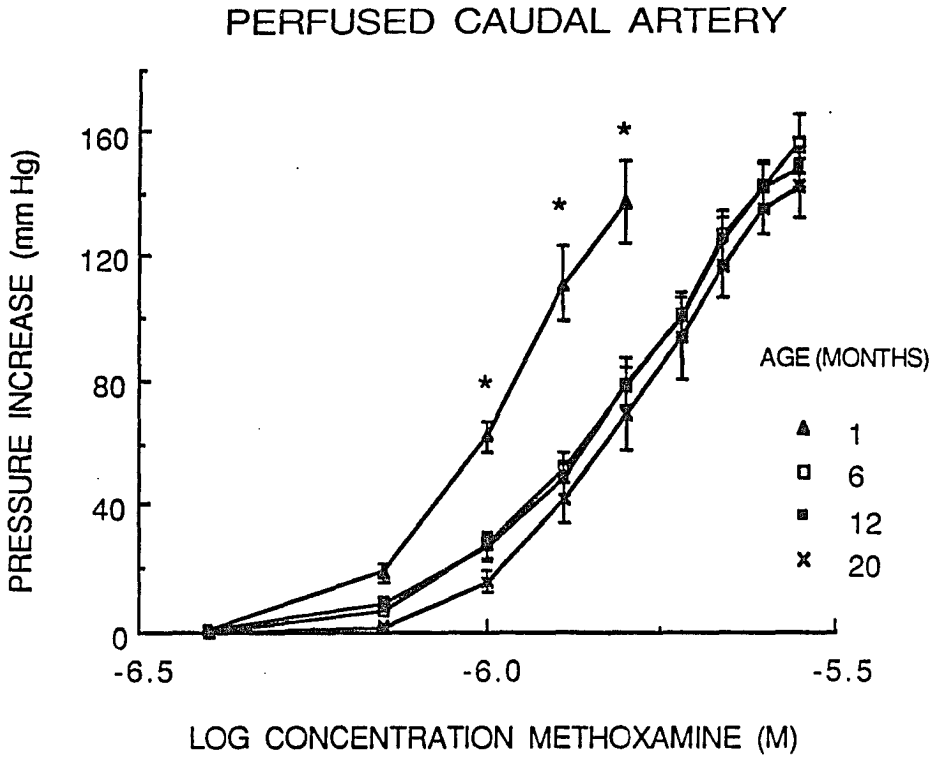

Figure 9. Concentration-response curves for the contractile response of perfused caudal artery segments from rats of different ages to increasing bath concentration of the alpha adrenergic agonist methoxamine. Symbols represent the mean values ± S.E.M. (n = 4 to 9) and are expressed as the increase in perfusion pressure (mm Hg) at a constant flow of 0.5 ml/min. Some standard errors were omitted for clarity. (* different from all other ages, $P < .05$ by ANOVA)

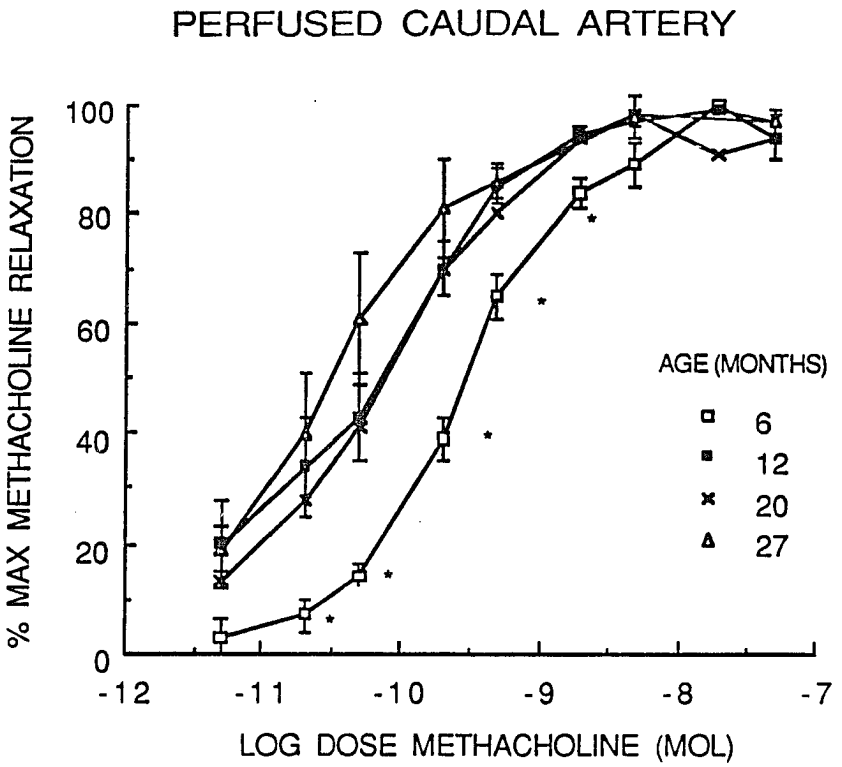

Figure 10. Dose-response curves for the endothelium-dependent relaxation of perfused caudal arteries from rats of different ages to bolus doses of methacholine. Vessels were precontracted to 100 mm Hg by methoxamine. Symbols represent the mean values ± S.E.M. (n = 5 to 11) and are expressed as percentage of the maximum relaxation to methacholine. Some standard errors were omitted for clarity. (* different from all other ages, $P < .05$ by ANOVA)

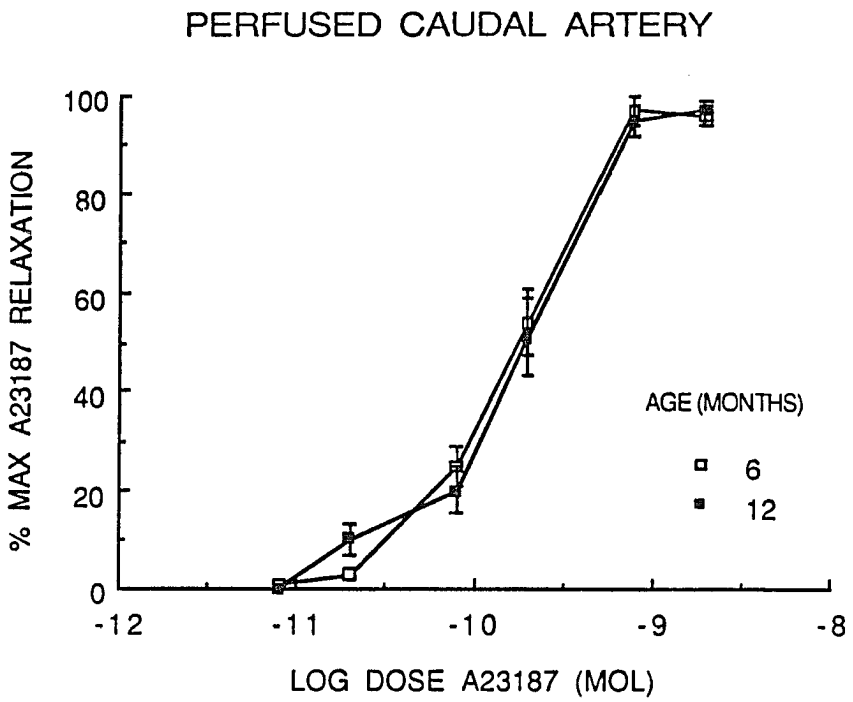

Figure 11. Dose-response curves for the endothelium-dependent relaxation of perfused caudal arteries from 6- and 12-month-old rats to bolus doses of the calcium ionophore A23187. Vessel were precontracted to 100 mm Hg by methoxamine. Symbols represent the mean values ± S.E.M. (n = 5) and are expressed as percentage of the maximum response to A23187.

Table 8

Influence of age on relaxation of perfused rat caudal artery to A23187.

Age (months)	Maximum (% papaverine)	-Log ED_{50} (mol)	N
6	87 ± 3	9.8 ± 0.1	5
12	88 ± 3	9.7 ± 0.1	5

Mean ± S.E.M.

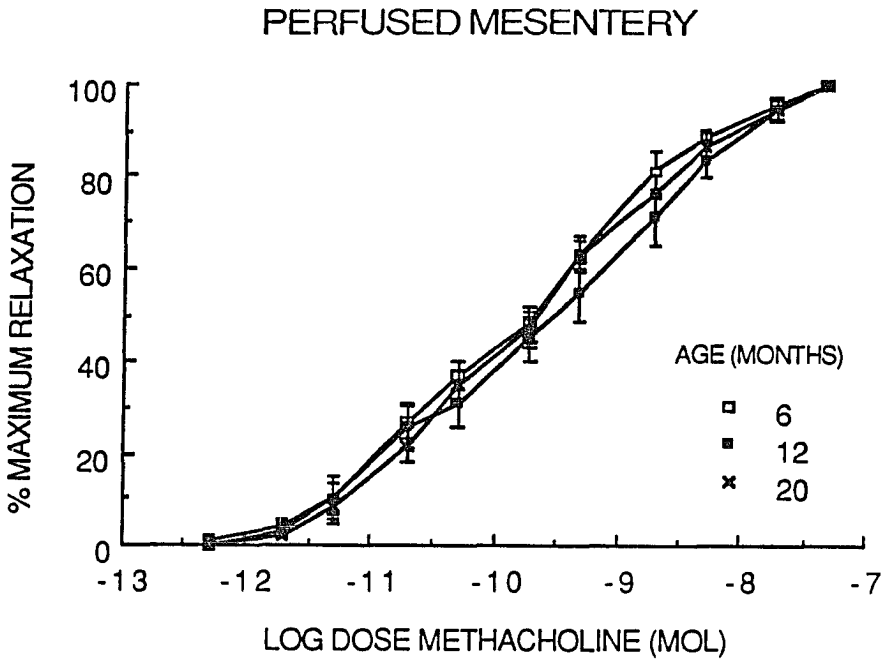

Figure 12. Dose-response curves for the endothelium-dependent relaxation to bolus doses of methacholine of the perfused mesenteric bed from rats of different ages. The mesenteric bed was precontracted to 100 mm Hg by methoxamine in the perfusate. Symbols represent the mean values ± S.E.M. (n = 5) and are expressed as percentage of the maximum response to methacholine.

Discussion

Increasing age does not affect the contractile response of the aorta or caudal artery to NE and methoxamine (Figs. 6 and 8). This finding is consistent with earlier reports from our laboratory which demonstrate that contractility to NE and KCl of isolated rat blood vessel ring segments remains constant from youth to senescence (Duckles et al., 1985; Duckles and Hurlbert, 1986). This consistency allowed us to eliminate contractility differences as a factor when examining altered relaxation response with age.

One exception to this is the apparent greater sensitivity of perfused caudal arteries from 1-month-old rats (Fig. 9). This difference is most likely due to the smaller initial diameter of these vessels rather than increased contractility of the smooth muscle. A vessel with smaller initial radius when perfused at a constant flow will have a greater increase in pressure with the same absolute change in radius than a vessel with larger initial diameter. Further, in the aortic ring preparation where developed tension is unaffected by vessel radius, contractile responses of segments from 1-month animals were not different from any other age. Therefore, the difference in perfused vessels is most likely mechanical in nature.

Of greater importance, however, is the finding that contractile responses of the aorta did not significantly increase when the endothelium was removed. This is surprising in view of recent observations that endothelial removal in rat aorta enhances both sensitivity (EC_{50}) and maximum response to α-adrenergic agonists (Lues and Schůmann, 1984; Eglème et al.,1984; Miller et al., 1984), an effect which appears to be due to spontaneous release of EDRF rather than stimulation of endothelial adrenergic receptors (Martin et al, 1986). It is possible that this

difference is due to differences in strain (F 344 vs Wistar in the above studies) or age. Although only the report by Miller et al. (1984) included animal ages (11-15 wks), the animal weights suggest an age range of 2 to 4 months, while in our experiments ages were 6 and 20 months. Thus it is possible that in the strain we used, or at the older ages, there is less spontaneous release of EDRF. Another explanation involves inadvertent damage to the endothelium. Some damage to the endothelium is inevitable in any experimental procedure, and, as shown in Table 7, there was substantial endothelial damage in all vessels studied. Again it is possible that in this strain of rats, or as a function of age, the endothelium is more susceptible to damage than in younger animals or other strains.

As observed by Furchgott and Zawadzki (1980) cholinergic endothelium-mediated relaxations can still be observed when only a fraction of the endothelium remains intact, although the maximum acetylcholine relaxation may be reduced, depending on the degree of endothelial damage. Tables 6 and 7 show that ring segments having less than 40% intact intima still gave greater than 70 % of maximum relaxation. As discussed above, it is not clear whether the degree of damage to the endothelium we observed was a result of our experimental procedures or was related to the strain and ages of our animals. Nevertheless, the results in Tables 6 and 7 demonstrate that using the relaxation to cholinergic stimulation as an indicator of intact endothelium makes "intact" a relative term, and any quantitative results may be compromised if there is variation in the degree of endothelial damage, for example with age.

The major age-related difference found in this study was the increased sensitivity of isolated vessels to endothelium-mediated cholinergic relaxation. Aortic segments from 20-month-old rats showed 2.5 and 5 fold differences in EC_{50}

when compared to 6- and 1-month (Fig. 8, Table 6). As with the contractions the potential effect of endothelial damage on the quantification of relaxations cannot be ignored, but in order to be responsible for the observed increase in sensitivity, endothelial damage would have to be less in the vessels from older animals. As seen in Table 7 there was no statistically significant difference in the level of observed damage, with older vessels tending to be slightly more susceptible to damage. It is clear, however, that endothelial damage can affect the nature of the relaxation response. At some point the ability of the segment to respond becomes severely impaired. When this occurs response threshold increases, maximum relaxation drops and the concentration-response curve becomes very flat. Fortunately, these segments were identified not only by a decreased response but also by a significantly lower (< 15 %) level of intact endothelium (Table 7), and could, therefore, be removed from the aging comparisons.

The results from the perfused caudal arteries confirmed the observation in the aorta and support the conclusion that these differences are not due to variations in degree of endothelial damage. Vessels from 12-, 20- and 27-month-old rats showed a significant increase in sensitivity compared to 6-month (Fig. 10, Table 6). No difference was seen as age increased from 12 to 27 months, and maximum relaxations were not affected by age.

The results from the aorta and caudal artery together suggest that the increasing sensitivity of the endothelium to cholinergic stimulation occurs during growth and development rather than old age. The sensitivity change occurs between 6 and 12 months of age when growth is still relatively rapid, and then stays constant from the time growth levels off at about 12 months (Masoro, 1980) until senility.

While the mechanism for cholinergic, endothelium-dependent relaxation is not fully understood, it is generally thought that the process begins with activation of muscarinic, cholinergic receptors on the endothelial cells (Furchgott and Zawadzki, 1980), most likely of the M_2 subtype (Hynes et al., 1986). Stimulation of these receptors produces the calcium dependent (Singer and Peach, 1982) release of an as yet unidentified substance referred to as endothelium-derived relaxing factor (EDRF). EDRF diffuses to the smooth muscle where it produces an increase in cyclic GMP (Rapoport and Murad, 1983) and relaxation. The greater sensitivity of vessels from older animals could be a result of changes anywhere along this proposed pathway. However, results from experiments using the calcium ionophore A23187 suggest the change is at the level of the muscarinic receptor or specific receptor coupling processes within the endothelial cell. A23187 stimulated relaxation results from an endothelium specific influx of calcium ion and subsequent EDRF production independent of the muscarinic receptors (Singer and Peach, 1983). Since these responses are not altered with increasing age it is likely that the age related changes in cholinergic relaxation are due to either an increase in receptor density or more efficient receptor coupling to effect a release of EDRF from endothelial cells.

Changes in cholinergic sensitivity were not seen in the isolated perfused mesenteric bed (Fig. 12, Table 6). Since the perfused mesentery is a more complicated system than either ring segments or a single perfused vessel, the differences measured in the simpler systems might be lost in the dynamics of flow through such a large number of vessels. Alternatively, if the function of endothelial cells of resistance vessels is different from larger vessels, as has been suggested by Rivers et al (1986), then the sensitivity increase observed in the large vessels may

be absent in the microcirculation. However, the alteration of sensitivity observed in larger vessels may be a physiologically significant change regardless of the extent it reaches into the microcirculation.

The physiological role that cholinergic, endothelium-mediated vasodilatation may play in the dynamics of blood flow control is still unknown. It is interesting to note, however, that the increased sensitivity to cholinergic stimulation occurs at approximatly the same age as systolic blood pressure begins to increase (Yu et al, 1985). The direction of the sensitivity change eliminates this as a possible contributor to the blood pressure increase. However, it might be a compensatory response, albeit an ineffective one, since blood pressure continues to increase with age (Yu et al, 1985), while cholinergic sensitivity does not.

CHAPTER 4

ENHANCED CONTRACTION OF ISOLATED RAT CAUDAL ARTERY WITH REMOVAL OF THE ENDOTHELIUM

Furchgott and Zawadzki (1980) demonstrated that the vasodilatory action of acetylcholine was totally dependent on the presence of an intact endothelium. In response to cholinergic stimulation the endothelial cells produce an unidentified, labile "endothelium-derived relaxing factor" (EDRF) which causes the smooth muscle cells to relax. Since Furchgott's discovery, vasodilatation produced by a variety of substances, including bradykinin, histamine, serotonin, catecholamines and others, has been shown to be partially or totally dependent on the presence of the endothelium (Furchgott, 1983; Vanhoutte and Miller, 1985)

Recently enhanced contractile responses to a-adrenoceptor agonists have been reported in vessels stripped of endothelium including rabbit coronary artery (Griffith et al., 1984c), dog iliac artery (Young and Vatner, 1986) and rat aorta (Eglème et al., 1984; Lues and Schümann, 1984; Miller et al., 1984; Godfraind et al., 1985). Of particular relevance to this study are the responses of the rat aorta where both α_1- and α_2-agonist induced contractions are enhanced; however, there appears to be a preferential enhancement of α_2-agonist mediated contraction. These observations, coupled with the existence of endothelial α_2-adrenoceptor mediated relaxations of canine arteries, apparently via EDRF release (Cocks and Angus, 1983; Angus et al., 1986), have led to speculation that EDRF is similarly released via α_2-adrenoceptor stimulation to inhibit contractions in rat aorta (Eglème et al., 1984).

An alternative proposal is that the endothelium of rat aorta spontaneously releases a basal level of EDRF producing a constant inhibition of contraction which disappears with removal of the endothelium. This proposal further suggests that while the response to any contractile agent should be enhanced by removal of the endothelium, contractions to α_2-agonists show a greater effect because they are acting as partial agonists in this tissue.

In this study we examined the effect of endothelial removal on contractile responses in the isolated, perfused rat caudal artery. Contractions were induced by selective α_1-and α_2-adrenoceptor agonists as in the aorta studies; however, the effect of endothelium removal on the response to electrical nerve stimulation was also examined. pK_B values for the α-agonists were determined to explore the possibility that any preferential enhancement of α_2-adrenoceptor agonist induced contractions were a result of partial agonist activity.

Methods

After decapitation a 3 cm segment of proximal caudal artery was carefully removed and placed into room temperature Krebs' solution which was composed as follows (mM): Na^+, 147.6; K^+, 6.4; Ca^{++}, 1.6; Mg^{++}, 1.2; Cl^-, 130; HCO_3^-, 26; SO_4^{--}, 1.2; $H_2PO_4^-$, 1.2; glucose, 11; disodium ethylenediamine tetracetate, 0.027. During all procedures care was taken not to stretch the vessels or damage the endothelium. Unless stated otherwise the Krebs' solution was at a temperature of 37° C and aerated with a gas mixture of 95% O_2-5% CO_2.

Contraction with Exogenous Agonists

The vessel segment was cannulated at each end with PE 50 tubing, placed in a tissue bath with Krebs' solution, stretched to the *in situ* length, and perfused

with Krebs' solution at a constant rate of 2 or 4 ml/min using a Buchler polystaltic pump (Model 2-6100). Perfusion pressure was monitored with a Statham P23 Db pressure transducer and recorded on a Soltec 1220 chart recorder. After an equilibration period of at least 45 min the segment was repeatedly exposed (2-3 times with 15 min intervals between exposures) to the α_1-adrenergic agonist methoxamine (added to the bath solution) until a stable response was obtained. Presence of an intact endothelium was determined by a relaxation response to methacholine added as a 10 µl bolus into the perfusing solution through an injection port proximal to the segment. A cumulative concentration-response curve to methoxamine or BH-T 920 was determined. The vessels were then removed from the bath and either stripped of endothelium by running a 2 inch length of doubled 5-0 silk through the lumen, or handled in a similar fashion with the exception of endothelial removal (sham stripped). The vessel segments were replaced in the tissue bath and again equilibrated and stabilized. A second concentration-response curve to methoxamine or BH-T 920 was then determined. Comparisons were made between the first concentration-response curve (intact endothelium) and the second curve (stripped or sham stripped).

Transmural Nerve Stimulation

Alternatively, after the initial equilibration period the vessel segment was contracted once by methoxamine and relaxed with a bolus dose of methacholine to determine endothelial integrity. This was followed be exposure (2-3 times with 10 min between exposures) to transmural nerve stimulation (2 Hz,10 sec, 40 V, 0.3 msec) until a stable response was obtained. Electrical stimulation was delivered from a Grass stimulator via two platinum electrodes, placed 5 cm apart and 1 cm from either end of the perfused segment. After stabilization, a

frequency-response curve was determined (0.1-4.0 Hz, 30 sec) with the maximum stimulation frequency limited to 4 Hz to both avoid the possibility of tissue damage at high pressures and to maintain consistent responses to repeated stimulation. The segment was removed and stripped or sham stripped, replaced, equilibrated and stabilized, as described above. A second frequency-response curve was determined. Some segments were then exposed to tetrodotoxin (1 μM) for 20 min and again stimulated to confirm that the contractions were nerve-mediated rather than due to a direct effect on the vascular smooth muscle.

Before transmural nerve stimulation the adrenergic nerves of some tissues were destroyed by pretreatment with 6-hydroxydopamine (Aprigliano and Hermsmeyer, 1976; Aprugkuabi et al., 1976). 6-Hydroxydopamine (1.5 mM) was dissolved in unbuffered Krebs' and glutathion (20 μM) solution at a pH of 4.9. A vessel segment was exposed to the 6-hydroxydopamine solution or the Krebs/glutathion vehicle for two 10 min periods separated by an interval of 30 min in buffered, aerated Krebs'. The segment was then cannulated and equilibrated, as described above. Contractile response to transmural nerve stimulation was tested every 30 min (2 Hz, 10 sec). When responses in the 6-hydroxydopamine treated tissues reached a stable minimum value (2-3 hr), the segment was contacted to approximately 100 mm Hg by methoxamine (2-4 μM) in the bath, and endothelial integrity was determined by a 10 μl bolus injection of methacholine (0.2 mmol) into the perfusion buffer. Then, while still contracted, responses to transmural nerve stimulation (1 - 8 Hz) were measured.

Determination of Antagonist Affinities

Dissociation constants of competitive antagonists (K_B), were determined for the α_1-adrenoceptor antagonist, prazosin, and the α_2-adrenoceptor antagonist,

yohimbine, against both methoxamine and BH-T 920. Vessels stripped of endothelium were used to avoid any possible endothelial-mediated effects. After the segments were stripped, equilibrated and stabilized by repeated exposure to methoxamine, as described above, an initial concentration-response curve was determined to either methoxamine or BH-T 920. The segment was then incubated with either prazosin (0.1 nM) or yohimbine (1-10 μM) for 30 min, and a second concentration-response curve was determined. From the dose-ratio of these concentration-response curves in the presence and absence of antagonist (B), the K_B was calculated, as described by Tallarida and Jacob (1979), from the following formula:

$$K_B = \frac{(B)}{(\text{dose ratio} - 1)}$$

with final results presented as pK_B values where $pK_B = -\log K_B$.

Drugs

The following drugs were used: methacholine chloride, tetrodotoxin, glutathione, 6-hydroxydopamine (Sigma Chemical Co., St. Louis, MO); methoxamine hydrochloride (Burroughs Welcome Co., Research Triangle Park, NC); and BH-T 920 (Boehringer Ingelheim KG).

Statistics

Results are expressed as the mean ± S.E.M. Statistical differences between three or more groups were evaluated by analysis of variance and Newman-Keuls Multiple Range Test ($P < .05$) (Snedecor and Cochran, 1967).

Results

Contractile Response to Exogenous Agonists

Both the α_1-adrenoceptor selective agonist methoxamine and the α_2-adrenoceptor selective agonist BH-T 920 produced dose-dependent increases in the perfusion pressure of isolated rat caudal arteries (Figs. 13 and 14). In the range of concentrations examined the response to methoxamine was significantly greater than that to BH-T 920; however, for both drugs only partial concentration-response curves were obtained. In the case of methoxamine this was to avoid tissue damage which can occur at higher pressures, and with BH-T 920 it was in an attempt to maintain α_2-adrenoceptor specificity.

Endothelium removal produced a significant increase in the contractile response to both methoxamine (Fig. 13) and BH-T 920 (Fig. 14). While the response to methoxamine was still much greater than that to BH-T 920, the relative increase in pressure observed with endothelial removal was considerably larger with BH-T 920. At the maximum concentrations compared, endothelial removal produced a 3-4 fold increase in BH-T 920 induced contractions while for methoxamine the increase with endothelium removal was approximately 2 fold. Contractile responses of sham stripped vessels were not different from initial responses to either methoxamine or BH-T 920 (Fig.13; Fig. 14).

Transmural Nerve Stimulation

Contractions in response to transmural nerve stimulation are shown in Fig. 15 as frequency-response curves. While no differences were observed when intact and sham stripped segments were compared, segments devoid of endothelium had significantly enhanced contractions at all frequencies tested from 0.25 to 4 Hz

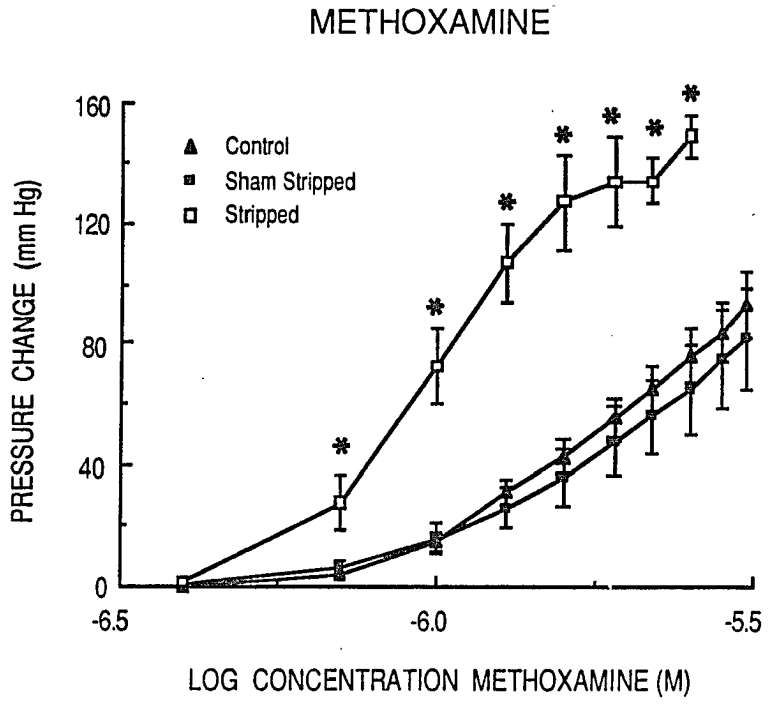

Figure 13. Concentration-response curves for the contractile response to methoxamine in perfused rat caudal arteries which were untreated, stripped of endothelium or sham stripped. Symbols represent mean values ± S.E.M. (n = 5 to 9) and are expressed as the increase in perfusion pressure (mm Hg) at a constant flow of 2 ml/min. (* $P < .01$ by ANOVA).

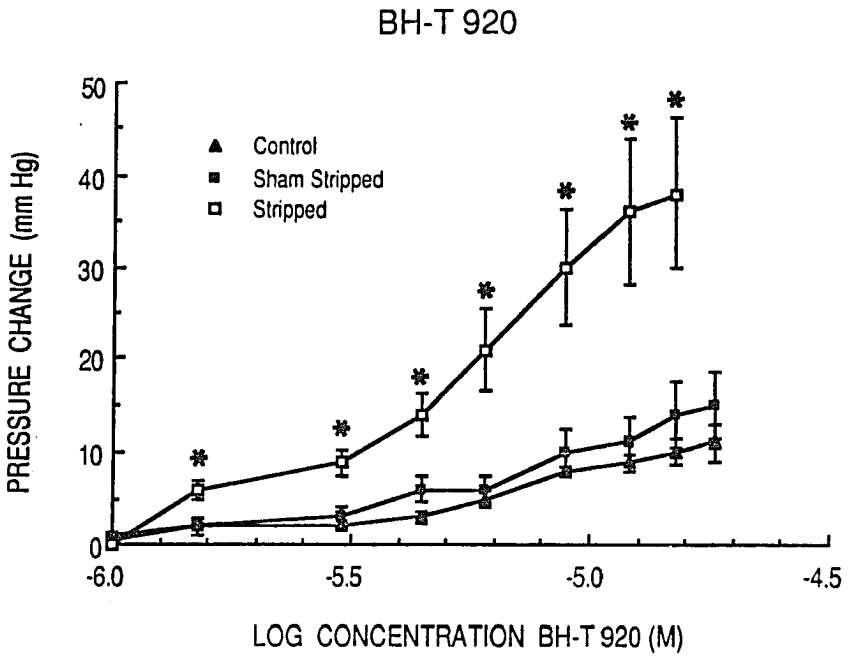

Figure 14. Concentration-response curves for the contractile response to BH-T 920 in perfused rat caudal arteries which were untreated, stripped of endothelium or sham stripped. Symbols represent mean values ± S.E.M. (n = 4 to 7) and are expressed as the increase in perfusion pressure (mm Hg) at a constant flow of 4 ml/min. (* $P < .01$ by ANOVA).

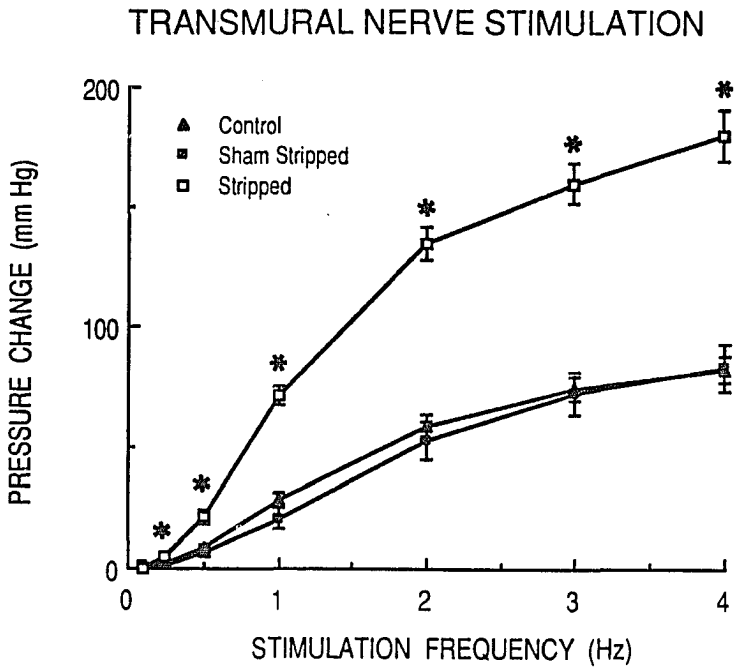

Figure 15. Frequency-response curves for the contractile response to transmural nerve stimulation (30 sec, 40 V, 0.3 msec) of perfused rat caudal arteries which were untreated, stripped of endothelium or sham stripped. Symbols represent mean values ± S.E.M. (n = 5 to 10) and are expressed as the increase in perfusion pressure (mm Hg) at a constant flow of 2 ml/min. (* $P < .01$ by ANOVA).

with an approximate 2 fold increase in pressure at 4 Hz. Exposure to tetrodotoxin abolished all responses to transmural nerve stimulation.

To determine if transmural nerve stimulation directly induced the release of a vasodilator substance, vessels were treated with 6-hydroxydopamine to destroy the contraction induced by stimulation of adrenergic nerves and unmask any relaxation effects. After 6-hydroxydopamine treatment the contractile responses to transmural nerve stimulation were decreased to less than 5% of controls, while the contraction to exogenous methoxamine and the endothelium-dependent relaxation to methacholine were well maintained (Fig. 16). When 6-hydroxydopamine treated tissues were contracted with exogenous methoxamine to a pressure of 100 mm Hg, transmural nerve stimulation produced small further increases in pressure. If contracted vessels were also exposed to tetrodotoxin even these small additional pressure increases were abolished, leaving transmural nerve stimulation with no observable effect (Fig. 16).

Antagonist Affinity

Contractile responses to methoxamine in perfused vessel segments devoid of endothelium were inhibited by both prazosin and yohimbine, producing parallel shifts in the concentration-response curves (Fig. 17). Dose-ratios were obtained at approximately 50% of maximum observed contraction and the resulting pK_B values calculated are shown in Table 9. Contractions to BH-T 920 were also inhibited by both prazosin and yohimbine; however, neither antagonist produced parallel shifts in concentration-response curves (Fig. 18). Both antagonists tended to produce a greater shift against lower concentrations of BH-T 920 than against higher concentrations. To determine approximate affinity of these antagonists for the receptors stimulated by BH-T 920, dose-ratios were obtained at three points along

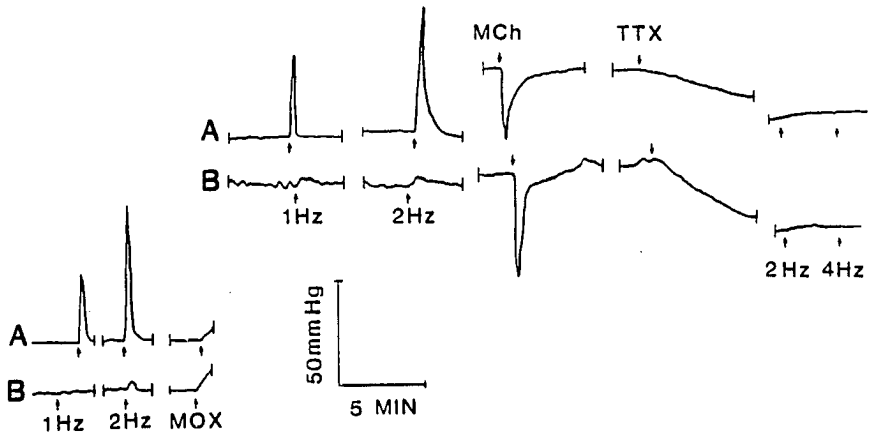

Figure 16. Effect of 6-hydroxydopamine treatment on contractile response of perfused rat caudal artery to transmural nerve stimulation. A. Control artery. B. Pretreatment with 6-hydroxydopamine. Vessels were stimulated (30 sec, 40 V, 0.3 msec) at basal tension (flow 2 ml/min), during exposure to methoxamine (MOX) (2 μM) and after additional exposure to tetrodotoxin (TTX)(1 μM). Presence of intact endothelium is indicated be response to bolus dose of methacholine (MCh) (0.2 mmol) in perfusion buffer.

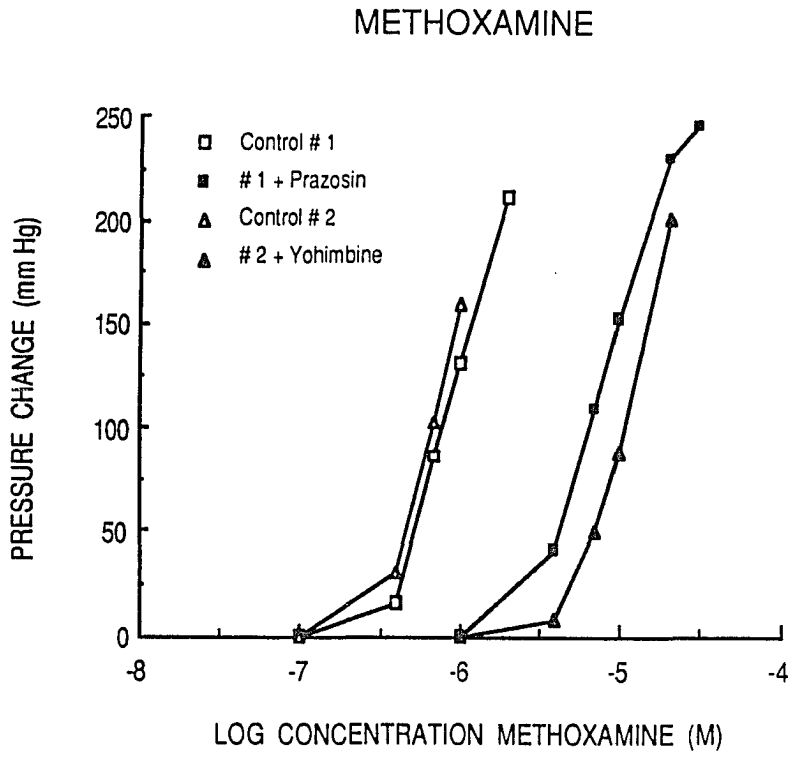

Figure 17. Representive concentration-response curves for the contractile response to methoxamine in perfused rat caudal arteries which were stripped of endothelium. Unfilled symbols represent initial responses and filled symbols represent responses after 30 min incubation with either prazosin (0.1 nM, squares) or yohimbine (10 μM, triangles) and are expressed as the increase in perfusion pressure (mm Hg) at a constant flow of 2 ml/min..

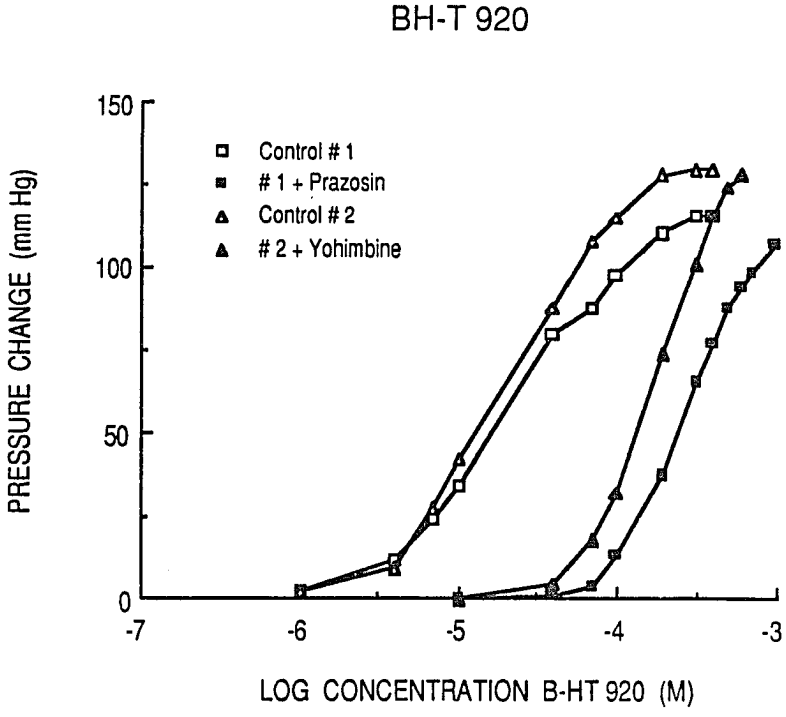

Figure 18. Representive concentration-response curves for the contractile response to BH-T 920 in perfused rat caudal arteries which were stripped of endothelium. Unfilled symbols represent initial responses and filled symbols represent responses after 30 min incubation with either prazosin (0.1 nM, squares) or yohimbine (10 μM, triangles) and are expressed as the increase in perfusion pressure (mm Hg) at a constant flow of 4 ml/min..

Table 9

Agonist	Prazosin pK_B	Yohimbine pK_B
Methoxamine	8.67 ± .08	6.27 ± .10
BH-T 920 (20 mm Hg)	9.21 ± .04 *	6.02 ± .04
BH-T 920 (50 mm Hg)	9.09 ± .06 *	5.86 ± .05 *
BH-T 920 (90 mm Hg)	8.92 ± .20 *	5.57 ± .09 *

* $P < .05$; Significantly different from value vs. methoxamine

the concentration-response curves (20, 50 and 90 mm Hg). The resulting pK_B values are shown in Table 9.

The affinity of prazosin for receptors activated by BH-T 920 was very similar to that found for receptors activated by methoxamine with a small, but statistically significant difference, indicating a greater affinity for receptors activated by BH-T 920. Yohimbine also demonstrated similar affinities against BH-T 920 and methoxamine; however, in this case there was a slightly greater affinity for receptors activated by methoxamine.

Discussion

The removal of endothelium from perfused rat caudal artery significantly enhanced the contractile response to transmural nerve stimulation (Fig. 15) as well as responses to methoxamine (Fig. 13) and BH-T 920 (Fig. 14). While the magnitude of responses to either transmural nerve stimulation or methoxamine were greater than those to BH-T 920, the relative increase in response with removal of endothelium was greater with BH-T 920 (3-4 fold) than with either transmural nerve stimulation or methoxamine (2 fold). These observations are consistent with previous results in rat aortic ring segments where endothelial removal has been shown to enhance the response to α-adrenoceptor agonists, showing a similar preferential enhancement of contractions produced by α_2- selective agonists (Eglème et al., 1984; Lues and Schûmann, 1984; Miller et al., 1984; Godfrained et al., 1985). Hemoglobin, which blocks the vasodepressor actions of EDRF (Martin et al., 1985), has also been shown to enhance responses to α-agonists. These observations have lead to the conclusion that EDRF released from endothelium inhibits the agonist induced contractions; therefore, with removal of endothelium greater contractions are

observed (Martin et al., 1986). It is likely that EDRF plays a similar role in the enhanced response with endothelial removal in the caudal artery.

Precontracted dog arteries have been shown to relax to catecholamines via the stimulation of endothelial α_2-adrenoceptors which release EDRF (Cocks and Angus, 1983; Angus et al., 1986). This observation, coupled with the preferential enhancement of selective α_2-agonist contractions in rat aorta, has led to speculation that the observed release of EDRF in the presence of α-agonists is stimulated via endothelial α_2-adrenoceptors (Eglème et al., 1984).

However, the results of the present study are not entirely consistent with this proposal. The use of ring segments in studies by others of the rat aorta allowed the α-agonists direct access to endothelium. The proposed stimulation of endothelium to produce EDRF would then be simultaneous with stimulation of smooth muscle contraction. In the current study exogenous agonists were added to the bathing medium surrounding the perfused segment with no direct access to the endothelium. Thus an agonist must diffuse through (and contract) smooth muscle cells prior to reaching the endothelium and stimulating EDRF release. It would then be necessary for EDRF to diffuse back into the smooth muscle layer to inhibit contraction. This series of events,while complex, is possible in the case of exogenous agents; however, it is less likely in the case of responses to transmural nerve stimulation. Norepinephrine released from adrenergic nerves during transmural nerve stimulation is susceptible to uptake into both nerve and smooth muscle cells, making it unlikely that sufficient norepinephrine would reach the endothelium and generate enough EDRF to inhibit its own contraction, especially at lower frequencies of stimulation. We have shown that electrical field stimulation does not directly induce the release of EDRF from endothelial cells or the release of some other vasodilator from non-adrenergic nerves since precontracted vessels

treated with 6-hydroxydopamine failed to relax during transmural nerve stimulation (Fig. 16).

As an alternative to α_2-adrenoceptor mediated release of EDRF, it has been proposed that endothelial cells spontaneously release a basal level of EDRF (Griffith et al., 1984a; Miller et al., 1984; Martin et al., 1985). This proposal is more consistent with current results since it eliminates the necessity for diffusion of the agonist through the entire smooth muscle layer in order to have actions on the endothelial cells.

This proposal of spontaneous release of EDRF to inhibit contractions does not initially appear consistent with the differential effects observed with α_1- and α_2-specific agonists. However, in rat aorta pretreated with dibenamine to irreversibly inactivate a large fraction of α-adrenoceptors, Martin et al. (1986) have demonstrated that the α_1-selective agonist, phenylepherine, which is normally a full agonist now behaves as if it were a partial agonist,and with removal of the endothelium, the responses to phenylephrine are now enhanced in a manner similar to α_2-selective agonists. It has been suggested, therefore, that the preferential enhancement with endothelial removal of contraction induced by α_2-agonists is due to their action as partial agonists at α_1-adrenoceptors rather than activity at α_2-adrenoceptors (Martin et al., 1986).

To examine the possibility that preferential enhancement of BH-T 920 contraction was due to its activity as a partial agonist at α_1-adrenoceptors, the affinities of prazosin and yohimbine for receptors stimulated by both methoxamine and BH-T 920 were determined. Vessel segments stripped of endothelium were used in these experiments in order to avoid any complications due to either basal or stimulated EDRF release. Methoxamine contractions were inhibited by both prazosin and yohimbine giving a parallel shift in the concentration-response curve (Fig.

17) The resulting pK_B values are consistent with those previously observed against selective α_1-agonists in vascular smooth muscle (see Agrawal et al., 1984). However, determination of pK_B values for these antagonists using BH-T 920 as agonist was not as straightforward. As shown in Figure 18 both agents inhibited contraction to BH-T 920, but non-parallel shifts in the concentration-response curves were produced. Estimates of affinity were made from dose-ratios taken at the lower, middle and upper parts of the concentration-response curves. The resulting pK_B values (Table 9) for both prazosin and yohimbine against BH-T 920 are very similar to those determined against methoxamine suggesting that both agonists are acting at the same binding site. The reason for the small but statistically significant higher affinity of prazosin against BH-T 920 and lower affinity for yohimbine against BH-T 920 are unclear at this time. However, it does seem clear that BH-T 920 in rat caudal artery does not mediate contraction via an α_2-adrenoceptor, but by actions as a partial agonist at the α_1-adrenoceptor. These results are consistent with the propsal that preferential enhancement of α_2-adrenoceptor specific agonists is a result of partial agonist activity at smooth muscle receptors rather than action at endothelial adrenergic receptors (Martin et al., 1986). They are, therefore, also consistent with the proposal of spontaneous rather than stimulated release of EDRF.

The enhanced contractile response to exogenous a-agonists and transmural nerve stimulation observed with endothelial removal appears, therefore, to be a result of the removal of a spontaneous, basal level of vasodilator, most likely EDRF, rather than a release stimulated via endothelial α_2-adrenoceptors. These results, particularly the significant enhancement of nerve stimulated contractility, suggest a physiological role for spontaneous release of vasodilator in the regulation of

vascular tone, and the possibility that an alteration in the level of release may be involved in pathological states such as hypertension or vasospasm.

CHAPTER 5

CONCLUSION

As explained at the end of the first chapter, chapters 2, 3 and 4 are composed of manuscripts which report on separate studies, including an in depth discussion of the results. Therefore, it is not the purpose of this final chapter to again analyze those results, but rather to briefly examine them in terms of the objectives and working hypotheses for each study, as stated in chapter 1.

Muscarinic Receptors of Smooth Muscle and Endothelium

The objective of this first study was to determine if muscarinic receptors mediating relaxation located on the endothelial cell were of the same subtype as those on smooth muscle which mediate contraction. Preliminary functional studies using the rabbit ear artery had suggested the presence of few, if any, smooth muscle muscarinic receptors. Therefore, ear artery appeared to be an excellent vessel to investigate endothelial muscarinic receptors. The initial hypothesis was: **Cholinergic, muscarinic receptors of the rabbit ear artery are primarily on the vascular endothelium and are of the low affinity, M_2 subtype.**

Results of the functional studies where relaxation was inhibited using the selective antagonist pirenzepine identified the endothelial muscarinic receptor as the low affinity M_2 subtype as predicted in the initial hypothesis. The choice of ear artery for these studies was fortuitous for three reasons: 1) as already mentioned, the smooth muscle showed no contractile response to any concentration of methacholine. If this had occurred, it would have made the analysis of inhibition of

methacholine. If this had occurred, it would have made the analysis of inhibition of relaxation more difficult if not impossible. 2) The endothelium of rabbit earartery is durable, remaining relatively intact and responsive in spite of occasional rough handling and lengthy experiments. 3) The vascular smooth muscle cells actually do have muscarinic receptors, although nonfunctional, which allowed determination of the subtype found on smooth muscle cells using this same tissue.

Based on the initial hypothesis, the density of [^{3}H](-)QNB binding sites (B_{max}) should have decreased when stripped vessels rather than intact vessels were used to prepare membrane homogenates. However, binding density was not decreased with endothelial removal leading to the conclusion that muscarinic receptors were present on smooth muscle cells but apparently nonfunctional. As mentioned above, this had the advantage of allowing a determination of the smooth muscle subtype using the same tissue. Inhibition of [^{3}H](-)QNB binding by pirenzepine identified the smooth muscle muscarinic receptor as the same low affinity M_2 subtype found on the endothelium.

While the initial hypothesis was only partially correct, the objective of this study, to determine if muscarinic receptors on endothelium and smooth muscle were the same, was accomplished. However, recent reports of subtypes of the M_2 receptor identified by the antagonist AF-DX 116 (Giachetti et al., 1986; Hammer et al., 1986) suggest that the characterization of vascular muscarinic receptors may still be incomplete.

Increasing Age and Endothelium-Dependent Vasodilation

The objective of this study was to determine if endothelium-dependent vasodilation was altered with advancing age. In retrospect, this objective appears unrealistically ambitious. Considering the tissue and species differences observed

in endothelium-dependent responses, as well as the large number of agents acting through this mechanism, to actually achieve this objective as stated would take many years of investigation. However, what was achieved was the determination that in some vessels of the rat, cholinergic endothelium-dependent relaxation is altered with increasing age.

The initial hypothesis was: **Cholinergic, endothelium-dependent relaxation declines in the rat vasculature from maturity to senescence.** The results indicate that this is not the case. Sensitivity to endothelium-dependent relaxation induced by methacholine was found to increase with increasing age in the aorta and caudal artery, while responses of the vessels of the mesenteric bed remained unchanged. The reason for this increase in sensitivity is not yet clear. However, responses to A23187 were unchanged in both caudal artery and perfused mesentery. This observation suggests that an alteration at the level of the muscarinic receptor or specific receptor coupling is involved in enhanced methacholine sensitivity rather than an increase in the ability of endothelium to release EDRF or of smooth muscle cells to respond to EDRF.

While these results indicate a relationship between increasing age and endothelium-dependent responses, a great deal of further research is required before reaching a complete understanding of these relationships. This research could approach the problem from many directions including: 1) a wider survey of tissues to determine the extent of age-related changes, 2) a more exact determination of the site (or sites) in the production and response to EDRF that are affected by aging, 3) expansion of the vasoactive agents tested to include other endothelium-dependent agents as well as those not dependent on the endothelium or an examination of spontaneous EDRF release. All of these approaches would be

worth consideration, however, a change in the level of spontaneous EDRF release, as discussed further in the next section, could have the greatest overall effect on vascular control and, therefore, should be explored first.

Enhanced Contraction and Removal of the Endothelium

The objective of this final study was to use the unique experimental opportunities presented with perfused vessels, in this case the rat caudal artery, to resolve the controversy regarding spontaneous versus α_2-adrenoceptor stimulated release of EDRF. The initial hypothesis was: **Removal of the endothelium enhances the contractile responses to both exogenous agonists and electrically stimulated nerve responses. This enhancement is a result of elimination of EDRF which is released from endothelium spontaneously as well as a result of α-adrenoceptor stimulation.** While the results as discussed in chapter 4 suggest a predominant role for spontaneous release of EDRF, clearly, the controversy was not, as hoped, resolved. Several approaches have yet to be explored which may yet clarify the roles of stimulated and spontaneous release. Differential exposure of endothelium and smooth muscle to selective agonists and antagonists, by addition of these agents to either the bathing or perfusion solution, has the potential to uncover a stimulated response, while exposure to hemoglobin could be used to confirm the existence of spontaneous release.

An alternative approach which could be used in conjunction with a continuation of the aging study would be the use of a perfusion cascade system. Preliminary results using a caudal artery to caudal artery cascade have, thus far, shown no success; however, other laboratories have shown good results using larger vessels to show spontaneous release, and this approach should be attempted. It

should be possible using this system to clearly separate spontaneous and induced release since the contractile agent for the test ring can be either perfused through or bypass the EDRF generating segment. Furthermore, experiments using different combinations of EDRF-generating and test rings from rats of different ages could determine the influence of increasing age on spontaneous release of EDRF. Since spontaneously released EDRF can affect the response to any contractile agent, this clearly can have a great influence on regulation of vascular tone. Therefore, a determination of normal levels of spontaneous EDRF release, as well as the release in pathological states, should have high priority in future studies.

A Final Comment

The endothelium-dependent response of blood vessels, which only a few years ago was unknown, has been revealed as an important part of the complex interaction between a wide variety of agents and the vasculature. The exact physiological and pathological, as well as potential therapeutic, roles of the endothelium are still being determined. Hopefully, the work presented in this dissertation will, to some small extent, aid in that pursuit.

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