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# THE ROLE OF NITRIC OXIDE IN SMALL INTESTINE FUNCTIONS IN VIVO AND IN VITRO

By

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## ABSTRACT

### THE ROLE OF NITRIC OXIDE IN SMALL INTESTINE FUNCTIONS IN VIVO AND IN VITRO

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Experiments were designed to determine whether nitric oxide plays a role in regulating intestinal blood flow and motility in vivo. The effect of nitric oxide synthesis inhibition on the vasodilation produced by substance P in vivo, and the relaxation produced by ACh, substance P, and bradykinin in vitro was also determined.

L-NAME, an analogue of L-arginine that inhibits nitric oxide synthesis, significantly increased jejunal vascular resistance and intestinal motility. The effect of L-NAME is long-lasting and remains for up to 50 minutes after L-NAME. The vasoconstriction produced by L-NAME was reversed by nitroglycerin, but not by L-arginine or D-arginine. The motility produced by L-NAME was abolished by arterial infusion of nitroglycerin, L-arginine, or atropine, whereas D-arginine was without effect. L-NAME did not affect the hyperemic response to vascular occlusion. Intraluminal placement of methylene blue, a specific inhibitor of nitric oxide action, also produced a significant increase in jejunal blood flow, oxygen uptake, and motility under resting conditions. Methylene blue also inhibited the hyperemic response to digested food, but did not affect the hyperemic response to vascular occlusion.

The relaxation of the superior mesenteric artery produced by acetylcholine, substance P, or bradykinin in vitro is inhibited by removal of the endothelial cells, methylene blue or L-NAME. Furthermore, the vasoconstriction and motility produced by L-NAME was associated with impaired vasodilator responses to these agents that were reversed after incubation with L-arginine. Conversely, the vasodilation produced by substance P in vivo was not inhibited by L-NAME.

These results are consistent with the concept that basal tone of mesenteric resistance vessels are regulated by the release of nitric oxide, and that nitric oxide is a mediator of the hyperemic response to digested food. Nitric oxide also plays a major role in suppressing cholinergic-mediated motility under resting condition. Nitric oxide derived from the endothelium also mediates the relaxation produced by acetylcholine, substance P, and bradykinin in vitro. In contrast, nitric oxide does not appear to mediate the hyperemic response to vascular occlusion or the dilation of mesenteric resistance arterioles produced by substance P in vivo.

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TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

# INTRODUCTION

The tunica intima is the innermost layer of the circulatory system. It is comprised of a single layer of endothelial cells that are supported to the vascular smooth muscle by connective tissue and a basement membrane. The average 70 kg human possesses approximately 1.5-2 kilograms of endothelium that covers an area of nearly 700 square meters. Although previously thought to simply provide a physical barrier that prevented the direct contact of blood constituents with vascular smooth muscle, the vascular endothelium is now known to play a more dynamic role in maintaining hemostasis. In addition to providing a physical barrier, the endothelial cells regulate vascular smooth muscle tone in response to mechanical changes in blood flow, shear stress, and several blood-borne vasodilator agents (30,50,53,132). Currently, the endothelium has been shown to relax vascular smooth muscle through the synthesis and release of prostacyclin from arachidonic acid and nitric oxide from L-arginine (52,131,132).

The demonstration in 1987 that the formation of nitric oxide by an enzyme in the vascular endothelial cells opened up a new field of biological research. Currect research indicates that the nitric oxide/cyclic GMP signal transduction mechanism is widespread and may play a role in cell function and cellular communication processes in a variety of tissues and organs. Like prostacyclin, nitric oxide biosynthesis is not restricted to the vascular endothelial cells. Nitric oxide is also synthesized from homogenates of rat brain tissue (61,103) and peripheral nerve fibers (14), released from activated phagocytic cells such as macrophages (78,79) and neutrophils (169,199). These findings have led to speculation that nitric oxide may also function as a neurotransmitter within the central and peripheral nervous systems, and function as a mediator of local inflammatory responses involving activated macrophages and neutrophils.

There is evidence suggesting that nitric oxide regulates vascular smooth muscle tone and may mediate the endothelium-dependent vasodilation produced by ACh in the rat gastrointestinal tract in vitro (57,58,60,133). In addition, nitric oxide appears to regulate intestinal smooth muscle

contractility in vitro (11,30,116,163) and may act synergistically with prostanoids to maintain gastric mucosal integrity after mucosal insult using ethanol (121,195). However, this evidence is based primarily on studies performed in vitro. To date, there are no studies that have simultaneously investigated the role that nitric oxide may play in regulating intestinal blood flow and motility in vivo.

In the following text, I will first review the current literature and data regarding the identification and mechanism of action of nitric oxide and then the vasodilator agents that are most commonly used to study the nitric oxide/cyclic GMP signal transduction system. In addition, the role of nitric oxide as an endogenous relaxant of vascular and intestinal smooth muscle will be discussed in detail. As will be described more completely in the objective section of this dissertation, the literature review led to the formulation of the hypothesis that nitric oxide may function to regulate intestinal blood flow, oxygen uptake, and motility in vivo. Also, nitric oxide may mediate the endothelium-dependent vasodilation produced by several humoral agents, including ACh, substance P, and bradykinin in the canine mesenteric vasculature in vitro and in vivo. Moreover, nitric oxide may account for the intestinal hyperemia produced by either vascular occlusion or placement of digested food in the lumen of the canine jejunum.

## The Discovery of Endothelium-Derived Relaxing Factor (EDRF)

Acetylcholine (ACh) has long been recognized as a vasodilator agent when injected into vascular beds of whole animals. However, the muscarinic agonists would invariably constrict strips of artery and vein in vitro. The reason for the paradoxical effect of ACh in vivo and in vitro was resolved in 1980 when Furchgott et al. (53) demonstrated that the endothelial cells play

an obligatory role in muscarinic cholinergic relaxation of rabbit thoracic aorta rings preconstricted with norepinephrine (NE). When care was taken to avoid damage to the endothelium, the preconstricted aortic rings produced excellent relaxation in response to the muscarinic agonists; ACh, carbachol and methacholine (48,50,53). Conversely, intentional rubbing of the intimal surface with a cotton applicator stick or pretreatment with collagenase selectively abolished the vascular smooth muscle relaxation in response to the muscarinic agonists but did not alter the relaxation produced by organic nitrates (48,50,53). These initial studies indicate that the lack of relaxation produced by ACh in previous studies was a result of damage to the endothelial cells.

Using a "sandwich" arrangement of 2 arterial strips of rabbit aorta, one without and one with endothelial cells, Furchgott (46) was able to demonstrate that activation of muscarinic receptors located on endothelial cells stimulated the release a diffusable factor (or factors) which in turn acts on the smooth muscle to produce relaxation. Experiments were preformed to test the response to ACh on a transverse aortic strip without endothelial cells when mounted alone or when sandwiched to a longitudinal strip of the same width and length with endothelial cells. Removal of the endothelial cells from the transverse strip by mechanical rubbing abolished the dose-dependent relaxations by ACh. Because of the orientation of the longitudinal strip of aorta with endothelial cells in relation to the force transducer, ACh failed to relax the longitudinal strip. When the longitudinal strip was "sandwiched" adjacent to the endothelial denuded transverse strip, ACh caused a dose dependent relaxation of the denuded transverse strip of vascular smooth muscle. Therefore, upon binding to muscarinic receptors located on the endothelium of the longitudinal strip, a factor is released from the endothelial cells that then diffuses to the vascular smooth muscle of the transverse strip and produces relaxation. Furchgott termed this relaxing factor endothelium-derived relaxing factor (EDRF) after it was shown to be distinct from prostacyclin ($PGI_2$).

The cholinergic receptors on the endothelial cells are of the muscarinic type and atropine is a potent competitive inhibitor of the endothelium-dependent relaxation of rabbit aorta produced by ACh (53). The dissociation constant ($K_b$) of the atropine-receptor complex on rabbit aortic endothelial cells was estimated at 0.35±0.04 nM after 30 min of incubation with 10 uM atropine (53). The relative potencies of the muscarinic agonists ACh, methacholine and carbachol were 1.0, 0.3 and 0.1, respectively (53). The range of concentration which ACh relaxes rabbit thoracic aorta precontracted with 0.3-0.1 uM norepinephrine is usually 0.01-1.0 uM ACh. Higher concentrations of ACh either have little effect or occassionally produce contraction of the precontracted aortic ring. Atropine abolishes the relaxation of rabbit aorta and canine mesenteric artery at low doses (0.01-0.1 uM) of ACh (53,107), and the contraction of rabbit aorta produced at high concentrations of ACh (1-10 uM) (53). The acetylcholinesterase inhibitor, physostigmine (0.3 uM), did not significantly increase the sensitivity to ACh (53).

In general, ACh is equally effective in relaxing rabbit aortic rings that were preconstricted to the same magnitude with norepinephrine, histamine, serotonin, and $PGF_{2a}$. In rabbit aortic rings preconstricted with norepinephrine (0.3-0.01 uM), half maximal relaxation usually occurs between 0.3-0.10 uM ACh and the degree of relaxation elicited by the addition of a fixed concentration of ACh tends to decrease as the level of initial tone of the aortic ring is increased with increasing doses of norepinephrine (53). 0.1 uM ACh is sufficient to produce 90-100% relaxation of rabbit thoracic aorta rings precontracted with norepinephrine or phenylephrine, but when these rings are maximally contracted with 30 uM norepinephrine, ACh produces only about 30% relaxation. However, there are differences between the endothelium-dependent vasodilation produced by ACh depending on the agonist used to preconstrict the arterial preparation (12). For example, vascular smooth muscle preparations preconstricted with potassium chloride are less sensitive to relaxation elicited by ACh, showing a maximal relaxation of 10-50% to those of rings preconstricted with

norepinephrine.

## Identification of EDRF as Nitric Oxide

Furchgott first suggested that EDRF was nitric oxide after noting the similarities between the relaxation of rabbit aorta produced by endothelium-dependent vasodilating agents and endothelium-independent organic nitrates (48). Furchgott demonstrated that methylene blue and oxyhemoglobin, agents that specifically inhibit relaxation of smooth muscle associated with an increase in cyclic GMP, blocked the relaxation produced by endothelium dependent vasodilating agents and organic nitrates (19,48,51). Subsequent biocascade/superfusion experiments utilizing bovine intrapulmonary artery and cultured porcine aortic endothelial cells demonstrated that the pharmacologic and biochemical properties of EDRF are identical to authentic nitric oxide (89,142). It was established, using biocascade techniques, that EDRF, like nitric oxide, was a very short lived substance with a half-life of ranging between 3 and 45 seconds in oxygenated physiologic solutions in vitro (72,73,88,142). In addition, the half-life and relaxation produced by EDRF and nitric oxide is readily decreased by superoxide anion (161), and is enhanced in the presence of superoxide dismutase (74,88,90,92,142). These results are consistent with the effect of pyrogallol, which generates superoxide anion when in oxygenated media (123), on the activity and action of EDRF and purified solutions of nitric oxide. The addition of pyrogallol to the media that superfuses isolated arterial smooth muscle decreases the half-life, relaxation, and accumulation of cGMP produced by EDRF and nitric oxide (74,88,92). Spectrophotometric analysis of the reaction of EDRF or nitric oxide to oxidized hemoglobin (88) and ozone (142) also provided evidence that nitric oxide was EDRF, and responsible for the relaxation of bovine pulmonary artery and rabbit aorta produced by ACh, bradykinin, and the calcium ionophore A23187.

## Biosynthesis of Nitric Oxide

It has been demonstrated that within vascular endothelial cell, nitric oxide is synthesized from the guanidino nitrogen group of L-arginine (141,155,162,168), and depletion of intracellular stores of L-arginine results in diminished release of nitric oxide from the bovine pulmonary artery endothelium when stimulated by bradykinin or the calcium ionophore A23187 (63,64). These results are consistent with the observations published by Palmer et al. (142). These investigators demonstrated that the release of nitric oxide from cultured porcine endothelial cells was significantly diminished after incubation for 24 hours in a media deficient of L-arginine (142). The addition of $^{15}N$-labelled L-arginine to the L-arginine deficient media enhanced the release of $^{15}N$-labelled nitric oxide in the presence of bradykinin or A23187 as measured using mass spectroscopy (142). Other basic amino acids similar in structure to L-arginine and their D-enantiomers had no effect on nitric oxide formation (142). Endothelial cells from arteries of most vascular beds have since been shown to synthesize nitric oxide in amounts sufficient to account for the vasodilation produced by several humoral vasodilator substances, including ACh, substance P and bradykinin (5,46,142,143). Nitric oxide synthesis can be inhibited in the presence of synthetic analogues of L-arginine such as $N^G$-nitro-L-arginine (L-NOARG), $N^G$-nitro-L-arginine methylester (L-NAME), or $N^G$-monomethyl-L-arginine (L-NMMA). L-NOARG, L-NAME, and L-NMMA have modified guanidino terminal nitrogens that cannot be metabolized and competitively inhibit the formation of nitric oxide from L-arginine (116,133). These analogues of L-arginine are also potent inhibitors of the vasodilation of rabbit aorta produced by ACh, bradykinin and substance P (133,158). The inhibitory action of L-NOARG, L-NAME, or L-NMMA can be reversed in the presence of excess L-arginine but not with D-arginine, indicating

that there is strict substrate specificity for the enzymatic process involved in nitric oxide synthesis. The inhibitory effect of L-NOARG, L-NAME, and L-NMMA is specific to the L-isomers and the D-enantiomers are without effect (133,155,156). These studies indicate that the synthesis of nitric oxide occurs within cells and only the transportable L-enantiomers are recognized by the enzyme that synthesizes nitric oxide.

The enzyme responsible for the synthesis of nitric oxide from L-arginine is called nitric oxide synthase. In the last year it has become apparant that there are two forms of this enzyme. One form of the enzyme is cytosolic, requires NADPH, is $Ca^{+2}$/calmodulin dependent, and produces nitric oxide in response to receptor or physical stimulation for short periods of time (15,132). The second form of the nitric oxide synthase enzyme is induced after interaction between endothelial cells and immunologically activated white blood cells, and allows for the release of nitric oxide for longer periods of time (132). This form of nitric oxide synthase is $Ca^{+2}$-independent and requires tetrahydrobiopterin and other cofactors. Using an immunohistochemical stain for purified nitric oxide synthase isolated from rat cerebellum, Bredt et al (14) demonstrated that nitric oxide synthase is located within the purkinje fibers of the brain, vascular endothelial cells, and within the cell bodies and nerve fibers of the myenteric plexus (14). However, it is unclear from this study whether the enzyme from these tissues represents the same or different isozymes of the enzymes located in the endothelial cells.

## The Mechanism of Nitric Oxide Release - The Role of Calcium

Early studies demonstrated that the generation of EDRF from the endothelial cells was a calcium-dependent process and that extracellular $Ca^{+2}$ plays a role in the endothelium-dependent

relaxation. First, the $Ca^{+2}$ ionophore A23187 was shown to be a potent endothelium-dependent vasodilator of vascular smooth muscle (46,51), suggesting that the release of EDRF from the endothelium was dependent on extracellular $Ca^{+2}$ flux into the endothelium. Secondly, eliminating $Ca^{+2}$ from the perfusate attenuates the endothelium-dependent relaxation of rabbit aorta produced by methacholine or A23187 by 67% and 92%, respectively (174). The reason for the lesser degree of inhibition of the relaxation induced by methacholine was attributed to the possible utilization of a pool of $Ca^{+2}$ by methacholine that was inaccessible to the ionophore, such as intracellular $Ca^{+2}$ stores.

The effect of calcium channel blockers on endothelium-dependent relaxation is variable. In preconstricted rabbit aortic rings, Singer and Peach (174) showed that the $Ca^{+2}$ channel blockers verapamil and nifedipine inhibit the relaxation of rabbit aorta by methacholine or A23187 by 40-45%. However, other investigators have failed to demonstrate any effect of $Ca^{+2}$ channel blockers on the relaxation of isolated dog coronary arteries produced by methacholine, substance P, or A23187 (6). It is not clear why $Ca^{+2}$ channel blockers inhibit A23187-induced relaxation of rabbit aorta, since the ionophore should transport $Ca^{+2}$ directly across the cell membrane without the involvement of activated channels. These inconsistencies led to the proposal that intracellular calcium stores may also play a role in EDRF release. Studies utilizing electrophysiologic recordings of $Ca^{+2}$ flux have shown that intracellular calcium stores do play a role in mediating the relaxation of vascular smooth muscle to endothelium-dependent agonists (95). In cultured bovine aortic endothelial cells, ATP and bradykinin both mobilize intracellular stores of calcium (95). Currently, it is thought that the initial $Ca^{+2}$ signal for the relaxation produced by EDRF is of intracellular origin, whereas the maintained release of EDRF is a result of calcium entry from the extracellular space (95,144).

## Mechanism of Vascular Smooth Muscle Relaxation Produced by Nitric Oxide

Prior to the discovery of EDRF in 1980 (53), organic nitrates were shown to relax vascular smooth muscle by directly activating soluble guanylate cyclase and increasing the cyclic GMP concentration within the smooth muscle (1,8,36). The increase in cyclic GMP stimulated by organic nitrates preceded the relaxation and after reaching a peak, decline toward initial concentrations even though the relaxation remained (135). It was suggested that nitric oxide, or a closely related nitrosothiol compound that releases nitric oxide, was the active metabolite of the organic nitrates because authentic nitric oxide was shown to activate soluble guanylate cyclase, enhance the accumulation cyclic GMP, and relaxed preconstricted bovine coronary arteries (48,67). However, the increase in cyclic GMP produced by nitroglycerin and other organic nitrates is somewhat greater, and the relaxation produced by these agents is longer in duration than that produced by authentic nitric oxide (48,67). The longer action of the organic nitrates compared to nitric oxide is probably because the lipophilic ester of organic nitrates permeates the cell membrane of smooth muscle cells and then degrades to nitric oxide, and thereby protects the organic nitrates from extracellular degradation to inactive nitrate compounds.

Like the organic nitrate vasodilating agents, endothelial-dependent relaxation of vascular smooth muscle exhibits a close relationship between mechanical relaxation of arterial smooth muscle and cyclic GMP accumulation (135,153). ACh, bradykinin, and A23187 produce a dose-dependent increase in arterial cyclic GMP concentration which preceeds the relaxation of bovine coronary and pulmonary arteries (84,89). However, ACh, A23187, thrombin and organic nitrates do not enhance cyclic AMP accumulation within arterial smooth muscle (135). Removal of the endothelium from bovine intrapulmonary arterial rings markedly reduces the ability of ACh to enhance cyclic GMP accumulation within smooth muscle and abolishes the relaxation produced

by ACh (153). Atropine abolishes the accumulation of cyclic GMP and vasodilation to ACh in bovine coronary (84) or intrapulmonary (89) arterial smooth muscle with intact endothelium. From these studies it seems that endothelium-dependent vasodilating agents like ACh, bradykinin and A23187 relax vascular smooth muscle by the same mechanism utilized by organic nitrate vasodilating agents and purified solutions of nitric oxide, i.e. by enhancing the activity of soluble guanylate cyclase within the smooth muscle. However, unlike organic nitrates and purified exogenous nitric oxide, the relaxation of vascular smooth muscle produced by endothelium-dependent vasodilating agents relies on the synthesis and release of nitric oxide from within the endothelial cells.

Methylene blue, an inhibitor of soluble guanylate cyclase, prevents the relaxation of vascular smooth muscle produced by organic nitrates and endothelium-dependent vasodilating agents. Methylene blue (5-100 uM) attenuates the accumulation of cyclic GMP and relaxation produced by organic nitrates, ACh, A23187, or bradykinin in bovine coronary and pulmonary arteries (67,84,88,89,93), rabbit aorta (126), and canine mesenteric artery (107). The inhibitory effect of methylene blue is selective for cyclic GMP-dependent relaxation of vascular smooth muscle and does not affect the relaxation of smooth muscle produced by the increase of cyclic AMP within vascular smooth muscle (90,123). Biocascade experiments demonstrate that the inhibitory effect of methylene blue on the relaxation of rabbit coronary arteries produced by 1 uM ACh is due to the direct effect of methylene blue on the vascular smooth muscle and not a result of nitric oxide inactivation in transit (71). Moreover, methylene blue lowers resting arterial levels of cyclic GMP and increases resting tension of rat aorta and bovine coronary artery produced by norepinephrine (67,126). These studies indicate that methylene blue inhibits the relaxation and accumulation of cyclic GMP produced by organic nitrates and several endothelium-dependent vasodilator compounds.

Studies using exogenous cyclic GMP and its 8-bromo analogue, 8-bromo-cyclic GMP, and inhibitors of cyclic GMP degradation also indicate that the relaxation of vascular smooth muscle produced by organic nitrates and endothelium-dependent vasodilating agents is cyclic GMP-dependent. Cyclic GMP and 8-bromo-cyclic GMP (1-100 uM) produced a dose-dependent relaxation of bovine coronary artery preconstricted with norepinephrine (111,139). 8-bromo-cyclic GMP also reduced the tension produced by KCl, but to a lesser extent than when the coronary artery was preconstricted to a comparable amount of tension with norepinephrine (139). MB-22948, an inhibitor of cyclic GMP phosphodiesterase, potentiated the relaxation of coronary artery produced by exogenous cyclic GMP analogues (84,111). Furthermore, MB-22948 augmented the relaxation and accumulation of cyclic GMP in rat cremastic arterioles and rabbit aorta produced by ACh (84,124). The ability of MB-22948 to augment endothelium-dependent relaxations can be abolished in the presence of methylene blue (84,111). These studies are consistent with the concept that guanylate cyclase mediates the relaxation produced by endothelium-dependent vasodilating agents.

The mechanism by which cyclic GMP relaxes vascular smooth muscle remains uncertain. Rapaport and Murad have suggested that cyclic GMP accumulation enhances cyclic GMP-dependent protein kinase activity in rabbit aortic smooth muscle (43), which results in the inactivation of myosin light chain kinase (36,151,152). The increase in cyclic GMP-dependent protein kinase activity and decrease in $^{32}P$ incorporation into myosin light chain correlate well with the vascular smooth muscle relaxation (36,151). Organic nitrates and endothelium-dependent vasodilators both enhance cyclic GMP-dependent protein kinase and alter the incorporation of $^{32}P$ into several proteins within vascular smooth muscle (43,135,151,152). The effect of nitroprusside is endothelium-independent (135) and can be mimicked with exogenous cyclic GMP or 8-bromo-cyclic GMP (151). As determined by gel electrophoresis, the phosphorylation patterns observed

with endothelium-dependent agonists in rat aortic spiral strips is similar to that produced by nitroprusside (135,151). Because cyclic GMP-dependent protein kinase activation alters the phosphorylation pattern of many still undetermined smooth muscle proteins (36,135,151), the inactivation of myosin light chain could result from decreased myosin light chain kinase activity or increased myosin light chain phosphatase activity. Another possible mechanism for the relaxation produced by organic nitrates and endothelium-dependent vasodilators includes the sequestration of free cytosolic $Ca^{+2}$ inside the smooth muscle, which is required for myosin light chain phosphorylation by myosin light chain kinase (1,135). Further studies are necessary to elucidate the changes in protein phosphorylation produced by relaxation mediated by nitric oxide.

## Endothelium-Dependent Vasodilator Agents

Since the initial discovery that the endothelium plays an obligatory role in the relaxation to ACh, several other vasodilating agents have been shown to relax vascular smooth muscle via an endothelium-dependent mechanism. These include the calcium ionophore A23187 (46,47,156), substance P (5,203,204), bradykinin (19,73), ATP and ADP (33,34), thrombin (33), vasoactive intestinal peptide (31), cholecystokinin (47), and histamine (182). Current evidence suggest that the arterial smooth muscle relaxation produced by these agents is mediated by endothelium-derived nitric oxide. However, there are variations in the mechanism of relaxation produced by these vasodilating agents within different vascular beds of a given species and depending on the species studied. I will briefly review the mechanism(s) of arterial smooth muscle relaxation produced by the calcium ionophore A23187, substance P, and bradykinin, which are the three agents most commonly used to study the mechanism of nitric oxide synthesis and release.

Calcium ionophore A23187

The calcium ionophore A23187 is a unique endothelium-dependent vasodilator in that it directly stimulates the synthesis and release of nitric oxide without binding to a membrane receptor. Like the relaxation produced by ACh, the relaxation produced by A23187 is endothelium-dependent and not affected by cyclooxygenase inhibition in rat and rabbit aorta (46), bovine pulmonary artery (92), and several peripheral arteries of dog and human (47,178). However, A23187 appears to be more potent than ACh and produces a greater relaxation at higher concentrations of norepinephrine than does ACh (53,58).

Biocascade studies demonstrate that A23187 enhances the release of $PGI_2$, $PGE_2$, and nitric oxide from cultured porcine endothelial cells that can directly relax rabbit aortic strips denuded of endothelium (73,141). The release of prostanoids and nitric oxide were quantified using specific radioimmunoassays and chemiluminescence, respectively. Perfusion of A23187 onto the smooth muscle directly did not produce relaxation and did not increase prostanoid or nitric oxide release detected in the effluent perfusate (73). However, the significance of the relaxation of rabbit aortic strips to prostanoids released from porcine endothelium by A23187 is unclear, since exogenous administration of $PGI_2$ does not normally relax porcine aorta (66).

The endothelium-dependent relaxation of preconstricted rabbit aortic rings produced by A23187 is mediated by nitric oxide (155), and incubation of the aorta with either methylene blue or L-NMMA inhibit the relaxation produced by A23187 (46,126,155). Methylene blue also inhibits the relaxation of human coronary artery and bovine intrapulmonary artery produced by A23187 (90,92,178), suggesting that the relaxation is dependent on increased levels of cyclic GMP. The relaxation of bovine intrapulmonary arteries produced by A23187 is abolished after depletion of intracellular L-arginine (64), suggesting that L-arginine-dependent nitric oxide synthesis mediates the relaxation to A23187. Whether the relaxation of human coronary artery

produced by A23187 is affected by inhibitors of nitric oxide synthesis has not been determined.

Substance P

Substance P has been shown to be a powerful vasodilator of several blood vessels and in all cases its action is strictly dependent on intact endothelial cells. The threshold concentration of substance P required elicit relaxation of vascular smooth muscle ranges from 30 pM in isolated rabbit aorta to 1 pM in canine celiac and superior mesenteric arteries (47,203,204). Removal of the endothelial cells from renal, celiac, or mesenteric arteries of rabbit, dog, cat, and guinea pig abolishes the relaxation produced by substance P in vitro (12,134,203,204). Likewise, Brizzolara and Burnstock (16) have shown the relaxation of preconstricted rabbit hepatic arterial rings is abolished after mechanical removal of the endothelial cells. The relaxation produced by substance P is not altered by 40 uM indomethacin, but is inhibited after incubation of the arterial rings with 10 uM methylene blue (53,204). These studies suggest that the relaxation produced by substance P is dependent on the activation of soluble guanylate cyclase within the vascular smooth muscle. This is supported by the results of Bolton and Clapp (12) utilizing strips of guinea pig mesenteric artery. These investigators have demonstrated that the relaxation produced by substance P is abolished in the presence of 10 uM hemoglobin (12), which has been shown to inactivate nitric oxide extracellularly (66,125,126). In the rabbit aorta, the relaxation produced by 10 nM substance P is inhibited after treatment of the aortic ring with the nitric oxide synthesis inhibitor L-NMMA (155). Therefore, nitric oxide may mediate the relaxation of substance P in the mesenteric artery of dogs and guinea pigs. However, the effect of nitric oxide synthesis inhibition on the relaxation produced by substance P in these vessels has not been determined.

The mechanism responsible for the vasodilation produced by substance P in vivo has not been thoroughly investigated. In studies using sonomicrometry crystals to measure canine femoral

artery diameter in vivo, Angus and Cocks (5,8) demonstrated that arterial infusion of 0.1-1 nM substance P produced concentration-dependent dilation of the femoral artery in situ. The dilation produced by substance P was abolished after endothelial cell removal by intraarterial balloon inflation, whereas systemic administration of indomethacin (5 mg/kg) was without effect (5,8). This would suggest that the dilation of canine femoral artery produced by substance P is mediated by the release of a non-prostanoid endothelium-derived factor, perhaps nitric oxide. There are no studies to determine whether nitric oxide mediates the relaxation produced by substance P in vivo.

Bradykinin

The relaxation produced by bradykinin is very heterogeneous and varies between species (19) and depending on the agonist used to constrict the arterial smooth muscle (28). In porcine coronary arteries preconstricted with the thromboxane analogue U-46619, the relaxation produced by bradykinin was not affected by 300 uM L-NMMA and was only slightly inhibited when incubated with 10 uM methylene blue (28). However, when porcine coronary arteries are constricted with 25 mM potassium chloride, the relaxation produced by bradykinin is effectively blocked by L-NMMA or methylene blue (28). In rings of superior mesenteric and celiac arteries from rabbit and cat (19), the relaxation produced by bradykinin is completely blocked by the inhibition of cyclooxygenase with indomethacin (19,53), whereas mechanical removal of the endothelium does not inhibit the relaxation produced by bradykinin. In contrast to cat and rabbit arteries, the relaxation of canine and human arteries produced by bradykinin is strictly endothelium-dependent and is not inhibited by indomethacin or flurbiprofen (19,50). In dog arteries, bradykinin is typically more potent than ACh in eliciting endothelium-dependent

relaxation, with a threshold concentration ranging from 0.1-1.0 nM (19). Similar to the relaxation elicited by ACh and the calcium ionophore A23187, the relaxation of canine arteries produced by bradykinin is inhibited after incubation of the arterial strips with ETYA (19), suggesting that ACh, A23187, and bradykinin stimulate the release of the same relaxing factor from canine endothelial cells.

Bradykinin has been shown to stimulate the release of more than one relaxing factor from endothelial cells from vascular beds of other species. In bovine intrapulmonary arteries, bradykinin produces an endothelium-dependent relaxation that is partially inhibited by methylene blue or indomethacin and completely abolished in the presence of both inhibitors (91,94). In the presence of indomethacin, bradykinin (20-100 uM) stimulates the release of nitric oxide from cultured porcine endothelial cells, as detected by the relaxation of rabbit aorta denuded of endothelium in biocascade studies (73,142). In addition, bradykinin (20-100 nM) stimulates the release of $PGI_2$ and $PGE_2$ from cultured porcine endothelium and the release of $PGI_2$ and $PGE_2$ is inhibited by indomethacin (73). However, it appears that $PGI_2$ does not account for the endothelium-dependent relaxation of rabbit aorta produced by bradykinin because exogenously added $PGI_2$ fails to relax this arterial smooth muscle preparation (66). Furthermore, cyclooxygenase inhibitors failed to attenuate the relaxation of rabbit aorta produced by bradykinin (66). It is not clear from these studies whether bradykinin also stimulated the release of an endothelium-derived hyperpolarizing factor. Nonetheless, as is the case with ACh and A23187, nitric oxide released from porcine endothelial cells upon stimulation with bradykinin is sufficient to account for the relaxation of rabbit aorta (142).

## Other Endothelium-Derived Vasorelaxant Factors

### Endothelium-derived hyperpolarizing factor (EDHF)

In addition to nitric oxide, there is evidence suggesting that ACh and bradykinin may stimulate the release of a second factor from endothelial cells called "endothelial-derived hyperpolarizing factor" (EDHF) (13,41,107,108,109). The release of EDHF from the endothelium produces a transient hyperpolarization of the smooth muscle cell membrane associated with the opening of $^{86}Rb$-permeable $K^+$ channels (65). The relaxation of canine mesenteric and renal arterial smooth muscle produced by EDHF can be blocked by 1 uM atropine but not by 10 uM methylene blue or oxyhemoglobin (107). This suggests that the relaxation produced by EDHF is not associated with an increase in cyclic GMP levels within the smooth muscle. The release of two endothelial-derived factors by ACh may a result of the activation of different muscarinic receptors ($M_1$ and $M_2$) located on the endothelial cells (108,109). In the rabbit saphenous artery, endothelium-dependent hyperpolarization was produced by ACh but not by the $M_2$ agonist oxofremorine; however, both ACh and oxofremorine cause endothelium-dependent relaxation (109). Therefore, the stimulation of the $M_1$ subtype with ACh causes a relaxation of vascular smooth muscle that is associated with smooth muscle hyperpolarization, whereas stimulation of the $M_2$ subtype releases a separate relaxing factor from the endothelial cells, most likely nitric oxide, that is not associated with hyperpolarization (108).

Studies using ouabain have suggested that the release or action of EDHF requires $Na^+/K^+$ pump activity. In biocascade studies, incubation of porcine endothelial cells (donor tissue) with ouabain (5 uM) inhibits the relaxation of coronary arteries without endothelial cells produced by bradykinin or A23187 (13). In contrast, incubation of the coronary arteries with ouabain does not effect the relaxation produced by bradykinin, A23187 or purified solutions of nitric oxide (13).

Incubation of endothelium denuded canine arterial rings with ouabain inhibits the relaxation produced by the basal release of EDRF or ACh-induced release of EDRF from left circumflex arteries with functional endothelium (41). However, the relaxation evoked by infusion of bradykinin into the perfusate is not altered by incubation of the bioassay rings with ouabain. These studies suggests that ACh and bradykinin stimulate the release of different relaxing factors from the coronary endothelium. The factor responsible for the relaxation under basal conditions and upon stimulation with ACh is most likely mediated through the activation of the $Na^+/K^+$ ATPase pump on vascular smooth muscle. In contrast, the EDRF released upon stimulation with bradykinin appear to requires $Na^+/K^+$ pumping at the level the endothelial cell rather than vascular smooth muscle.

Based on the effect of ouabain on the endothelium-dependent relaxation produced by ACh and bradykinin (13,41,83), and since exogenously added nitric oxide did not hyperpolarize canine mesenteric arterial smooth muscle (107), it was suggested that nitric oxide and endothelium-derived hyperpolarizating factor were separate entities. However, exogenously added organic nitrates and purified solutions of nitric oxide can hyperpolarize rat thoracic aorta and uterine smooth muscle, respectively (154,177). Furthermore, it has been recently shown that ouabain may inhibit the relaxation of human resistance arteries produced by ACh in vitro by impairing either the synthesis and/or release of nitric oxide from the vascular endothelium (198). In addition, studies have shown that very low concentrations of ouabain (10 pM) can inhibit the relaxation produced by nitroprusside (154). These studies imply that the factor that leads to the hyperpolarization of vascular smooth muscle may somehow be linked to nitric oxide synthesis and/or release, rather than existing as a separate entity.

Prostanoids

Exogenous arachidonate can induce endothelium-dependent vasodilation in a number of isolated arteries. DeMey and Vanhoutte have demonstrated that exogenous arachidonic acid (0.1-10 uM) produced a dose-dependent relaxation that was eliminated or reduced when the endothelial cells of canine femoral, saphenous, pulmonary and splenic arterial rings are removed (44). The relaxation of the femoral artery produced by arachidonic acid is abolished by incubation with either indomethacin and 5,8,11,14, eicosatetraenoic acid (ETYA) (33). Using [$^{14}$C] arachidonic acid, these investigators demonstrated that the major product of arachidonate was 6-keto $PGF_{1a}$, the inactive metabolite of prostacyclin (33). Therefore, prostanoids act as mediators of endothelium-dependent relaxation in certain vascular preparations. Although prostanoids released from endothelial cells can relax the underlying vascular smooth muscle, a prostanoid cannot account for the relaxation produced by EDRF since, by definition, the relaxation produced by EDRF is not inhibited by cyclooxygenase blockade.

As eluded to previously, some humoral vasodilator agents that relax vascular smooth muscle through the release of nitric oxide, may also produce an endothelium-dependent vasodilation mediated through the release of prostanoids from the vascular endothelial cells in certain preparations. For example, bradykinin stimulates the release of both prostanoids and nitric oxide from the endothelial cells of bovine intrapulmonary artery (91,94). In the rabbit aorta, ACh can stimulate the production of $PGI_2$ and $PGE_2$ from the endothelium in a dose-dependent manner. However, $PGI_2$ or $PGE_2$ do not appear to account for the ACh-induced vasodilation since the concentration of ACh needed to produce a significant stimulation of $PGI_2$ release is an order of magnitude greater than those needed to produce 50% relaxation. Furthermore, several chemically diferent cyclooxygenase inhibitors do not impair the vasodilation produced by ACh (44).

Nonprostaglandin Metabolites of Arachidonic Acid

Before the discovery that the major EDRF released from endothelial cells was nitric oxide, it was postulated that EDRF may be a lipoxygenase or cytochrome P-450 monooxygenase derivative of arachidonic acid metabolism based on the inhibition produced by several antagonists of arachidonic acid oxidation (53). 5,8,11,14 eicosatetraenoic acid (ETYA), an inhibitor of both cyclooxygenase and lipoxygenase, is an effective inhibitor of the relaxation of rabbit aorta produced by ACh (47,53,72). Incubation of the rabbit aorta with ETYA (100 uM) for 30-60 minutes will completely and irreversibly abolished the relaxation of rabbit thoracic aorta in response to ACh (19). Subsequent studies have demonstrated that ETYA blocks the endothelium-dependent relaxation of rabbit and rat aorta produced by A23187 (50,203), ATP in the rabbit aorta (46), bradykinin in canine pulmonary, renal, coronary and mesenteric arteries (46,50), substance P in dog renal and celiac arteries (204), and the relaxation produced by vasoactive intestinal peptide in rat aorta (31). In contrast, ETYA does not effect endothelium-independent relaxation of rabbit aorta produced by isoproterenol, $NaNO_2$, glyceryl trinitrate, adenosine or AMP. Nordihydroguiaretic acid (NDGA), an agent that inhibits lipoxygenase, produces effects similar to those of ETYA. NDGA (30-100 uM) completely abolishes the relaxation of rabbit aorta produced by ACh or methacholine (173). NDGA also inhibits the relaxation produced by A23187 in rabbit aorta (173) and human mesenteric arteries (46,50), the relaxation produced by bradykinin in dog arteries (19,46), and by arachidonic acid in the rabbit aorta (172,173). Furthermore, arachidonic acid (53,172,173) and mellitin, a protein in bee venom that activates the calcium-sensitive phospholipase $A_2$ and stimulates the metabolism of arachidonic acid (45), elicit an endothelium-dependent relaxation that is not inhibited by blockade of cyclooxygenase. These studies suggested that EDRF was a lipoxygenase derivative. However, more recent bioassay studies (72,130) have shown that ETYA, NDGA, and other inhibitors of arachidonic acid

oxidation also possess oxidant properties, and inhibit nitric oxide-mediated relaxation of vascular smooth muscle by extracellular inactivation of nitric oxide while in transit from the endothelial cells to the smooth muscle.

## Physiologic Implications of Nitric Oxide in the Cardiovascular System

### Regulation of Arterial Blood Pressure

Nitric oxide has been proposed to play a physiologic role in the regulation of mean arterial blood pressure and the distribution of regional blood flow in vivo (57,60,196). Intravenous infusion of L-NMMA or L-NAME induces a dose-dependent, enantiomer-specific hypertension in rats (57,59,60,196), rabbits (134,156) and guinea pigs (3). In conscious rats chronically instrumented for recording of arterial blood pressure and blood flow through the carotid, mesenteric, renal, and femoral artery, Gardiner et al (57,59,60) demonstrated that administration of either L-NMMA or L-NAME increased arterial blood pressure and decreased vascular conductance in the renal, mesenteric, carotid, and hindquarters vascular beds. The vasoconstrictive effects produced by injection of L-NMMA or L-NAME were not observed after injection of D-NMMA or D-NAME (57,59,60). In addition, the hypertensive and vasoconstrictive effect of L-NMMA and L-NAME were partially reversed by L-arginine, but not D-arginine (57,59,60). The hypertensive response to L-NMMA is associated with an inhibition of endothelium-dependent vasodilation induced by ACh in vivo (196). Furthermore, the selectivity of L-NMMA in the rat was confirmed by its failure to inhibit the vasodepressor responses to nitroglycerin (196). Similarly, Rees et al (155,156) has demonstrated that systemic administration of L-NMMA is accompanied by inhibition of nitric oxide release from the isolated strips of rabbit thoracic aorta in vitro and selectively inhibited the endothelium-dependent relaxation of rabbit aorta produced

by ACh and substance P in vitro. Infusion of L-arginine fully restored the released of nitric oxide and the relaxation produced by ACh in the rabbit aorta (155,156). These studies are consistent with the proposal that nitric oxide generated from L-arginine plays an important role in the control of arterial blood presssure, regional vascular blood flow, and the relaxation of vascular smooth muscle produced by ACh. Unfortunately, these studies do not determine whether the source of nitric oxide that is responsible for regulating systemic blood pressure and regional blood flow is derived from endothelial cells or other sources, such as PMNs, macrophages, or nerves.

It also appears that nitric oxide may function to regulate myocardial contractility. Evidence shows that removal of the endothelium from isolated papillary muscles of ferrets and pigs induces a negative inotrophic effect (175). The negative inotropic effect of endocardial cell removal were reversed in bioassay experiments where an endocardium-denuded papillary muscle was exposed to the effluent from a column of porcine endocardial cells cultured on microcarrier beads (175). In addition, cultured endocardium has been shown to possess the enzyme responsible for nitric oxide synthesis and does release nitric oxide in vitro (114,175). These studies imply that nitric oxide released from endocardial cells may function as a positive inotroph that increases myocardial contractility. This is supported by studies indicating that systemic injection of L-NMMA or L-NAME decreased cardiac output and the maximum positive slope of aortic flow (dF/dt) in conscious rats (59,60). While it is feasible that the reduction in cardiac output was a direct consequence of the increase in afterload produced by the hypertensive effect of L-NMMA and L-NAME, the marked reduction in stroke volume, peak thoracic aortic outflow, and dF/dt are consistent with negative inotrophic changes following inhibition of nitric oxide synthesis (59).

Conduit Arteries

Endothelial cells, either bathed in blood or Kreb's solution, release a dilator factor in response to different mechanical stimulation such as increases in pulsatile flow and shear stress on the luminal surface of the blood vessel (147,160). This endothelium-dependent, flow-induced vasodilation has been described in several vascular beds, including the canine coronary (81,202), femoral and iliac arteries (100,128,201), and in the resistance vessels perfused by the central artery in the rabbit ear (69). Since the relaxation is not blocked by inhibitors of cyclooxygenase (148,201,202), but is inhibited by agents that inhibit the relaxation to ACh or substance P, the flow-dependent vasodilation has been attributed to an increase in the synthesis and/or release of nitric oxide. In conscious dogs, the vasodilation of the iliac artery produced by graded increases in blood flow or arterial infusion of ACh under constant flow conditions can be inhibited by removal of the endothelium (201). Similarly, removal of the endothelium from the femoral artery abolished the dilation produced by exogenously administered ACh, but did not affect the dilation produced by either nitroprusside or nitroglycerin (6,100). Moreover, topical application of methylene blue to the adventitial surface of the femoral artery or continuous infusion into the femoral artery attenuates the vasodilation to arterial infusion of ACh, substance P, nitroglycerin, and increases in blood flow per se in vivo (96,176). These studies suggest that nitric oxide may mediate flow-mediated vasodilation in large conduit arteries in vivo. However, the effect of nitric oxide synthesis inhibition on flow-dependent dilation has not yet been determined. Hence, the exact role that nitric oxide may play in regulating flow-dependent vasodilation of conduit arteries is not clear.

Resistance Arteries

Damage to the endothelium or suffusion of isolated resistance arterioles with methylene blue decreases arteriole diameter and inhibits the dilation produced by ACh or bradykinin (99,158). Oxyhemoglobin, an agent that inactivates nitric oxide extracellularly (66,125,126), also attenuates the dilation produced by ACh, substance P, or the increase in perfusate flow rates in the isolated vascular bed of the central artery in rabbits (69). The importance of nitric oxide in the regulation of basal tone in resistance vessels has been demonstrated in the isolated perfused rabbit heart using L-NMMA (4). In this preparation, L-NMMA causes an increase in coronary perfusion pressure and attenuates the decrease in coronary perfusion pressure induced by ACh, and was accompanied by a decrease in the nitric oxide released into the coronary effluent (4). Levi and colleagues have reported similar results using isolated perfused guinea pig hearts (113).

Inhibition of nitric oxide synthesis produces a vasoconstriction and inhibits the endothelium-dependent relaxation of resistance arterioles to ACh and substance P in isolated, blood perfused vascular beds in vivo (37,146,185,186). L-NMMA has been shown to produce a dose-dependent vasoconstriction and inhibit the relaxation of arterioles to ACh in rat cremasteric and spinotrapezius muscle vascular bed (138,146). Using intravital microscopy to measure arteriole diameter, Nakamura and Prewitt (138) reported that superfusion of L-NMMA (100 uM) over the arcade arterioles of the spinotrapezius muscle reduced arteriolar diameter by 64% and attenuated the relaxation produced by ACh by about 50%. L-NMMA did not affect the relaxation produced by nitroprusside (138). These effects of L-NMMA were partially reversed by 1 mM L-arginine (138). The vasoconstriction produced by L-NMMA was not affected by tetrodotoxin, indomethacin, or propranolol (138). In addition, suffusion of the arterioles with L-NMMA significantly constricted the arterioles even in the presence of prazosin (138). Therefore, nerve conduction, prostanoids, $alpha_1$- and beta-adrenergic receptors are not responsible for the

vasoconstriction produced by nitric oxide synthesis inhibition.

Similar results have been reported after arterial infusion of L-NMMA in blood perfused resistance beds in anesthetized cats (37) and conscious humans (185,186). Infusion of L-NMMA (1-4 umol/min) for 5 minutes into the brachial artery of healthy human subjects produces a dose-dependent reduction in resting forearm blood flow and attenuates the vasodilation produced by ACh, whereas the dilation to nitroglycerin are unaffected (185,186). L-NMMA (25-330 uM plasma concentration) reduced human forearm blood flow by 40% and the constrictor response of L-NMMA was reversed by arterial infusion of L-arginine at a rate of 100 nmol/min (186). These studies suggest that resting arteriolar tone is locally modulated by endogenous nitric oxide biosynthesis, and that the endothelium-dependent vasodilation produced by ACh is mediated by the formation of endogenous nitric oxide in vivo.

Just as flow-dependent EDRF release can alter large artery diameter, so Griffith et al. have demonstrated a similar mechanism in the resistance vessels of the rabbit ear (70). They used hemoglobin as an inhibitor of flow-mediated release of nitric oxide to demonstrate that basal nitric oxide release coordinates the hemodynamic flow distribution in an intact network of vessels whose diameter ranged from 87 to 548 um. Using angiography to measure the vessel diameter in this preparation, hemoglobin was shown to increase the pressure-fall across these vessels and caused constriction of the first and third branched vessels (70). Moreover, Griffith et al. reported that the endothelium-dependent vasodilation in response to ACh was enhanced at increased flows (70). These authors subsequently showed that the activity of endogenous nitric oxide was greatest in the arterioles in which the hydraulic resistance and shear stress are greatest (68). Hence, these authors suggest that nitric oxide mediates the flow-dependent and ACh-mediated vasodilation of resistance vessels in situ.

Active and Reactive Hyperemic Responses

The de novo synthesis of nitric oxide from L-arginine is thought to contribute importantly, although not exclusively, to the regulation of peripheral vascular resistance and the vasodilatory effects produced by ACh in vivo (37,57,60,156,185,186,196). Therefore, one may reason that nitric oxide may also participate in the reactive hyperemia in response to vascular occlusion and/or the functional hyperemia in response to an increase in tissue metabolism. Studies using nonspecific inhibitors of relaxations mediated by EDRF support this hypothesis (197). However, there are only two publications that have addressed this issue using specific inhibitors of nitric oxide synthesis in rats (146,197), and neither study found nitric oxide to play a significant role in the hyperemic responses to vascular occlusion (197) or increased muscle activity (146).

Currently, there is evidence supporting a role for both prostaglandins and adenosine as local mediators of the reactive hyperemic response to vascular occlusion. In humans, the reactive hyperemia produced after long periods of occlusion (3-5 minutes) of the brachial artery is mediated in part by prostaglandins and adenosine (18). Inhibition of prostaglandin synthesis using ibuprofen reduced the reactive hyperemic response by 70%. The attenuation was due to both a reduction of the peak post-occlusive flow and to a shortening of the duration of the post-occlusive hyperemia (18). The adenosine receptor antagonist theophylline reduced the reactive hyperemia following 5 minutes of arterial occlusion by 35%. In contrast, infusion of dipyridamole, a drug that inhibits cellular adenosine uptake, potentiated the reactive hyperemic response by 45% (18). Combined treatment with ibuprofen and theophylline did not reduce the reactive hyperemia more that either drug alone (18). The lack of additive effects of ibuprofen and theophylline suggests a link between the vascular relaxation produced by prostaglandins and by adenosine.

In isolated rat cremasteric third order arterioles, Messina and Kaley have shown that

prostaglandins are partially responsible for the vasodilation produced after arterial occlusion for 15 seconds, and indomethacin inhibited the vasodilation by about 50% (127). However, adenosine does not appear to mediate the reactive hyperemia in rat cremasteric arterioles since theophylline inhibited the relaxation produced by adenosine, but was without effect on the post-occlusive hyperemia (197). The reactive hyperemia in rat skeletal muscle arterioles has both an endothelium-dependent component and endothelium-independent component (104). Koller and Kaley (104) reported that the peak post-occlusive hyperemia produced after short (20 seconds) or long (80 seconds) periods of occlusion was significantly diminished after functional impairment of the endothelium by laser/dye treatment. The duration of the hyperemic response following impairment of the endothelium was significantly reduced only on release of the short occlusion periods (104). The laser/dye technique utilized by the authors to impair endothelial cell function has been previously shown to specifically inhibit endothelium-dependent vasodilation produced by ACh, but does not alter the dilation produced by either adenosine or nitroprusside, agents that directly relax vascular smooth muscle (105).

Wolin et al. (197) used methylene blue and L-NMMA to block guanylate cyclase activity and nitric oxide synthesis, respectively, to determine if the endothelium-dependent component of the reactive hyperemia was mediated by nitric oxide. These authors found that suffusion of the arterioles with methylene blue or L-NMMA significantly attenuated the dilation produced by ACh, and methylene blue was also found to significantly attenuate the reactive hyperemia (197). Conversely, L-NMMA had no effect on the reactive hyperemia to vascular occlusion (197). These authors concluded that the inhibitory effect of methylene blue on the reactive hyperemia resulted from blocking soluble guanylate cyclase activation by hydrogen peroxide, which may accumulate within endothelial cells during the reperfusion period after release of the occlusion. This suggests that the endothelium component of the reactive hyperemia in isolated cremasteric arterioles is

mediated by the release of prostaglandins (127) and hydrogen peroxide (197), but not by nitric oxide.

The effect of L-NMMA treatment on the functional (active) hyperemia has been investigated in the rat teniussimus muscle using motor nerve stimulation to produce a submaximal hyperemia (146). Relative to control responses, there were no significant alterations by L-NMMA in the peak blood flow or the subsequent post contraction hyperemia observed (146). L-NMMA did produce a slight increase in resting vascular resistance significantly inhibited the dilatory response to ACh in this preparation (146). This study suggests that nitric oxide does not play a role in the functional hyperemia in isolated preparations of rat skeletal muscle.

## The Role of Nitric Oxide in the Gastrointestinal Tract

### Intestinal Blood Flow

There is evidence suggesting that nitric oxide may mediate the relaxation of mesenteric arteries produced by ACh or substance P in many different species. For example, removal of the endothelial cells abolishes the relaxation of mesenteric arterial smooth muscle produced by ACh in cats, rabbits, dogs, guinea pigs, and humans (12,19,107). Similar results have been obtained using substance P (12,50,203,204). Methylene blue, an inhibitor of soluble guanylate cyclase (126), or oxyhemaglobin, which inactivates nitric oxide in transit (69), attenuate the relaxation produced by ACh or substance P in mesenteric arteries of dogs and guinea pigs (12,86,87,107). Methylene blue and oxyhemoglobin also attenuated the relaxation of mesenteric arteries produced by exogenously added nitric oxide or organic nitrates and prevented the acculumation of cyclic GMP within the smooth muscle (12,19,107). Recently, Hwa and Chatterjee (87) reported that the relaxation of guinea pig mesenteric arteries produced by ACh (0.3 uM) is inhibited after

incubation of the artery with 1 mM L-NMMA. These studies suggest that nitric oxide synthesis mediates the dilation produced by ACh in mesenteric arterial rings in vitro. The effect of nitric oxide synthesis inhibition on other endothelium-dependent vasodilators, such as substance P, bradkinin and ATP, have not yet been investigated.

Endothelium-derived nitric oxide also appears to mediate the dose-dependent vasodilation produced by arterial infusion of ACh in isolated rat mesenteric vascular bed (133,150,192). Arterial infusion of ACh produces a dose-dependent vasodilation in the isolated rat mesenteric vascular bed constantly perfused with Kreb's solution (5-7 mls/min) in the presence of indomethacin (133,192). Chemical destruction of the endothelial cells by brief exposure to sodium deoxycholate or 3-(3-cholamidopropyl)1-propanesulphonate (CHAPS) selectively blocks the relaxation produced by ACh but did not affect the endothelium-independent vasodilation produced by nitric oxide or sodium nitroprusside (133,192). Furchgott and his colleagues (49) used collagenase (0.2%) to remove the endothelial cells or hemoglobin to abolish the dilation produced by ACh in the rat perfused mesentery. Similarly, Randall and Hiley (150) have shown that infusion of CHAPS abolished the dilation produced by carbachol in the rat isolated mesenteric bed perfused with whole blood. In addition, these investigators demonstrated that continuous infusion of methylene blue (1% wt:vol) abolished the dilation produced by carbachol (150). Recently, Moore (133) demonstrated that the addition of L-$N^G$-nitro arginine (L-NOARG) or L-NMMA to the Kreb's perfusate significantly inhibited the vasodilator response in the rat mesenteric bed produced by ACh, but did not affect the vasodilation produced by sodium nitroprusside. The addition of L-arginine to the perfusate, but not D-arginine, partially reversed the effect of L-NOARG and L-NMMA (133).

The lack of effect of indomethacin of the relaxation produced by ACh (38,150) suggests that prostaglandins do not mediate the relaxation produced by ACh and are consistent with the

findings in isolated mesenteric arterial rings in vitro. Furthermore, suffusion of tetrodotoxin (3 uM) over isolated mesenteric arterioles does not suppress the dilation produced by ACh applied directly to the arteriolar wall (38). Therefore, the dilation produced by ACh in rat mesenteric vascular bed does not appear to involve local neural reflexes or nerve stimulation. Hence, nitric oxide derived from the vascular endothelial cells appears to mediate the relaxation produced by exogenous administration of ACh in the mesenteric vascular bed.

It is not currently clear whether basal release of nitric oxide regulates the resting vascular tone of the intestinal vascular bed. Studies using inhibitors of nitric oxide synthesis have produced conflicting results. Systemic administration of L-NMMA (100 mg/kg iv) or L-NAME (10 mg/kg iv) to conscious rats significantly decreases mesenteric blood flow (57,60). The constrictive effect of L-NMMA and L-NAME in the mesenteric vascular bed was early in onset and returned slowly toward control levels over a period of 90 minutes. Infusion of L-arginine (100 mg/kg iv) either 10 minutes prior to or 30 minutes after administration of the nitric oxide synthesis inhibitor significantly attenuated the constrictor response to L-NMMA or L-NAME in the mesenteric bed (57,60). Similarly, Moore et al. (133) demonstrated that the addition of L-NOARG or L-NMMA to the perfusate resulted in a rapid and graded increase in the perfusion pressure of the isolated rat mesenteric vascular bed. Under constant flow conditions, L-NOARG and L-NMMA increased the perfusion pressure of the isolated mesenteric bed by 55% and 34%, respectively. However, the vasoconstriction was not maintained in the continued presence of the inhibitors, declining to control levels within 5-20 minutes (133). Under these conditions, L-NOARG and L-NMMA inhibited the dilation to ACh without affecting the dilation produced by nitroprusside (133). Other studies have shown that nitric oxide synthesis inhibition has variable effects in different regions of the gastrointestinal tract (85). Using radiolabelled $^{103}Rb$ to determine blood flow distribution, Humphries has demonstrated that intravenous infusion of L-NOARG at

a rate of 0.5 mg/kg/min for 20 minutes to conscious rabbits significantly decreased blood flow to the stomach, duodenum, and colon (85). In contrast, blood flow to the ileum was slightly increased 20 minutes after infusion of L-NOARG, although the increase was not stastistically significant (85). Therefore, nitric oxide may have differential effects on blood flow within the gastrointestinal tract.

Nitric oxide, or a compound closely resembling nitric oxide, has been proposed as a perivascular neurotransmitter released in response to electric field stimulation in canine and bovine mesenteric arteries in vitro (2,182). In isolated canine mesenteric arteries perfused at a constant rate (1 ml/min) with Ringer-Locke solution produced a constriction that is blocked by tetrodotoxin or phentolamine (182). Suffusion of L-NMMA over the extraluminal surface of the mesenteric artery potentiates the constrictive effect of electric field stimulation (182). In addition, L-arginine applied extraluminally attenuates the constriction produced by electric field stimulation and prevents the potentiation elicited by L-NMMA (182).

In the presence of 5 uM guanethidine, the constriction of bovine mesenteric arteries produced by electric field stimulation is reversed to a relaxation (2). The relaxation is not affected by removal of the endothelial cells but is abolished in the presence of tetrodotoxin (2). The smooth muscle relaxation to electric field stimulation is also correlated with an increase in cyclic GMP, whereas cyclic AMP levels are not affected (2). Methylene blue and the compound LY 83583 inhibit the relaxation by 60% and 50%, respectively (2). Zaprinast, a selective inhibitor of cyclic GMP degradation potentiates the relaxation produced by electric field stimulation (2). However, preincubation of the mesenteric arterial strips with L-NMMA or L-NOARG were without effect on the relaxation (2). The reason for the lack of effect by L-NMMA or L-NOARG is not clear and requires further investigation.

Intestinal Motility

Motility of the mammalian intestine is regulated by a complex network of neurones and nerve fibers of both intrinsic and extrinsic origin. Experiments utilizing isolated strips of gastrointestinal smooth muscle have established that nonadrenergic, noncholinergic (NANC) nerves exist in intestinal smooth muscle in addition to the cholinergic and noncholinergic excitatory nerves. NANC neurones are believed to provide the major inhibitory autonomic nerve supply to gastrointestinal smooth muscle and are thought to function in receptive relaxation to enable bolus progression in an aboral direction. The exact nature of the neurotransmitter released by these nerves is still unknown. ATP and VIP are considered as the two main candidates depending in the tissue and/or species (120). Evidence has recently been presented indicating that nitric oxide, or a closely related substance that releases nitric oxide, is involved in the NANC relaxation of mammalian smooth muscle. Nitric oxide has been proposed to play a role in the NANC relaxation produced by low frequency electric field stimulation in the rat anococcygeus (62,82,115,164) and gastric fundus (116), the canine lower esophageal sphincter (32), duodenum (181), ileocolonic junction (11), and proximal colon (30,163,179), opossum lower esophageal sphinctor (136,140,183,184), and ileal circular smooth muscle of human and guinea pig (122,171). Furthermore, immunohistologic studies using antisera for the purified nitric oxide synthase enzyme has shown that nitric oxide synthase is located within cell bodies and nerve fibers of the myenteric plexus of the rat intestine (14).

The NANC relaxation of visceral smooth muscle produced by electric field stimulation is neurally mediated since the nerve conduction inhibitor tetrodotoxin abolished the relaxation (32,164,171). Incubation of intestinal smooth muscle strips with either L-NAME or L-NMMA (10-100 uM), but not their D- enantiomers, significantly inhibits the NANC relaxation produced by electric field stimulation (62,82). The inhibitory effects of L-NAME and L-NMMA can be

reversed by the addition of excess L-arginine (183). However, the stereoisomer D-arginine, which is not a substrate for nitric oxide synthesis, has no effect on the inhibition produced by L-NAME or L-NMMA (183). In contrast, the relaxation produced by exogenous nitric oxide, nitrosocysteine, or nitroglycerin mimics the relaxation of intestinal smooth muscle produced by electric field stimulation, and is not inhibited by tetrodotoxin or after incubation with inhibitors of nitric oxide synthesis (11,179). 10 uM oxyhemoglobin, which rapidly binds nitric oxide (94), reduces the amplitude of the NANC relaxation without affecting the ability of the intestinal smooth muscle to relax to isoproterenol (171). There does not appear to be any basal release of nitric oxide from nerve terminals since L-NAME or oxyhemoglobin do not affect the amplitude of constriction to histamine (171).

Bioassay experiments have shown that canine ileocolonic sphinctor (donor tissue) is able to release a substance upon nerve stimulation that relaxes rabbit aorta denuded of endothelium and has biologic characteristics similar to nitric oxide (11). The release of the substance can be reduced by tetrodotoxin or inhibition of nitric oxide synthesis (11). Furthermore, the biologic activity of the transferable substance can be enhanced in the presence of superoxide dismutase and eliminated by hemoglobin (11). Exogenous nitric oxide elicited a relaxation of the rabbit aorta similar to that elicited by the transferable substance released from the ileocolonic sphincter upon electric field stimulation (11). ATP and VIP also relaxed the detector tissue, however the relaxation produced by ATP or VIP did not resemble the relaxation produced by electric field stimulation (11). This study strongly suggests that nitric oxide may mediate the NANC relaxation in canine ileocolonic smooth muscle.

The second messenger involved in the relaxation of intestinal smooth muscle elicited by nitric oxide or electric field stimulation has not been thoroughly investigated. Although, it appears that soluble guanylate cyclase is the second messenger. Cyclic GMP content within opossum LES

temporally increases in a frequency-dependent manner in response to field stimulation, whereas cyclic AMP content is not affected (183). Furthermore, the increased level of cyclic GMP precedes the relaxation produced by field stimulation or exogenous nitric oxide (17,140), and tetrodotoxin eliminates both the relaxation and accumulation of cyclic GMP is response to field stimulation (183). Methylene blue or cystamine, both of which inhibit soluble guanylate cyclase (126,190,191), attenuate the relaxation to field stimulation or nitroprusside (10). Moreover, 8-bromo-cyclic GMP and dibutyrl cyclic GMP, analogues of cyclic GMP, and MB 22948, a selective inhibitor of the cyclic GMP phosphodiesterase, produce a dose-dependent relaxation of opossum LES (9,17,140). These studies strongly suggest that the activation of guanylate cyclase with the smooth muscle mediates the nitric oxide-induced relaxation of opossum LES. However, whether cyclic GMP is the second messenger of visceral smooth muscle relaxation produced by nitric oxide in other parts of the gastrointestinal tract remains to be elucidated.

Electrophysiologic studies in canine proximal colon suggest that nitric oxide is responsible for the hyperpolarization in response to electric field stimulation. L-NAME causes a progressive decrease in the inhibitory junction potential (IJP) associated with the mechanical event of NANC relaxation (30). The inhibitory effect of L-NAME on the IJPs produced by field stimulation is reversed by excess L-arginine, but not D-arginine (30). In addition, exogenous nitric oxide and the nitric oxide carrier S-nitrosocysteine mimic the hyperpolarization of canine proximal colon produced by field stimulation (179). Oxyhemoglobin blocked both the IJPs produced by field stimulation and the hyperpolarization of the colonic smooth muscle in response to nitric oxide or S-nitrosocysteine (179). Also, incubation of colonic smooth muscle with 10 uM methylene blue results in smooth muscle depolarization and enhances the tonic contracture of the circular smooth muscle of the proximal colon (163).

# OBJECTIVES

As I have described in the literature review, there is sufficient evidence to suggest that nitric oxide plays an important role in the regulation of intestinal blood flow and motility, and may mediate the vasodilatory response to endothelium-dependent agents such as ACh, bradykinin and substance P. However, a comprehensive study investigating the physiologic role of nitric oxide in the small intestine has not been done. My central hypothesis is that endogenous nitric oxide does regulate intestinal blood flow and motility in anesthetized dogs. In addition, I predict that nitric oxide mediates the relaxation of isolated mesenteric arterial rings elicited by ACh, substance P, and bradykinin in vitro and that nitric oxide mediates the vasodilatory response of the mesenteric vascular bed produced by substance P in vivo. Lastly, I postulate that nitric oxide is a mediator of the reactive hyperemic response to brief periods of vascular occlusion, and that nitric oxide is a mediator of the active hyperemic response to placement of digested food into the lumen of an isolated jejunal segment.

Experiments were designed to test the following hypotheses:

**#1:** Nitric oxide mediates the relaxation of the superior mesenteric artery produced by exogenous administration of acetylcholine, substance P, or bradykinin in vitro. Hence, removal of the endothelium or incubation of superior mesenteric arterial rings with methylene blue, a specific inhibitor of soluble guanylate cyclase, or $N^G$-nitro-L-arginine methylester (L-NAME), a specific inhibitor of nitric oxide synthesis, will inhibit the relaxation produced by these humoral agents.

**#2:** Nitric oxide mediates the vasodilation produced by arterial infusion of substance P into the isolated jejunal segment and systemic administration of L-NAME inhibits the substance P-induced vasodilation.

**#3:** Nitric oxide regulates resting jejunal blood flow in vivo. Therefore, arterial infusion of nitroglycerin, which relaxes vascular smooth muscle through the spontaneous release of nitric oxide, or L-arginine, the precursor to nitric oxide synthesis, enhance resting jejunal blood flow. In contrast, systemic administration or local arterial infusion of the L-arginine analogue L-NAME, which inhibits nitric oxide synthesis, decreases resting jejunal blood flow.

**#4:** Endogenous nitric oxide functions to supress intestinal motility through a soluble guanylate cyclase-dependent mechanism. Thus, the administration of L-NAME or methylene blue enhance intestinal motility in vivo. The motility produced by inhibition of endogenous nitric oxide synthesis inhibition can be reversed by L-arginine or nitroglycerin.

**#5:** The motility produced after inhibition of nitric oxide synthesis is mediated by cholinergic nerves. As a result, the motility can be abolished using the muscarinic antagonist atropine.

**#6:** Nitric oxide is the mediator responsible for the reactive hyperemic response to brief periods of vascular occlusion and is the mediator of the active hyperemia during luminal placement of digested food into the jejunum. Therefore, inhibition of nitric oxide-mediated vasodilation will attenuate the hyperemic response to these stimuli.

## The Role Of Nitric Oxide In The Relaxation Of Superior Mesenteric Artery Produced By Acetylcholine, Substance P, and Bradykinin In Vitro

### Introduction

Intestinal blood flow increases after ingestion of a meal (21,25) or when digested food is placed into the lumen of an isolated segment of canine jejunum in vivo (24,26). Several endogenous vasodilating agents, including substance P (149), bradykinin (170), and adenosine (165) have been proposed to function as a mediator of the food-induced increase in intestinal blood flow and oxygen consumption. For example, using a canine ileal preparation, Preman et al (149) demonstrated that arterial infusion of substance P produced a significant dilation within the canine ileum and may play a role in the postprandial intestinal hyperemia observed in this tissue. Other studies have implicated bradykinin in the functional hyperemia associated with nutrient absorption (75,170), because local arterial infusion of bradykinin significantly increases intestinal blood flow (22,75). Furthermore, bradykinin levels are elevated in the venous portal blood of conscious dogs after intraduodenal instillation of hypertonic glucose solutions (170). Similarly, arterial infusion of adenosine also produces vasodilation in the canine jejunum and ileum (167) and the plasma adenosine concentration in the venous effluent draining an isolated jejunal segment is increased after luminal placement of digested food (166). These studies suggest that substance P, bradykinin, and adenosine may regulate the functional hyperemia in canine small intestine associated with nutrient absorption.

It is now known that ACh, substance P and bradykinin relax arterial smooth muscle from various organ vascular beds through an endothelium-dependent mechanism (50,52,53). Nitric oxide, which is synthesized from L-arginine (141), has been identified as one of the endothelium-derived relaxing factor responsible for the relaxation of vascular smooth muscle produced by

acetylcholine, substance P, and bradykinin in several mammalian species (53,142,156). $N^G$-monomethyl-L-arginine (L-NMMA) and $N^G$-nitro-L-arginine methylester (L-NAME), analogues of L-arginine that inhibits nitric oxide synthesis (94,156), attenuate the endothelium-dependent relaxation produced by acetylcholine, substance P, and bradykinin in the rabbit aorta (155,157).

In the canine superior mesenteric artery, the relaxation produced by ACh, substance P, or bradykinin is abolished by removal of the endothelium (19,203,204). The inability of indomethacin to inhibit the relaxation of mesenteric arteries produced by these vasodilating agents suggests that factors other than prostanoids are released from the endothelium and account for the relaxation (19,204). Methylene blue, which inhibits guanylate cyclase (126), and oxyhemoglobin, which inactivates nitric oxide extracellularly (125,126), attenuate the relaxation of canine mesenteric arteries produced by ACh or exogenous nitric oxide to the same degree (107). Recently, L-NMMA and L-NAME were shown to attenuate the relaxation produced by ACh in the mesenteric artery of guinea pigs (87) and the isolated rat perfused mesenteric vascular bed (133). These studies suggest that nitric oxide may mediate the canine superior mesenteric artery relaxation produced by ACh, and possibly substance P and bradykinin in vitro.

However, there is a substantial degree of heterogeneity in endothelium-dependent responses depending on the endothelium-dependent vasodilator agent used (12,19,93), the animal species being studied (19), the type of vessel from a given species (35), and the agonist used to preconstrict the vascular smooth muscle prior to testing relaxant responses (12,19,23). For instance, substance P is a potent endothelium-dependent vasodilator of canine mesenteric, renal, coronary, and femoral artery (7,47). However, substance P does not relax bovine intrapulmonary arteries (76,93). Bradykinin-induced relaxation of vascular smooth muscle has been shown to be mediated by prostaglandins and/or nitric oxide, depending on the species and type of vessel under investigation. The relaxation of cat and rabbit mesenteric artery by bradykinin is independent of

the vascular endothelial cells and is completely blocked after inhibition of cyclooxygenase (19). Conversely, the relaxation of the canine and human superior mesenteric artery produced by bradykinin is strictly endothelium-dependent and not inhibited by cyclooxygenase inhibitors (19). Whereas in isolated rings of bovine intrapulmonary artery, bradykinin produces an endothelium-dependent relaxation that is partially inhibited by L-NMMA or indomethacin and is completely blocked by their combination (91). There is also heterogeneity in the vascular responses produced by adenosine. Adenosine has been shown to relax porcine, rat, and guinea pig aorta through a mechanism that is partially endothelium-dependent (34,65,77), whereas adenosine relaxes canine arteries by a mechanism that independent of the endothelium (35,50). These studies suggest that the endothelial-derived relaxing factor(s) released by different vasodilating agonists may differ from each other. Because the effect of nitric oxide synthesis inhibition on the relaxation of canine mesenteric arteries has not been investigated, the mechanism by which ACh, substance P, bradykinin, or adenosine relax canine mesenteric artery is not clear, the present study was designed to clarify the mechanism by which ACh, substance P, bradykinin, and adenosine relax the canine mesenteric artery in vivo. The primary objective of this study was to determine if nitric oxide mediates the relaxation elicited by acetylcholine, substance P, or bradykinin in canine mesenteric artery in vitro. To determine this we evaluated the effect of endothelial cell removal, methylene blue (a soluble guanylate cyclase inhibitor), L-NAME (a nitric oxide synthesis inhibitor), and meclofenamate (a cyclooxygenase inhibitor) on the relaxation of preconstricted canine superior mesenteric arterial rings produced by ACh, substance P, or bradykinin in vitro.

Methods

37 dogs of either sex were bled through a cannula in the femoral artery after being anesthetized with pentobarbitol sodium (30 mg/kg i.v.). Peripheral blood smears were examined for microfilariae and macroscopic inspection of the pulmonary artery and right ventricle for adult

worms was done to assure that the dogs were not infected with heartworms, since heartworms have been shown to impair endothelium-dependent relaxation in arteries (97). No dog was found to be infected with heartworms using these criteria. The superior mesenteric artery (SMA) was excised and placed in a modified Kreb's-Ringer bicarbonate solution, the composition (in mM) is: 118.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 $KH_2PO_4$, 25.0 $NaHCO_3$, 0.026 calcium disodium EDTA, and 11.1 glucose, 0.8 sodium pyruvate. The blood vessels were cleaned of connective tissue and cut into 4 rings (3-4 mm wide) with special care not to damage the intimal surface. The rings were taken from the root of the SMA proximal to the first jejunal artery branch. In some experiments, the endothelium was removed from SMA rings by inserting a cotton tipped applicator into the lumen and gently rolling it back and forth for 90 seconds. This method has been previously shown to effectively remove endothelial cells from the intimal surface of vascular smooth muscle (19). The rings were then suspended in separate organ chambers by surgical silk to allow recordings of isometric contraction. Tension was continuously measured by force transducers (model FT 10C, Grass Company, Quincy, Mass.) connected to a Grass polygraph (model 7D). The organ chambers were filled with 30 ml of modified Kreb's-Ringer solution maintained at 37° C and continuously aerated with a 95% $O_2$-5% $CO_2$ gas mixture.

To determine the optimum length for active contraction, the rings were gradually stretched in a stepwise manner and contracted with 0.6 uM norepinephrine (NE). The passive tension necessary to achieve optimal length for active isometric contraction of mesenteric arterial rings ranged from 11.6 to 21.7 grams (mean $\pm$ SE, 18.3 $\pm$ 0.3 grams). The rings were then repetitively washed and allowed to equilibrate for a minimum of 90 minutes. The rings were preconstricted to 60-70% of the maximal active isometric contraction using 0.6 uM NE and thereafter relaxed with ACh (1 uM), substance P (20 nM), bradykinin (10 uM), nitroglycerin (0.2 uM), or adenosine (0.1 mM). Control responses for each vasodilating agent were obtained prior to the removal of

the endothelium, or before incubation with methylene blue, L-NAME, or mefenamic acid. Removal of the endothelium was confirmed by the lack of relaxation to 1 uM ACh. To determine the effect of guanylate cyclase inhibition, NO synthesis inhibition, and cyclooxygenase inhibition on the relaxation responses, arterial rings with intact endothelium were incubated with either 10 uM methylene blue, 30 uM $N^G$-nitro-L-arginine methylester (L-NAME), or 10 uM meclofenamate, respectively. In the arterial rings that were incubated with L-NAME (30 uM) for 20 minutes, either 300 uM L-arginine or 300 uM D-arginine was added to the organ chamber and allowed to incubate with the preconstricted arterial ring for another 20 minutes. No more than one vasodilating agents was added to the preconstricted arterial ring at any time. Appropriate time controls for the vasodilating agents were performed, where at least one arterial ring with intact endothelium was not exposed to MB or L-NAME in each experiment. The volume of solution containing any chemical added into the 30 ml organ chambers never exceeded a total of 60 uL.

**Preparation of drugs** Methylene Blue, $N^G$-nitro-L-arginine methylester (L-NAME), (L)- and (D)- arginine hydrochloride, acetylcholine, substance P, bradykinin, adenosine, ascorbic acid, mefenamic acid, and sodium carbonate were obtained from Sigma Chemical Co. (St. Louis, Mo.). Nitroglycerin (Nitrostat, 0.4mg tablets, Parke-Davis, Morris Plains, NJ.) and norepinephrine bitartrate (Levophed, Winthrop) were purchased from a local apothecary. All drugs were prepared the day of the experiment. Stock solutions of acetylcholine, substance P, and bradykinin were dissolved in distilled water then stored at -20° C. The appropriate concentrations were then serially diluted into Kreb's-Ringer solution the day of the experiment. Mefenamic acid (0.24 g) and adenosine (0.27 g) were separately dissolved into 100 ml of distilled water in the presence of sodium carbonate (0.96 g) to yeild stock solutions of 10 mM and 0.1M, respectively. The vehicle for all other agents used was modified Kreb's-Ringer solution, except for norepinephrine, which was diluted into 0.1 % ascorbate in distilled water.

**Statistics** Relaxation or contraction of the SMA rings was measured as the decrease or increase, respectively, in tension below or above the active tension elicited by 60-70% maximal contraction of norepinephrine (0.6 uM). The relaxation produced by acetylcholine, substance P, bradykinin, nitroglycerin, and adenosine were expressed as the percent relaxation of the active tension produced by 0.6 uM norepinephrine. Values in figures are expressed as mean $\pm$ SE, and represent paired or unpaired data. In each series of experiments, n indicates the number of dogs from which the SMA was obtained. Stastistical significance for paired observations was determined using a paired Student's T test. Where multiple comparisons with a common control were made, the difference between the mean of each experiment was evaluated by analysis of variance. If the analysis of variance showed a significant difference among the means, then statistical differences between individual groups were determined using the least significant difference test. Statistical significance was assessed at the 95% confidence level.

## Results

Figure 1 is a representative tracing illustrating the relaxant effect of ACh, substance P, bradykinin, and nitroglycerin on 4 mesenteric arterial rings preconstricted with NE prior to and after mechanical removal of the vascular endothelial cells. Removal of the endothelial cells abolished the relaxation produced by ACh, substance P, or bradykinin. In contrast, removal of the endothelial cells did not alter the relaxation produced by nitroglycerin. The effect of endothelial cell removal on the percent relaxation of preconstricted mesenteric arterial rings produced by ACh, substance P, bradykinin, nitroglycerin, or adenosine are summarized in Figure 2. Removal of the endothelial cells significantly inhibited the relaxation produced by ACh, substance P, and bradykinin. In contrast, nitroglycerin and adenosine relaxations were not inhibited by removal of the endothelial cells.

Figure 3 is a representative tracing illustrating the effect of 10 uM methylene blue on the relaxation produced by ACh, bradykinin, substance P, nitroglycerin, and adenosine. Prior to incubation with methylene blue, ACh, substance P, bradykinin, nitroglycerin, and adenosine effectively relaxed the preconstricted mesenteric arteries. Methylene blue produced a marked increase in the resting active tension produced by norepinephrine. In addition, the relaxation produced by ACh, bradykinin, substance P, and nitroglycerin was markedly attenuated after incubation of the mesenteric arterial rings with methylene blue for 30 minutes. The decrease in tension elicited by adenosine was not affected by methylene blue. The effect of methylene blue on the percent relaxation of preconstricted mesenteric arterial rings produced by ACh, substance P, bradykinin, nitroglycerin, and adenosine are summarized in Figure 4. The percent relaxation of mesenteric arterial rings elicited by ACh, substance P, bradykinin, and nitroglycerin was significantly inhibited in the presence of methylene blue, whereas the percent relaxation produced by adenosine was not affected.

Figure 5 summarizes the effect of 30 uM L-NAME, followed by incubation of the SMA rings with 300 uM L-arginine or D-arginine on the percent relaxation of preconstricted mesenteric arterial rings produced by ACh, substance P, bradykinin, and nitroglycerin. Incubation of the rings with L-NAME significantly inhibited the relaxation produced by ACh, substance P, and bradykinin. The inhibitory effect of L-NAME was reversed after incubation of the rings with L-arginine, but not with D-arginine. The percent relaxation produced by nitroglycerin was not affected by incubation of the SMA rings L-NAME, L-arginine, or D-arginine.

To determine whether the relaxation produced by nitric oxide could be enhanced in the presence of excess substrate for nitric oxide synthesis, the effect of 300 uM L-arginine or D-arginine on the active tension produced by norepinephrine and the relaxation of superior mesenteric artery produced by ACh or nitroglycerin was evaluated using 4 adjacent mesenteric

arterial rings from two separate dogs (data not shown). Incubation of the mesenteric artery for 15 minutes with L-arginine or D-arginine did not significantly alter the active tension produced by norepinephrine or the relaxation produced by ACh or nitroglycerin. Prior to incubation with (L)- or (D)- arginine, ACh relaxed the mesenteric arterial rings by 68±3%. The relaxation produced by ACh after incubation with 300 uM L-arginine or D-arginine was 67±4% and 62±5%, respectively. Nitroglycerin relaxed the preconstricted mesenteric artery by 75±2% during control, by 83±3% after incubation with L-arginine, and by 79±3% after incubation with D-arginine. Therefore, L-arginine or D-arginine did not significantly enhance the relaxation produced by ACh or nitroglycerin.

Figure 6 is an experimental recording demonstrating the effect of cyclooxygenase inhibition using 10 uM meclofenamate on the relaxation of preconstricted mesenteric arterial rings produced by ACh, substance P, bradykinin, or nitroglycerin in one dog. Like methylene blue, meclofenamate markedly enhanced the resting active tension produced by norepinephrine. However, the decrease in tension produced by ACh, substance P, bradykinin, or nitroglycerin was not inhibited by incubation of the mesenteric arterial rings with meclofenamte. The percent relaxation of preconstricted mesenteric arterial rings elicited by ACh, substance P, bradykinin, nitroglycerin, and adenosine before and in the presence of meclofenamate is summarized in Figure 7. The relaxation produced by ACh, bradykinin, nitroglycerin and adenosine were not inhibited 30 minutes after exposure of the rings with meclofenamate. In contrast, the relaxation produced by substance P was significantly decreased after incubation of the rings with meclofenamate. Substance P relaxed the mesenteric arterial rings by 82±2% before incubation with meclofenamate, and by 62±3% in the presence of meclofenamate, resulting in a slight, yet stastistically significant inhibition of 19±3%.

The absolute decrease in tension of the preconstricted arterial rings produced by ACh,

bradykinin, nitroglycerin, or adenosine was significantly increased after 30 minutes of incubation with meclofenamate. Prior to incubation with meclofenamate, ACh, bradykinin, nitroglycerin, and adenosine decreased the active tension by 8.9±0.6 grams, 9.4±0.7 grams, 8.5±1.1 grams, and 3.6±0.3 grams, respectively. After incubation with meclofenamate, ACh, bradykinin, nitroglycerin, and adenosine decreased the active tension by 10.6±0.4 grams, 10.1±0.8 grams, 10.2±0.9 grams, and 6.4±0.7 grams, respectively. In contrast, the absolute decrease in tension produced by substance P was not significantly greater after incubation of the arterial rings with meclofenamate. The decrease in tension produced by substance P before meclofenamate was 9.0±0.7 grams compared to 9.3±0.6 grams after incubation with meclofenamate.

Figure 8 summarizes the effect of endothelial cell removal, or incubation of mesenteric arterial rings with L-NAME, MB, or meclofenamate on the active isometric tension produced by a norepinephrine (0.6 uM). Removal of the endothelial cells or incubation of the arterial rings with L-NAME did not significantly enhance the active isometric constriction produced by norepinephrine. In contrast, methylene blue significantly enhanced the active isometric constriction produced by norepinephrine by 33±5%. The enhanced constrictive effect produced by methylene blue was rapid in onset and maximum about 5-10 minutes after exposure of the arterial rings to methylene blue (Figure 3). Similarly, meclofenamate significantly enhanced the active isometric constriction produced by norepinephrine by 27±3% (n=20). The active tension produced by norepinephrine following washout of methylene blue or meclofenamate remained greater than control and was similar to that achieved in the presence of either methylene blue or meclofenamate. Neither methylene blue or meclofenamate affected the baseline tension of the arterial rings after washout of the chemicals from the organ chamber. The effect of endothelial cell removal on the ability of methylene blue or meclofenamate to enhance the active isometric constriction of mesenteric arterial rings was also determined using 4 separate dogs (data not

shown). Methylene blue enhanced the norepinephrine-induced constriction of arterial rings without endothelial cells by 49±7% (n=4), and meclofenamate increased the NE constriction of arterial rings without endothelium by 38±9% (n=4). The enhanced constriction of the preconstricted arterial rings without endothelium produced by methylene blue or meclofenamate was slightly greater than that observed in arterial rings where the endothelium was intact, although the difference was not statistically significant.

## Discussion

The primary objective of our study was to determine if endothelial-derived nitric oxide mediates the relaxation of canine superior mesenteric arterial rings produced by ACh, substance P, bradykinin, adenosine, or nitroglycerin in vitro. Our data demonstrate that mechanical removal of the endothelial cells abolished the relaxation produced by ACh, substance P, and bradykinin but did not inhibit the relaxtion elicited by adenosine or nitroglycerin (Figures 1 and 2). The lack of relaxation produced by ACh, substance P, and bradykinin after mechanical rubbing of the intimal surface cannot be attributed to damage of the smooth muscle cells since the constriction produced by NE and the relaxation produced by nitroglycerin or adenosine were not affected. Methylene blue significantly inhibited the relaxation produced by ACh, substance P, and bradykinin (Figures 3 and 4). Likewise, incubation of the mesenteric arterial rings with 30 uM L-NAME, a specific inhibitor of nitric oxide synthesis (94,156), significantly inhibited the relaxation elicited by ACh, substance P, or bradykinin (Figure 5). The inhibitory effect of L-NAME on the relaxation produced by ACh, substance P, and bradykinin was reversed with excess L-arginine. However, D-arginine, which is not a substrate for nitric oxide synthesis, did not reverse the effect of L-NAME. In contrast to the endothelium-dependent relaxation produced by ACh, substance P, and bradykinin, the endothelium-independent relaxation produced by

nitroglycerin was not affected by L-NAME, L-arginine, or D-arginine. Therefore, nitric oxide mediates the endothelium-dependent relaxation produced by ACh, substance P, and bradykinin in the canine mesenteric artery in vitro.

Like the relaxation produced by nitric oxide, organic nitrates such as nitroglycerin also relax vascular smooth muscle by enhancing soluble guanylate cyclase activity (1,126). The relaxation produced by ACh, substance P, bradykinin, nitroglycerin, and purified solutions of nitric oxide are associated with an increase of cyclic GMP within the vascular smooth muscle (51,126). This data is consistent with the proposal that the accumulation of cyclic GMP within smooth muscle is responsible for the relaxation produced by ACh, substance P, bradykinin, and nitroglycerin in the canine mesenteric artery. Methylene blue, an inhibitor of soluble guanylate cyclase activity (126), significantly attenuates the endothelium-dependent relaxation produced by ACh, substance P, and bradykinin, and the endothelium-independent relaxation produced by nitroglycerin (Figures 3 and 4). In contrast, methylene blue did not affect the relaxation produced by adenosine. Other investigators have reported similar results. Using a microscopic technique to visually measure changes in arteriolar diameter, Falcone and Bohlen (38) demonstrated that methylene blue selectively inhibits the dilation of rat mesenteric arterioles produced by ACh but does not inhibit the dilation produced by adenosine. Similarly, Komori et al. (107) has shown that the relaxation produced by ACh and authentic nitric oxide in canine mesenteric arteries preconstricted with $PGF_{2\alpha}$ is inhibited by methylene blue or oxyhemoglobin.

Incubation of cultured porcine aortic endothelial cells (141) or bovine intrapulmonary artery with intact endothelial cells (62,64) with L-arginine does not potentiate the relaxation produced by ACh or bradykinin, increase the resting cyclic GMP concentration, or enhance the release of nitric oxide from the endothelial cells. However, incubation of endothelial cells for 24 hours in a media deficient of L-arginine leads to a depletion of L-arginine, and attenuates the

relaxation produced by ACh or bradykinin, a significant decrease in resting cyclic GMP concentration, and inhibits the release of nitric oxide (63,64,141). Under these conditions where L-arginine has been depleted from the endothelial cells, exogenous L-arginine will enhance the relaxation produced by ACh or bradykinin, increase the resting cyclic GMP concentration, and enhance the release of nitric oxide from cultured endothelial cells (63,64,141). Our finding that incubation of mesenteric arterial rings with L-arginine failed to potentiate the relaxation produced by ACh is consistent with the proposal that under normal conditions L-arginine is present within vascular endothelial cells in sufficient quantity to saturate nitric oxide synthase. Nevertheless, conflicting results regarding the effect of exogenously added L-arginine on the relaxation of rabbit thoracic aorta produced by ACh have been reported. Using a biocascade of rabbit aortic rings and chemiluminescence to detect nitric oxide release, Rees et al. (156) have reported that infusion of 100 uM L-arginine through the aortae did not significantly affect the release of nitric oxide induced by ACh. These investigators had previously demonstrated that incubation of rabbit aortic rings with 100 uM L-arginine produced a marginal, but significant potentiation of the relaxation and release of nitric oxide elicited by ACh (155). Other studies have reported that incubation of rabbit thoracic aorta with L-arginine slightly enhanced the relaxation produced by ACh (133). However, the effect of L-arginine was inconsistent and did not occur in each aortic ring (133). The reason for the potentiation of the relaxation of rabbit thoracic aorta produced by ACh after incubation with L-arginine is not clear, but may reflect a depletion of L-arginine within the endothelial cells or a difference in methodology.

Incubation of the mesenteric rings with meclofenamate did not affect the relaxation elicited by ACh, bradykinin, nitroglycerin or adenosine (Figure 6 and 7). In contrast, the relaxation produced by substance P was decreased by approximately 20% after inhibition of cyclooxygenase with meclofenamate. The attenuation of the relaxation produced by substance P was not expected

since indomethacin and other cyclooxygenase inhibitors have been reported not to inhibit the relaxation produced by substance P in canine or guinea pig mesenteric arteries (12,204). The degree of endothelium-dependent relaxation of preconstricted arterial rings is dependent on the initial tension of the arterial preparation and is decreased at higher resting tension than at lower resting tension (53). Furthermore, when canine mesenteric arterial rings treated with cyclooxygenase inhibitors are preconstricted to a similar amount of tension by using a lower dose of phenylephrine, the relaxation produced by substance P is similar to that achieved prior to inhibition of cyclooxygenase (50,204). However, since the relaxation produced by ACh, bradykinin, nitroglycerin, or adenosine was not inhibited by meclofenamate in this study, inhibition of the relaxation produced by substance P cannot be attributed to an increase in tension produced by meclofenamate. Our study does not exclude the possibility that substance P stimulates the release of some prostanoid from the endothelial cells of the mesenteric artery. Although our results suggest that prostanoids may mediate part of the endothelium-dependent relaxation of the mesenteric artery produced by substance P, the portion of the substance P-induced relaxation dependent on prostanoid release is considerably less than the portion mediated by nitric oxide.

Unlike the relaxation produced by ACh, substance P, or bradykinin, a large body of evidence indicates that the endothelium plays no role in the adenosine-mediated relaxation of canine or rat arterial smooth muscle from various organ beds (34,39,105,106). Furthermore, methylene blue does not inhibit the relaxation produced by adenosine in arterioles of the skeletal and mesenteric vascular beds, suggesting that the relaxation produced by adenosine is not a result of soluble guanylate cyclase activation (f-1). Conversely, the relaxation of porcine, rat and guinea pig aorta produced by exogenous adenosine is partially dependent on the endothelium (65,77,200). In the guinea pig aorta, the endothelium-dependent response to adenosine appears unrelated to

nitric oxide or prostanoid release since oxyhemoglobin and indomethacin do not alter the endothelium-dependent response to adenosine (77). In this study, we demonstrate that relaxation of canine mesenteric artery produced by adenosine is not mediated by the endothelium. In addition, the relaxation produced by adenosine is not altered in the presence of meclofenamate, an inhibitor of prostanoid synthesis, or methylene blue, a soluble guanylate cyclase inhibitor. Thus, the relaxation of canine superior mesenteric artery produced by adenosine is endothelium-independent and not mediated by enhanced prostanoid synthesis or increased levels of cyclic GMP within the arterial smooth muscle.

At the concentrations used in this study, L-NAME or methylene blue did not completely inhibit the relaxation produced by ACh or bradykinin, whereas the relaxation produced by substance P was essentially abolished after incubation with L-NAME or methylene blue (Figures 4 and 5). Although we did not investigate the effect of L-NAME or methylene blue at higher concentrations, other studies have shown that higher concentrations of L-NAME failed to completely inhibit the relaxation produced by ACh in rabbit aorta and femoral arteries or when ACh was arterially infusion into an isolated rat mesenteric vascular bed (133,134,155). These results suggest that ACh and bradykinin may release a factor from endothelial cells other than nitric oxide. There is evidence suggesting that ACh and bradykinin may also stimulate the release of a hyperpolarizing factor distinct from nitric oxide in endothelial cells. In canine mesenteric arteries, ACh produces a hyperpolarization of the mesenteric arterial smooth muscle that is endothelium-dependent, but is not inhibited by methylene blue or oxyhemoglobin, inhibitors of the relaxation produced by nitric oxide (107). Nitric oxide does not appear to account for the hyperpolarization since exogenous nitric oxide did not hyperpolarize the mesenteric artery (107). Likewise, the muscarinic agonist carbachol, but not substance P, produced an endothelium-dependent hyperpolarization of guinea pig mesenteric arteries that could not be blocked by

oxyhemoglobin (12). Utilizing a biocascade assay system of porcine aortic endothelial cells and strips of canine coronary artery denuded of endothelium, Boulanger et al. (13) distinguished separate endothelium-dependent relaxing factors upon stimulation with bradykinin. Ouabain, which prevents the hyperpolarization of vascular smooth muscle produced by ACh (83), attenuates the endothelium-dependent relaxation produced by bradykinin (13). In some cases, the portion of the relaxation produced by bradykinin that remains after inhibition of nitric oxide synthesis can be blocked by indomethacin (91,94). Since meclofenamate did not inhibit the relaxation produced by ACh or bradykinin we can exclude a possible role for $PGI_2$ or $PGE_2$. Thus, bradykinin, like ACh, may stimulate the release of a hyperpolarizing factor from the endothelial cells of the canine mesenteric artery. Nonetheless, our study indicates that nitric oxide accounts for the majority of the relaxation of canine mesenteric arteries produced by ACh, substance P, and bradykinin in vitro.

Recent studies indicate that electric field stimulation (EFS) of preconstricted bovine and canine mesenteric arteries causes the release of a neurotransmitter that produces a transient relaxation of vascular smooth muscle (2,182). The tetrodotoxin sensitive relaxation produced by EFS in bovine mesenteric arteries is associated with an increase in cyclic GMP, but not cyclic AMP (2). Methylene blue and LY 83583, inhibitors of soluble guanylate cyclase, and Zaprinast, a selective inhibitor of cyclic GMP phosphodiesterase, potentiate the relaxation of bovine mesenteric artery produced by electric field stimulation (2). Incubation of canine mesenteric arteries with L-NMMA attenuated the relaxation produced by electric field stimulation (182). The relaxation produced by electric field stimulation is independent of the endothelial cells (2). Based on our results, ACh and substance P do not appear to be the neurotransmitter released upon electric field stimulation because the relaxation produced by these agents is an endothelium-dependent phenomemon. It is possible that nitric oxide or a nitroso compound that releases nitric

oxide acts as the neurotransmitter released from perivascular nerve terminal in response to electric field stimulation. Nitric oxide has been proposed as neurotransmitter synthesizes within the cell bodies of myenteric nerve cells (14) and is thought to mediate the nonadrenergic-noncholinergic relaxation of intestinal smooth muscle produced by electric field stimulation (11,16,30,32,181).

Inhibition of nitric oxide synthesis with L-NMMA or L-NAME has been previously reported to increase the resting tension of preconstricted rat aorta, rabbit thoracic aorta, and rabbit femoral artery (133,134,155). In addition, administration of L-NMMA or L-NAME to conscious rats produces a dose-dependent increase in systemic arterial blood pressure and mesenteric vascular resistance in vivo (57,59,60,133). These studies suggest that nitric oxide plays an important role in the regulation of the arterial blood pressure and mesenteric vascular tone in vivo. However, in this study we were unable to demonstrate a significant constrictor effect on the resting active tension of the mesenteric artery in response to the concentration of L-NAME used (Figure 8). Moreover, removal of the vascular endothelium failed to significantly enhance the isometric tension of mesenteric arterial rings produced by norepinephrine. Hence, endothelium-derived nitric oxide does not appear to significantly influence the active tension of canine superior mesenteric artery in vitro. The reason for this discrepancy is unclear and may reflect heterogeneity between species or differences between the type or size of vessel.

Our finding that methylene blue significantly increased the tension of the preconstricted mesenteric artery (Figures 3 and 8) suggests that nitric oxide may modulate the resting tension of canine mesenteric artery since nitric oxide is the only endogenous substance currently known to activate soluble guanylate cyclase. However, the enhanced norepinephrine constriction produced by methylene blue occurs in mesenteric arteries with and without endothelial cells, indicating that the effect of methylene blue on canine mesenteric arteries does not depend on endothelial cells. A more likely explanation for the increase in resting tension produced by methylene blue is due

to an enhanced release of norepinephrine from intramural nerves. Pretreatment of rabbits with reserpine prior to extraction of the aorta prevents the constrictive action of methylene blue in this vessel (126). Likewise, meclofenamate potentiated the active tension produced by norepinephrine by 27±3%, suggesting that prostanoids regulate the resting tension of vascular smooth muscle. The augmentation in resting tension produced by meclofenamate was observed in mesenteric rings with and without endothelial cells, indicating that the source of prostanoids that influence the resting tension of mesenteric arteries is probably from the smooth muscle. Other cyclooxygenase inhibitors, including indomethacin and fluorbiprofen have been shown to potentiate the resting tension of canine and human mesenteric arteries (19). The constrictive effect of cyclooxygenase inhibitors on mesenteric smooth muscle implies that prostanoid may play a role in regulating resting tone of the mesenteric bed in vivo. Indeed, Chou et al. (55) has shown that inhibition of prostanoid synthesis with indomethacin or meclofenamate produced a significant vasoconstriction in the canine jejunal vascular bed in vivo.

Recently, inhibition of nitric oxide synthesis was shown to selectively inhibit the vasodilation produced by arterial infusion of ACh in the rat isolated perfused mesenteric bed (133), suggesting that nitric oxide mediates the endothelium-dependent vasodilation produced by ACh in mesenteric resistance vessels. In this study, we demonstrate that endothelium-derived nitric oxide is responsible for the relaxation of preconstricted canine mesenteric arteries produced by ACh, substance P, or bradykinin in vitro. This suggests that nitric oxide may mediate the relaxation produced by ACh, substance P, or bradykinin in the mesenteric vascular bed in vivo. In contrast, the relaxation produced by adenosine occurs independent of nitric oxide since removal of the endothelium or incubation of the mesenteric arterial rings with methylene blue did not inhibit the relaxation. Substance P and bradykinin have been proposed to play a role in modulating the postprandial intestinal hyperemia (149,170). Our finding that nitric oxide mediates

the relaxation produced by these vasodilating agents would suggest that nitric oxide may be responsible for the hyperemic response observed after placement of digested food into the lumen of the small intestine. Therefore, the results of this study may be of significance in explaining the mechanism of vasodilation produced by ACh, substance P, and bradykinin as well as the acitve hyperemic response elicited by digested food in the small intestine.

Summary

Experiments were designed to determine whether nitric oxide or prostanoids mediate the endothelium-dependent relaxation of canine superior mesenteric artery produced by ACh, substance P, bradykinin, or adenosine. Removal of the endothelium abolished the relaxation produced by ACh, substance P, and bradykinin, but did not affect the relaxation produced by nitroglycerin or adenosine. Incubation or the arterial rings with methylene blue, an inhibitor of soluble guanylate cyclase (126,135,191), or the L-arginine analogue L-NAME, an inhibitor of nitric oxide synthesis (155,156), markedly attenuate the relaxation produced by ACh, substance P, and bradykinin. The inhibitory effect of L-NAME was reversed after incubation of the mesenteric arterial rings with 300 uM L-arginine, but not 300 uM D-arginine. The cyclooxygenase inhibitor meclofenamate did not effect the relaxation produced by ACh or bradykinin, whereas the substance P-induced relaxation was slightly attenuated after incubation of the arterial rings with meclofenamate. The relaxation produced by adenosine was not inhibited by removal of the endothelial cells, methylene blue, L-NAME, or meclofenamate. In conclusion, the relaxation produced by ACh or bradykinin is mediated by endothelium-derived nitric oxide. The relaxation produced by substance P was mediated primarily by nitric oxide. Prostanoids may also mediate a portion of the substance P-induced relaxation. In contrast, the relaxation produced by adenosine is endothelium-independent and is not mediated by nitric oxide or prostanoids. These results suggest that nitric oxide may mediate the vasodilatory action of ACh, substance P and bradykinin, but not adenosine, in the canine mesenteric vascular bed in vivo.

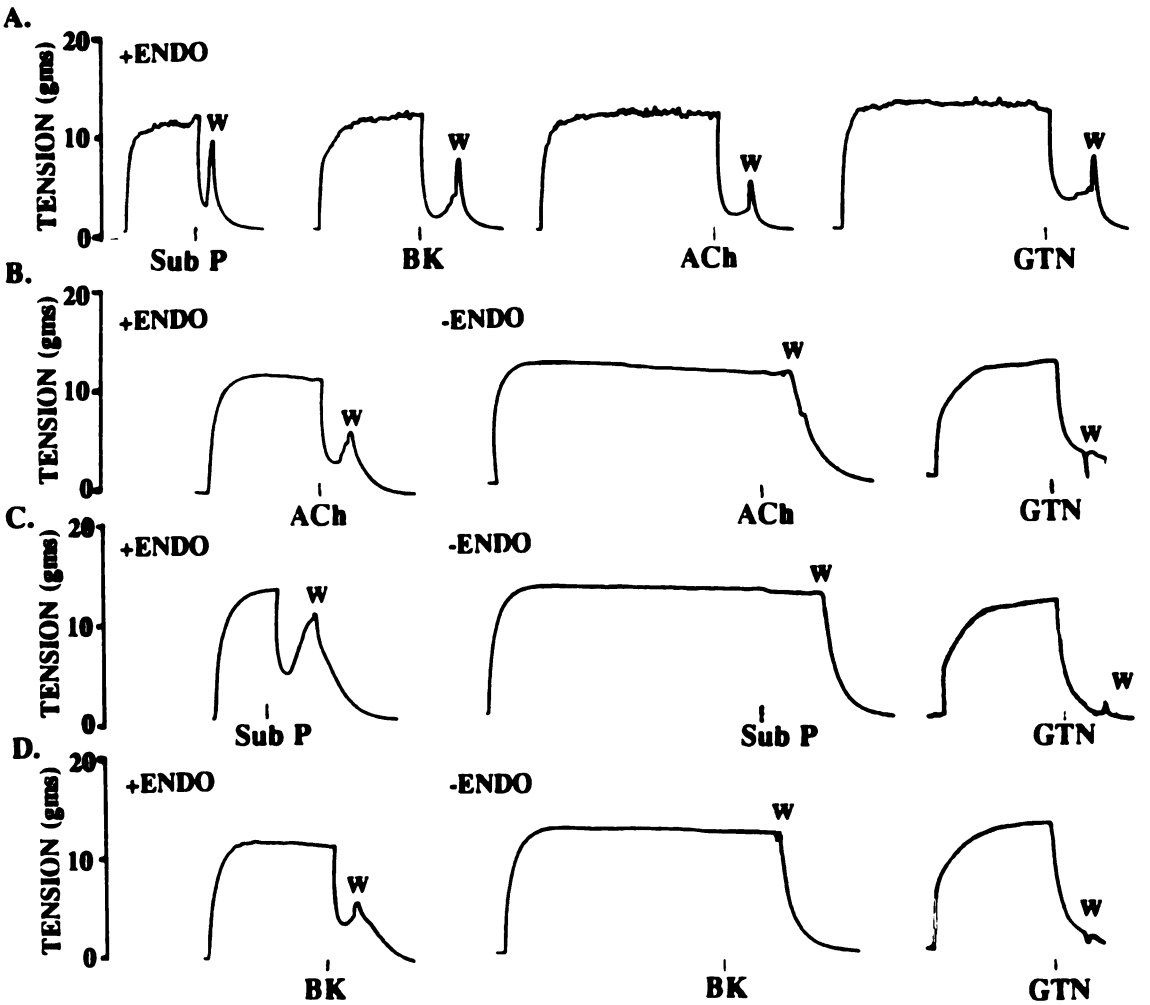



Figure 1. Experimental recording of the effect of endothelial cell removal on the relaxation produced by acetylcholine (ACh; 1 uM), substance P (sub P; 20 nM), bradykinin (BK; 10 uM) and nitroglycerin (GTN; 0.2 uM) in four adjacent canine mesenteric arterial rings in vitro. Each arterial ring was preconstricted with 0.6 uM norepinephrine. Removal of the endothelial cells abolished the relaxation produced by ACh, sub P, and BK, but did not effect the relaxation produced by GTN. W indicates washout of Kreb's media bathing the arterial rings.

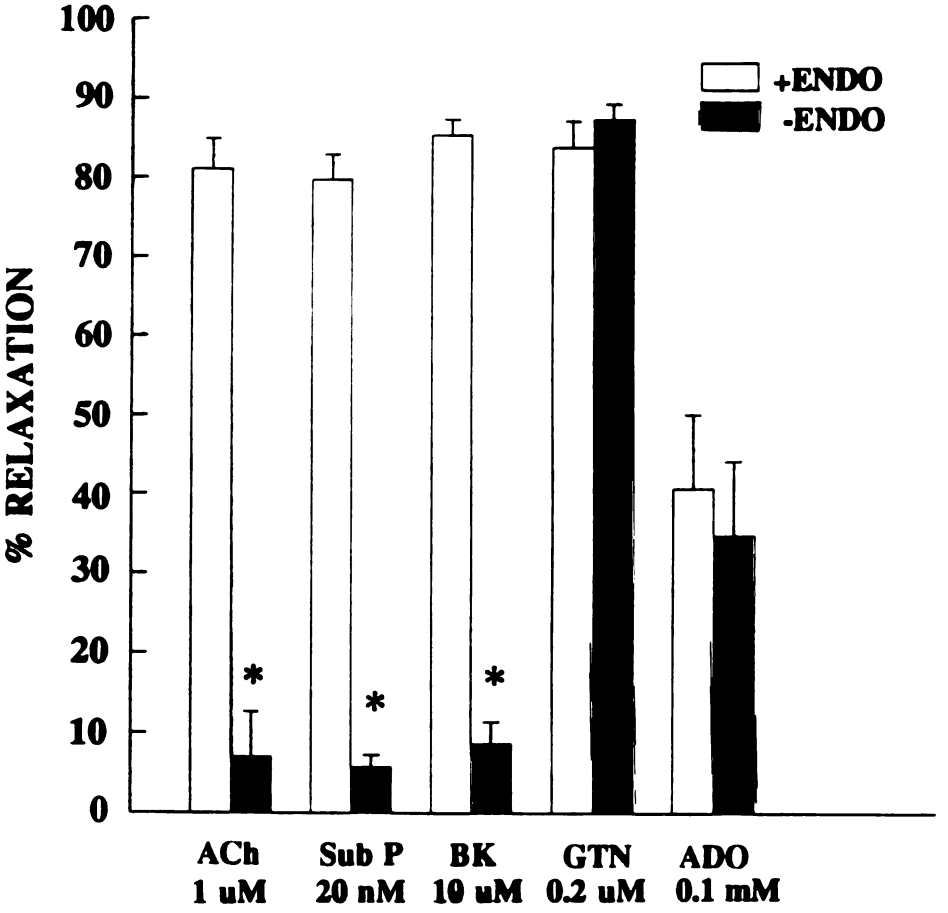



Figure 2. Effect of endothelial cell removal (-ENDO) on the relaxation of canine mesenteric arteries produced by acetylcholine (n=8), substance P (n=9), bradykinin (n=9), nitroglycerin (n=10), and adenosine (n=7) in vitro. Vascular rings were preconstricted with 0.6 uM norepinephrine (NE). Responses to each agent were tested prior to and after mechanical removal of the endothelial cells from the same ring. Values are means $\pm$ SE and expressed as percent relaxation from the active tension produced by NE. * indicates a significant difference from control responses (prior to endothelial cell removal). $P<0.05$.

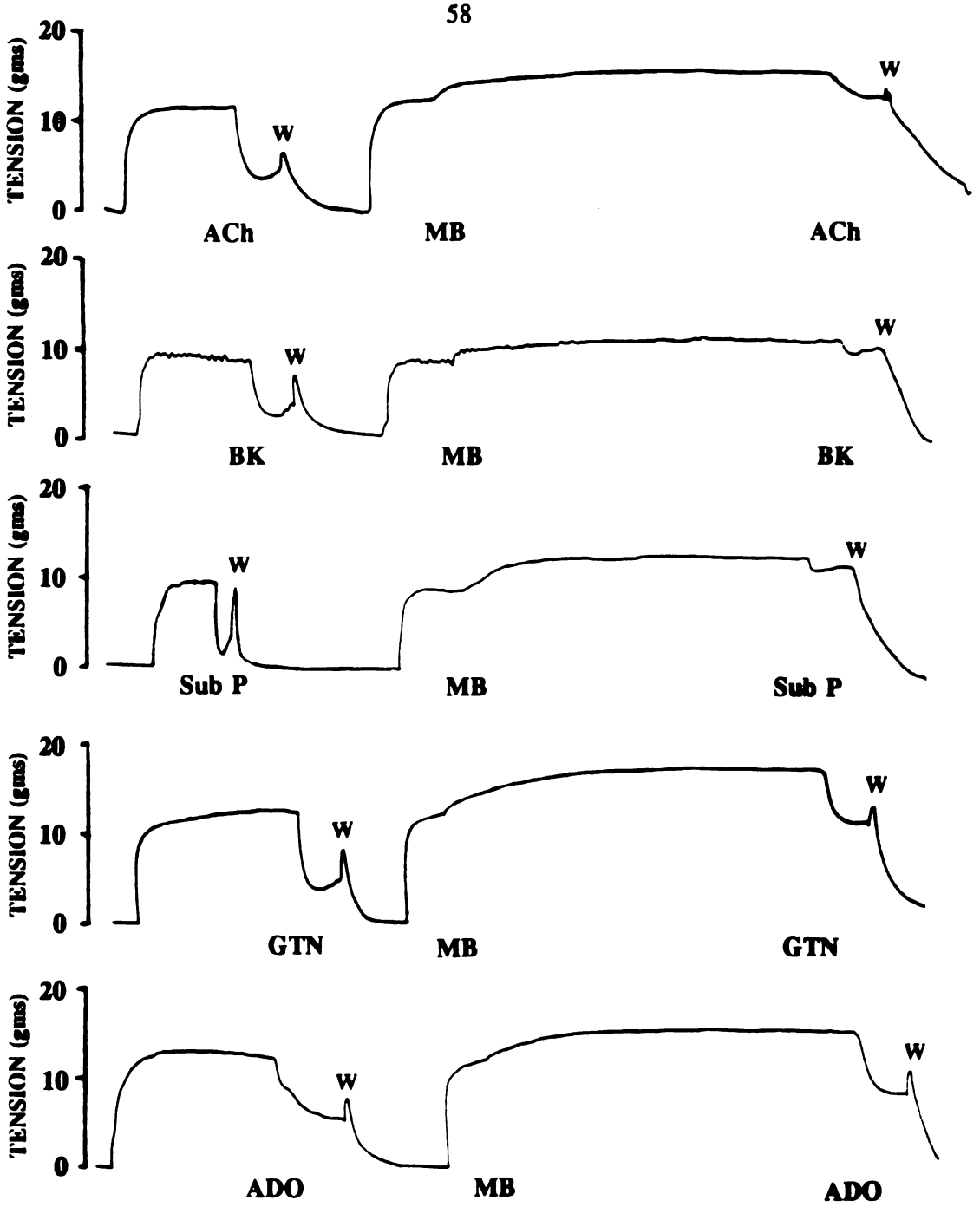



Figure 3. Experimental recording of the effect of 10 uM methylene blue (MB) on the relaxation produced by acetylcholine (ACh; 1 uM), substance P (sub P; 20 nM), bradykinin (BK; 10 uM), nitroglycerin (GTN; 20 uM), and adenosine (ADO; 0.1 mM) in five adjacent mesenteric arteries obtained from one dog in vitro. Each arterial ring was preconstricted with 0.6 uM norepinephrine (NE). MB enhanced the resting tension of each arterial ring and attenuated the relaxation produced by ACh, sub P, BK, and GTN, but did not effect the relaxation produced by ADO. W indicates washout of Kreb's media bathing the arterial rings.

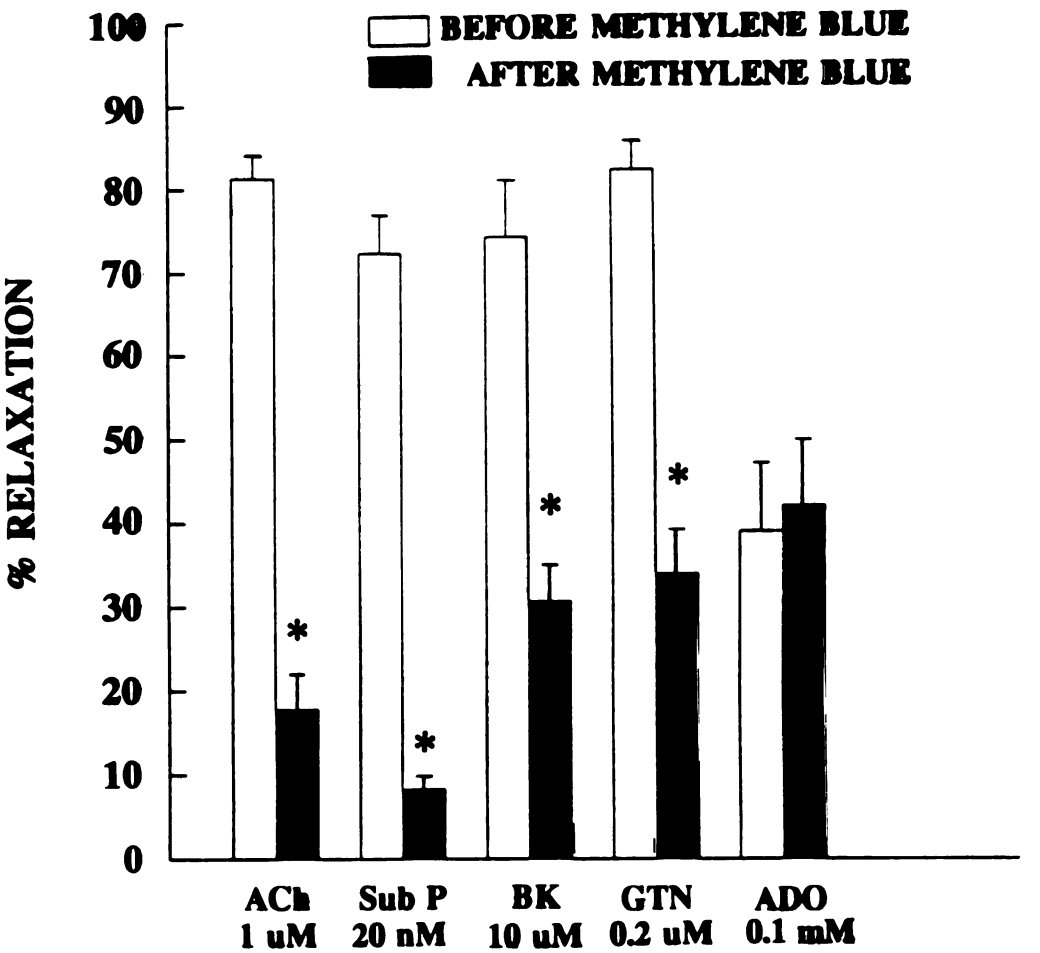



Figure 4. Effect of 10 uM methylene blue (MB) on the relaxation of canine mesenteric arteries produced by acetylcholine (n=10), substance P (n=6), bradykinin (n=9), nitroglycerin (n=9), and adenosine (n=6) in vitro. Vascular rings were preconstricted with 0.6 uM norepinephrine (NE). Responses to each agent were tested prior to (control) and 30 minutes after incubation with MB. Values are means $\pm$ SE and expressed as percent relaxation of the active tension produced by NE. * indicates a significant difference from control responses (prior to incubation with MB). $P<0.05$.

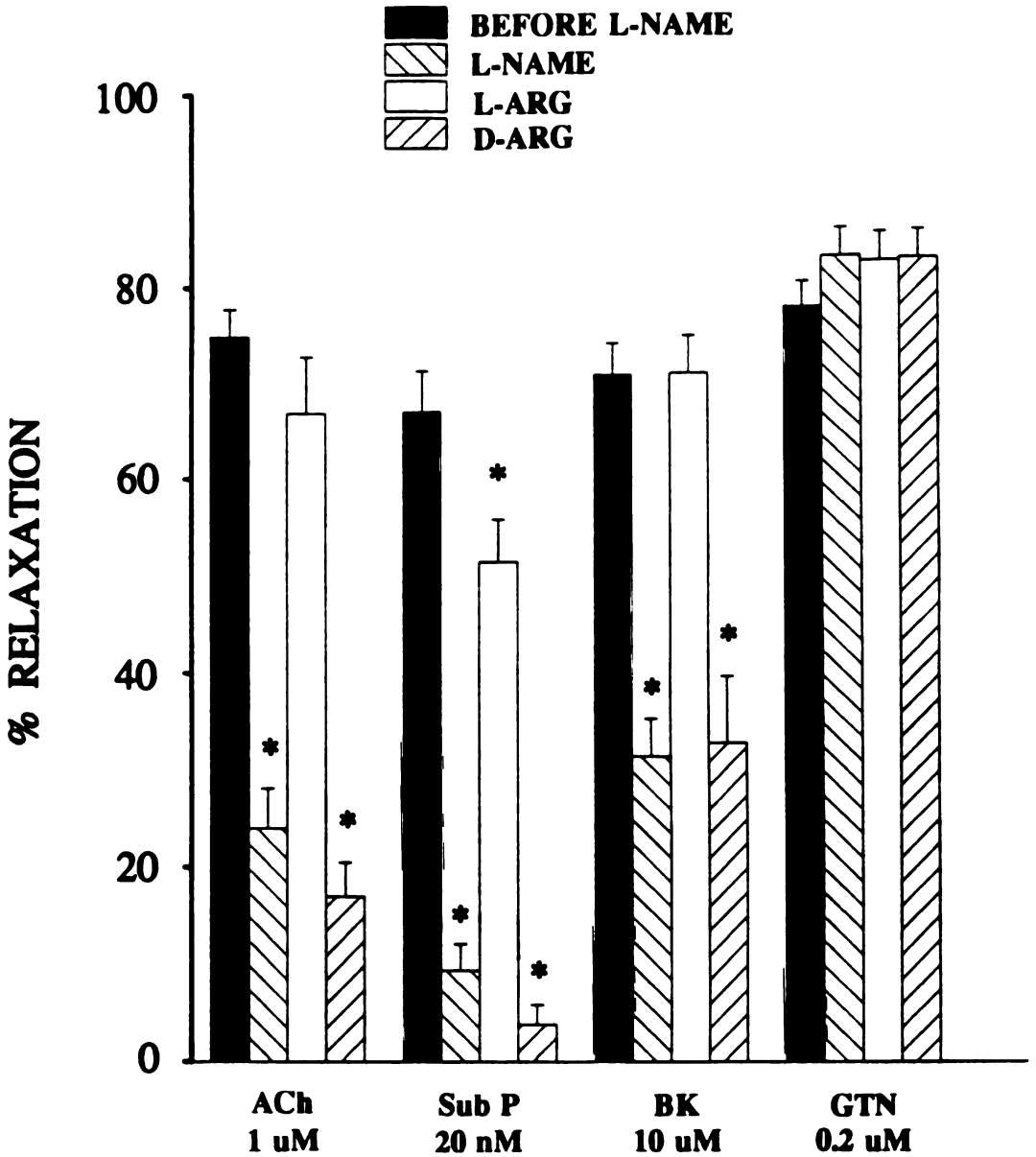



Figure 5. Effect of L-NAME, L-arginine, and D-arginine on the relaxation of canine mesenteric arteries produced by acetylcholine (n=7), substance P (n=5), bradykinin (n=6), and nitroglycerin (n=6) in vitro. Vascular rings were preconstricted with 0.6 uM norepinephrine (NE). Responses to each agent were tested prior to incubation with 30 uM L-NAME (control), after incubation with L-NAME, and after incubation with L-arginine or D-arginine in the same sequence. Values are means $\pm$ SE and expressed as percent relaxation of the active tension produced by NE. * indicates a significant difference from control responses (prior to incubation with L-NAME). $P<0.05$.

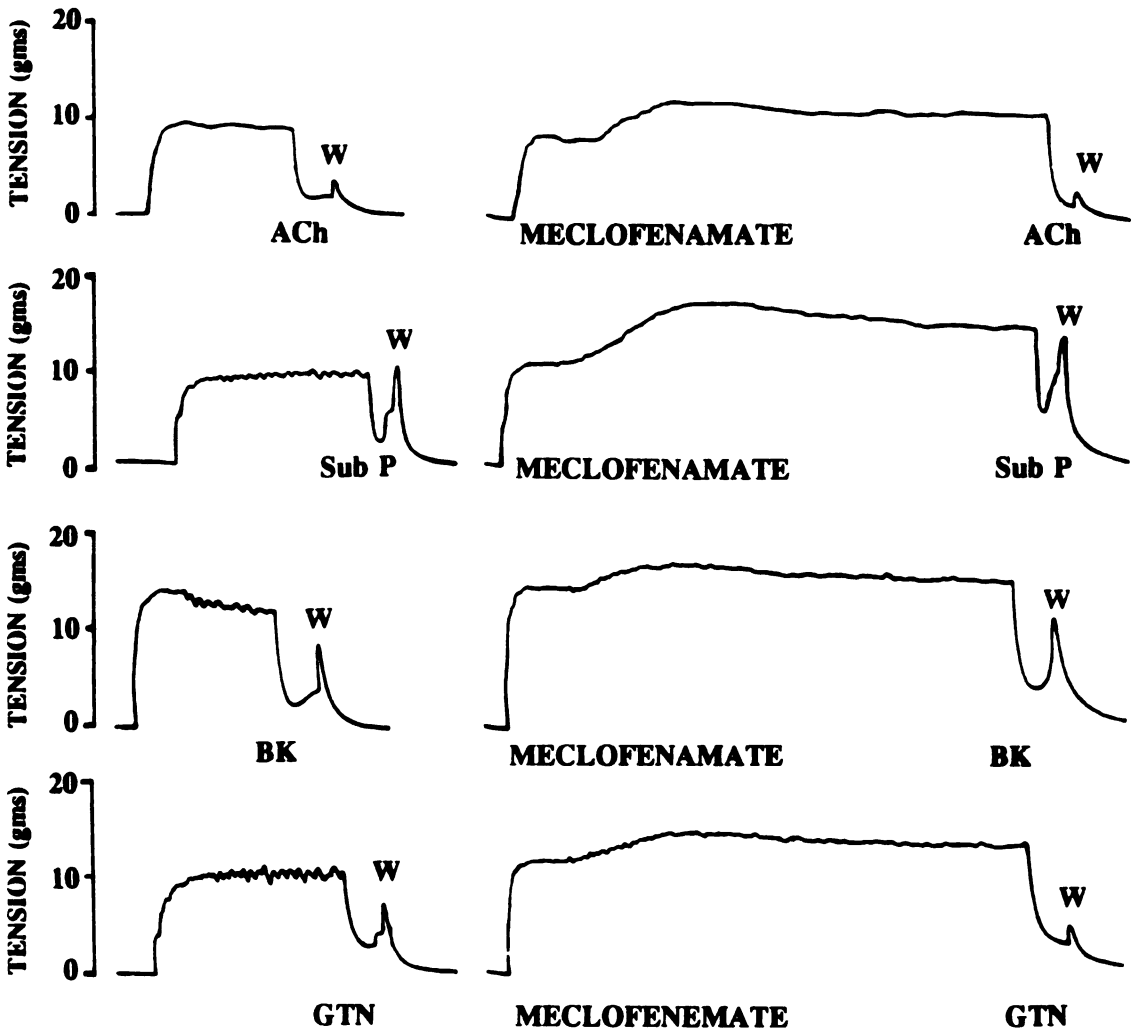



Figure 6. Experimental recording of the effect of 10 uM meclofenamate on the relaxation produced by acetylcholine (ACh; 1 uM), substance P (sub P; 20 nM), bradykinin (BK; 10 uM), nitroglycerin (GTN; 20 uM), in four adjacent mesenteric arteries obtained from one dog in vitro. Each arterial ring was preconstricted with 0.6 uM norepinephrine (NE). Meclofenamate enhanced the resting tension of each arterial ring and attenuated the relaxation produced by ACh, sub P, BK, and GTN, but did not effect the relaxation produced by ADO. W indicates washout of Kreb's media bathing the arterial rings.

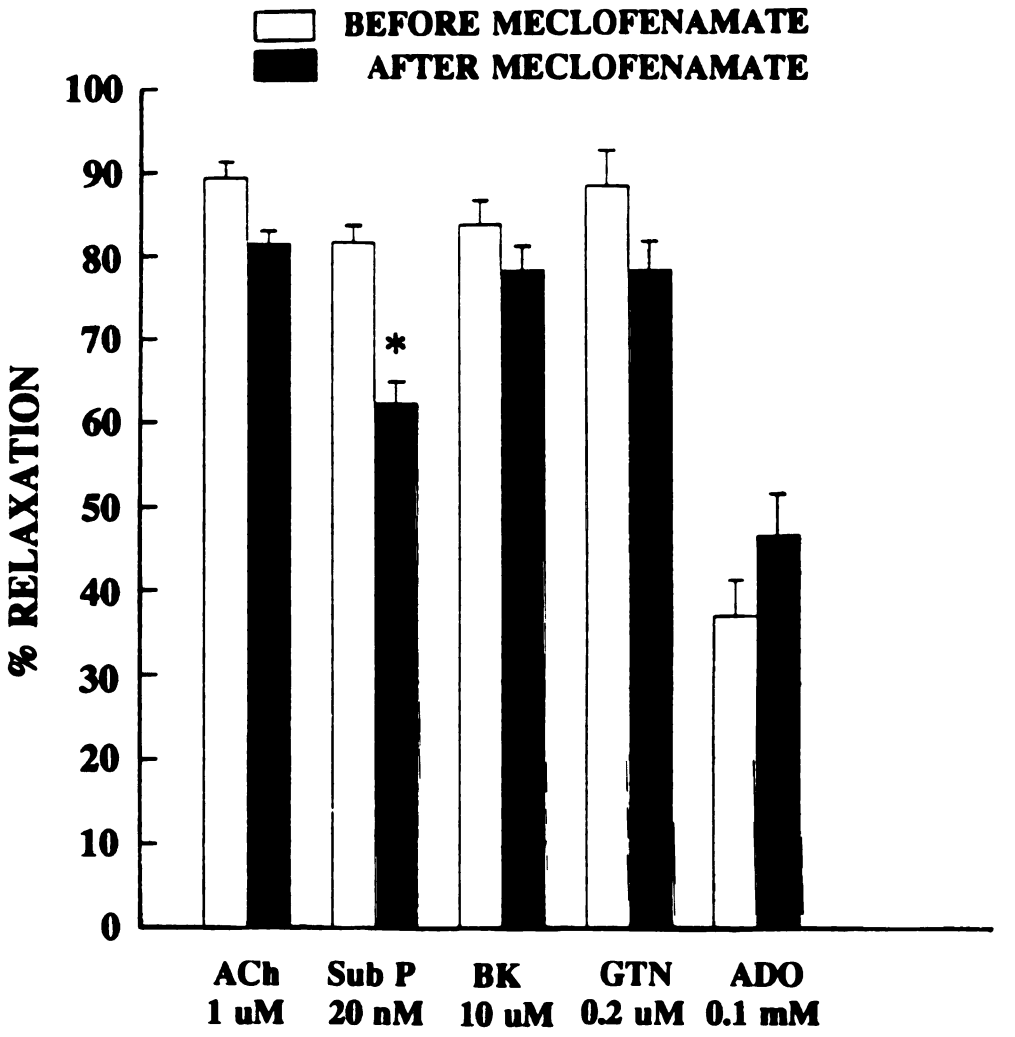



Figure 7. Effect of 10 uM meclofenamate on the relaxation of canine mesenteric arteries produced by acetylcholine (n=5), substance P (n=5), bradykinin (n=5), nitroglycerin (n=5), and adenosine (n=5) in vitro. Vascular rings were preconstricted with 0.6 uM norepinephrine (NE). Responses to each agent were tested prior to and 30 minutes after incubation with meclofenamate. Values are means $\pm$ SE and expressed as percent relaxation of the active tension produced by NE. * indicates a significant difference from control responses (before meclofenamate). $P<0.05$.

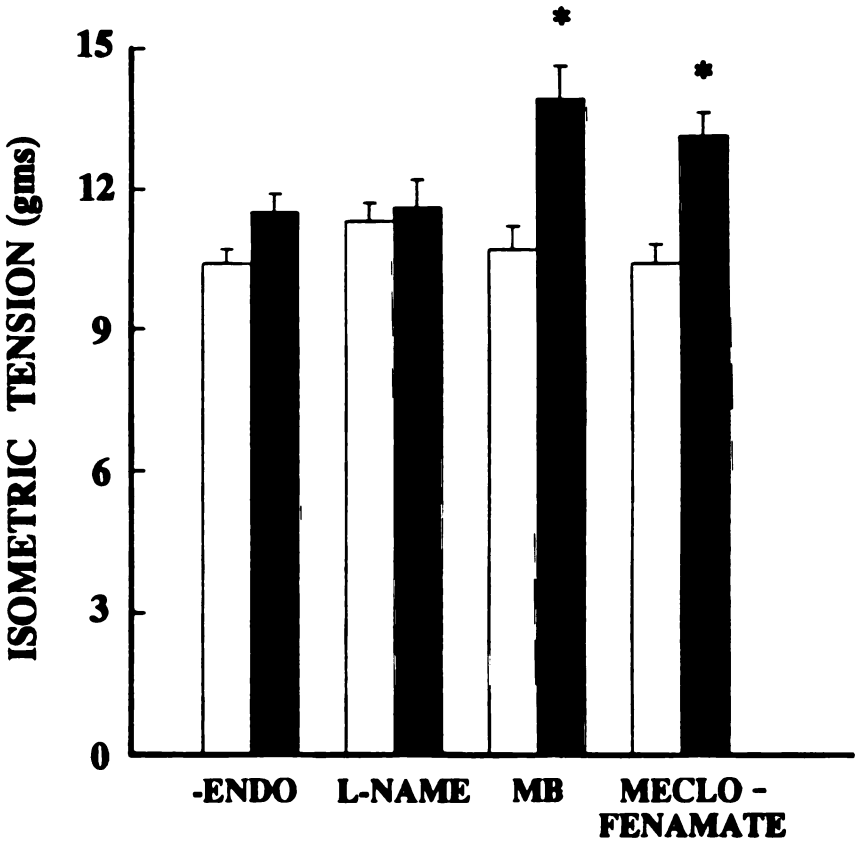



Figure 8. Effect of endothelial cell removal (n=41), or incubation of SMA rings with 30 uM L-NAME (n=41), 10 uM methylene blue (MB; n=30), or 10 uM meclofenamate (n=20) on the active tension produced by norepinephrine (NE; 0.6 uM). Values are mean $\pm$ SE and expressed in grams (gms.) tension. * indicates a significant difference from control responses (before endothelial cell removal). $P<0.05$.

# The Role of Nitric Oxide in the Regulation of Jejunal Blood Flow and Motility in vivo

## Introduction

Nitric oxide is a mediator of the endothelium-dependent relaxation of vascular smooth muscle produced by ACh in several mammalian species including man (142,158,185,187). Recently, nitric oxide has been proposed to play an important role in the regulation of arterial blood pressure and regional blood flow to several peripheral vascular beds under resting conditions (37,57,59,60,85,185,187). Nitric oxide is synthesized from the guanidino nitrogen atoms of L-arginine and its synthesis can be inhibited by $N^G$-monomethyl-L-arginine (L-NMMA) or $N^G$-nitro-L-arginine methyl ester (L-NAME), analogues of L-arginine with nonmetabolizable guanidino nitrogen atoms (91,153,155). In conscious rats and anesthetized rabbits, inhibition of nitric oxide synthesis in vivo by intravenous injection of L-NMMA or L-NAME produces a dose-dependent hypertension and widespread vasoconstriction of the mesenteric, renal, hindquarter, and internal carotid vascular beds. (57,60,156). The effect of L-NMMA and L-NAME are partially reversed by high concentrations of L-arginine, but not by D-arginine (57,156).

Nitric oxide also appears to modulate the neural-mediated relaxation of canine lower esophageal sphinctor (32), duodenum (181), ileocolonic junction (11), and proximal colon (30,179) evoked by electric field stimulation. L-NMMA and L-NAME inhibit the tetrodotoxin-sensitive relaxation of intestinal smooth muscle produced by nerve stimulation (11,30,32,179,181). In addition, the experimental tracings of the relaxation of canine intestinal smooth muscle produced by exogenous nitric oxide or nitroglycerin is similar to the relaxation produced by electric field stimulation, and is not blocked by L-NMMA or L-NAME (11,32). Utilizing a biocascade assay

system, Boeckxstaens et al. (11) has demonstrated that ileocolonic smooth muscle releases a transferable vasorelaxant factor in response to nerve stimulation which behaves pharmacologically like nitric oxide. Furthermore, immunohistochemical staining has shown that the enzyme that synthesizes nitric oxide is localized in cell bodies and nerve fibers within the myenteric plexus of the rat intestinal tract (14). These studies suggest that nitric oxide may play a physiologic role in regulating intestinal motor activity in vivo.

Although the literature suggests that nitric oxide may play a role in regulating both canine intestinal blood flow and motility, the effect of nitric oxide synthesis inhibition on intestinal blood flow and motility in vivo has not yet been determined. In this study, we investigated the effect of L-arginine, the physiologic precursor to nitric oxide formation, and the L-arginine analogue L-NAME, an inhibitor of nitric oxide synthesis, on mean arterial blood pressure, heart rate, jejunal blood flow, oxygen uptake and intestinal motility in anesthetized dogs. To demonstrate the efficacy of nitric oxide synthesis inhibition, the effect of systemic administration of L-NAME on the relaxation of superior mesenteric artery (SMA) produced by ACh, substance P and bradykinin were also tested in vitro. The goal of this investigation was to determine whether nitric oxide plays a role in the regulation of canine jejunal blood flow or motility in vivo.

Methods

*Surgical preparation.* 28 mongrel dogs (15-25 kg) of either sex were fasted for 24 hours, anesthetized with pentobarbital sodium (30 mg/kg iv), and ventilated with a positive-pressure respirator (Harvard Apparatus, Dover, MA.) at a rate and volume that maintain arterial blood pH between 7.38 and 7.43. Systemic arterial pressure was continuously monitored with a Statham pressure transducer (P23GB) through a cannula in the right femoral artery. A segment of jejunum (18.5-39.8 g) approximately 25 cm aboral to the ligament of Treitz was exteriorized. The single vein draining the jejunal segment was isolated with care to avoid any damage to nerves. The vein

was cannulated after administration of heparin sodium (500 U/kg). In experiments where L-arginine or D-arginine were infused (series 2 and 3), the single jejunal artery perfusing the segment was also cannulated and the jejunal segment was perfused naturally with aortic blood by interposing an extracorporeal shunt between the jejunal artery and left femoral artery. Jejunal blood flow was determined by timed measurements of venous outflow using a stop watch and graduated cylinder. In addition, venous intestinal blood flow was continuously monitored using an extra-corporeal flow transducer (BL 2048-E04, Biotronex Laboratories, Silver Springs, MD) placed into the venous outflow cannula and connected to an electromagnetic flowmeter (BL610, Biotronex Laborotories). Arterio-venous oxygen content difference ($AVO_2$) was continuously monitored by perfusing femoral arterial blood and a portion of the venous outflow (5-6 ml/min) through separate cuvettes of an arteriovenous oxygen content difference analyzer (AVOX Systems, San Antonio, TX) with a Gilson pump (Minipuls 2, Gilson Medical Electronics, Middleton, WI). The venous outflow and the outflow from the analyzer were directed to a reservior initially containing 200 ml of normal saline. The collected blood was returned by a pump to the animal through a femoral vein at rates equal to the total outflow. Both ends of the jejunal segment were tied and the mesentery cut to exclude collateral blood flow. A rubber tube was placed in the lumen for instilling and withdrawing normal saline and for measuring luminal pressure. The luminal contents were removed and replaced periodically prior to beginning the experimental protocol, after which 10-15 mls of saline was placed in the lumen and remained for the entire protocol. This amount of saline did not stimulate intestinal motility per se. In experiments of series 1, intraluminal motility was determined by recording changes in pressure of a saline-filled balloon condom placed into the jejunal segment. This was done to prevent the absorption of saline from the lumen which could reduce the detection of phasic contractions of the jejunal segment. Systemic arterial blood pressure, jejunal arterial perfusion pressure, and luminal pressure

were measured continuosly throughout the experiment using Statham pressure transducers (P23GB). Jejunal venous blood flow, lumen pressure, and arterial pressure were continuously recorded with a Grass polygraph (Model 7D). The preparation was covered by a plastic sheet to prevent evaporative water loss and kept between 37-38°C with a thermistor-controlled heat lamp. After the preparation had stabilized for 20-40 minutes, three series of experiments were performed under natural flow conditions. Figure 8 illustrates the surgical preparation used in this study.

Since heartworms are toxic to endothelial cells (102), and have been shown to attenuate the endothelium-dependent vascular smooth muscle relaxation mediated by acetylcholine in vivo (97) and in vitro (98), all dogs were screened for heartworms by microcapillary tube, slide examination, or necropsy. Based on these criteria, no dogs were excluded from this study as a result of heartworm infection.

Jejunal oxygen uptake was assumed to be equal to the product of venous blood flow and arteriovenous oxygen difference [(a-v)$O_2$]. The time required for venous blood to reach the (a-v)$O_2$ analyzer was 30 seconds and was compensated for in the calculation of oxygen uptake in every experiment. Jejunal vascular resistance was calculated by dividing the mean arterial pressure by intestinal blood flow and is expressed in total peripheral resistance (TPR) units. Motility was quantitated from the intraluminal pressure recording. The motility index was calculated by dividing the sum of all the pressure waves during a 1 minute period by the number of contractions in the same time period and was expressed in mm Hg. The motility index is a reflection of the average strength of the intestinal contractions (194). The dogs were euthanized with an overdose of pentobarbital and the jejunal segment was removed and weighed to standardize blood flow and oxygen uptake.

*Series 1.* In this series of experiments (n=8), dose reponse relationships to L-NAME (0.1-20 mg/kg) were obtained to determine the minimum and maximum dose of L-NAME necessary

to produce significant changes in jejunal blood flow, oxygen uptake, or jejunal motility. Experiments were begun after a control period of at least 30 minutes in which mean arterial blood pressure, venous outflow, oxygen uptake, and luminal pressure remained steady. Stock solutions of L-NAME (1.0 and 10.0 mg/ml) were dissolved in normal saline and injected into the femoral vein to achieve concentrations of 0.1, 0.2, 0.5, 1.0, 3.0, 7.0, 10, and 20 mg/kg in each dog. Mean arterial blood pressure, heart rate, jejunal blood flow, and motility were allowed to reach a steady state after each dose of L-NAME before the next dose of L-NAME was given. The average time between doses of L-NAME averaged between 8-12 minutes. After the effect of 20 mg/kg L-NAME was determined, 3 mls of nitroglycerin (40 ug/ml) was topically applied to the serosal surface and the effect on mean arterial pressure, jejunal blood flow and vascular resistance, oxygen uptake, and motility was evaluated.

*Series 2.* The temporal effect of L-NAME (10 mg/kg iv) on mean arterial pressure, heart rate, jejunal blood flow and vascular resistance, oxygen uptake, and motility was determined in 6 dogs. After a period of at least 30 minutes in which mean arterial blood pressure, heart rate, jejunal blood flow and motility remained steady, L-NAME was dissolved in 5 mls of saline and injected into the femoral vein. This dose of L-NAME was chosen based of the results of series 1 which indicated that 10 mg/kg L-NAME was sufficient to produce a maximal response in intestinal motility. Mean arterial blood pressure, heart rate, jejunal blood flow, oxygen extraction, and lumen pressure were continuously monitored for 50 minutes after administration of L-NAME.

*Series 3.* The effect of arterial infusion of either (L)- or (D)- arginine on jejunal blood flow, vascular resistance, oxygen uptake, or luminal pressure was determine in 4 dogs. Once a steady state in mean arterial pressure, jejunal blood flow, and oxygen uptake was achieved a dose response to L-arginine (12, 29, 58, 120, and 290 mg/min) was obtained. A dose response for D-arginine, which is not a precursor to nitric oxide, was also obtained and served as a control for

L-arginine. L-arginine and D-arginine (300 mg/ml NS) were individually administered with a Harvard infusion pump into the single artery perfusing the jejenal segment for 3 minutes at each infusion rate. The volume rate of infusion ranged between 0.04 and 0.97 mls/min and the total time of infusion of either amino acid was 15 minutes. The vehicle normal saline was also infused at the volume rates of infusion used for (L)-arginine and (D)- arginine.

*Series 4.* The effect of arterial infusion of L-arginine or D-arginine on the motility produced by L-NAME (10 mg/kg iv) was determined in 12 dogs. The objective of this study was to determine if the motility and hemodynamic effects produced by systemic administration of L-NAME could be reversed in the presence of L-arginine, the precursor to nitric oxide. When the increased motility reached a steady state (time 20 minutes after injection of L-NAME), L-arginine (n=6) or D-arginine (n=6) was infused into the artery perfusing the jejunal segment using a Harvard apparatus infusion pump at a rate of 97 mg/min for 20 minutes. The volume rate of infusion of (L)-arginine or (D)- arginine was 0.39 mls/min. All parameters were continuously recorded prior to, during and after infusion of L-NAME, (L)-arginine or (D)-arginine. The entire experimental protocol was completed within 40 minutes after intravenous administration of L-NAME.

A second objective of this study was to determine if inhibition of nitric oxide synthesis attenuated the reactive hyperemic response to arterial and venous occlusion. Prior to administration of L-NAME (10 mg/kg) and 15 minutes after intravenous injection of L-NAME, the single artery perfusing and the single vein draining the jejunum were occluded with rubber tipped forceps for 30 seconds and the peak hyperemic response was determined using the extracorporeal flow probe.

*Series 5.* The objective of this study was to determine the efficacy of L-NAME as an inhibitor of nitric oxide synthesis when administered systemically to anesthetized dogs. The

methods used to analyze the endothelium-dependent relaxation produced by ACh, substance P, bradykinin, adenosine, and nitroglycerin were the same as that previously described in chapter 4. The superior mesenteric arteries (SMA) were extracted from 6 dogs twenty minutes after administration of L-NAME (10 mg/kg iv) and the relaxation produced by ACh, substance P, and bradykinin were evaluated in vitro. Four adjacent mesenteric arterial rings from each dog that was pretreated with L-NAME were constricted with a submaximal concentration of NE (0.6 uM) and thereafter relaxed with ACh (1 uM), substance P (20 nM), bradykinin (10 uM), GTN (0.2 uM), or adenosine (0.1 mM). After the effect of L-NAME (10 mg/kg iv) on the responses to the vasodilating agents was determined, either 300 uM L-arginine or 300 uM D-arginine was added to the organ chamber and allowed to incubate with the preconstricted arterial ring for 20 minutes. Responses to the vasodilating agents were then repeated to determine if L-arginine or D-arginine reversed the inhibitory effect of L-NAME. The arterial rings that were initially incubated with D-arginine were subsequently incubated with L-arginine. Of the 24 mesenteric arterial rings studied 16 were incubated with L-arginine and 8 were incubated with D-arginine. Control responses for each vasodilating agent were obtained using 5 arterial rings taken from 5 separate dogs that were treated identically except that they did not receive L-NAME. No more than one vasodilating agents was added to the preconstricted arterial ring at any time. The volume of solution containing any chemical injected into the organ chamber never exceeded 60 uL.

**Preparation of chemicals** $N^G$-nitro-L-arginine methyl ester hydrochloride (L-NAME), L-arginine hydrochloride, D-arginine hydrochloride, acetylcholine hydrochloride, substance P, bradykinin, adenosine, sodium carbonate and L-ascorbic acid sodium salt were purchased from Sigma Chemical (St. Louis, MO). Norepinephrine bitartrate (distributed by Winthrop Pharmaceutical) and nitroglycerin (Nitrostat;0.4 mg tablets, Parke-Davis, Mount Plains, NJ.) were obtained from a local apothecary. The vehicle for all compounds used in vivo was normal saline

(NS). All drugs were prepared the day of the experiment. Stock solutions of acetylcholine, substance P, and bradykinin were dissolved in distilled water then stored at -20°C. The appropriate concentrations were serially diluted into Kreb's-Ringer solution the day of the experiment. Adenosine (0.27 g) was dissloved into 100 ml distilled water and 0.96 g sodium carbonate to yield a a stock solution of 0.1 M. The vehicle for all other chemicals was Kreb's-Ringer solution, except for norepinephrine, which was diluted in 0.1 % sodium ascorbate in distilled water.

**Statistics.** Values in figures are expressed as mean $\pm$ SE and represent paired or unpaired data depending on the specific series of experiment. The relaxation of superior mesenteric arteries produced by ACh, substance P, bradykinin, nitroglycerin, or adenosine was expressed as the percent relaxation of the active tension produced by 0.6 uM NE. In each series of experiments, n indicates the number of dogs. Statistical significance for paired observations were determined using a paired Student's T test. Where comparisons with a common control were made, the difference between the means of each experiment were evaluated by analysis of variance. If the analysis of variance showed a significant difference among the means, then statistical differences between individual groups were determined using the least significant difference test. In situations where there was no common control, a Student's T test for unpaired observations was used.

## Results

*Series 1.* The effect of cumulative doses of L-NAME (1-20 mg/kg iv) on mean arterial blood pressure, heart rate, jejunal blood flow, oxygen uptake, and vascular resistance, are summarized in Table 1. Systemic administration of L-NAME produced a dose-dependent increase mean arterial blood pressure and decrease heart rate that was statistically significant at concentrations of 1 mg/kg L-NAME or greater. L-NAME also produced a progressive decrease

in jejunal blood flow and progressive increase in jejunal vascular resistance at doses ranging between 0.1 and 5.0 mg/kg. However, at higher concentrations (10-20 mg/kg) L-NAME did not significantly affect jejunal blood flow or vascular resistance. The effect of L-NAME on the intestinal motility index is shown in Table 1 and Figure 10. There was a significant, dose-dependent increase in the motility index, with a maximal effect occurring between 10-20 mg/kg. At high concentrations of L-NAME (10-20 mg/kg), the increase in motility index was associated with a significant increase in oxygen uptake.

The effect of topical application of nitroglycerin on jejunal blood flow and motility produced after L-NAME (20 mg/kg iv) is shown in Figure 11. Nitroglycerin increased jejunal blood flow and abolished the motility elicited by L-NAME. The effect of topical nitroglycerin was transient, lasting for no more than 1 minute. The effect of nitroglycerin on the L-NAME-induced changes in mean arterial pressure, jejunal blood flow, vascular resistance, oxygen uptake, and motility index are summarized in Table 2. When topically applied to the serosal surface nitroglycerin did not alter the mean systemic arterial pressure, but significantly significantly increased jejunal blood flow and oxygen uptake, and significantly decreased the jejunal vascular resistance. The motility produced by L-NAME was abolished by topical application of nitroglycerin. The effect of nitroglycerin was transcient and lasted for about 40 seconds after topical application.

*Series 2.* Figure 12 is a typical recording of the effect of L-NAME (10 mg/kg iv) on mean arterial blood pressure, jejunal blood flow and intestinal motility. Bolus injection of L-NAME produced an immediate increase in mean arterial pressure that was rapid in onset, reaching a plateau within 2-4 minutes. The increase in blood pressure elicited by L-NAME ranged between 15-40 mm Hg (mean $28\pm4$ mm Hg, n=6) in each dog. The hypertension produced by L-NAME was accompanied by a significant decrease in heart rate from $167\pm5$ to $124\pm12$ beats/min. The

effect of L-NAME on mean arterial blood pressure and heart rate was long-lasting and remained for the entire duration of the experiment. At 50 minutes after injection of L-NAME, mean arterial blood pressure was increased by an average of 22±4 mm Hg (from 124±5 to 147±6 mm Hg) and heart rate was decreased by an average of 28±4 beats/min (from 167±5 to 139±7 beats/min). There was also a significant decrease in jejunal blood flow that was concomitant to the increase in mean arterial pressure and decrease in heart rate produced after injection L-NAME.

Shortly after the hemodynamic effects of L-NAME had stabilized, there was a marked increase in motility. The onset of motility varied between each dog, occurring between 1 and 4 minutes after injection of L-NAME. The motility produced by L-NAME was associated with an increase in the frequency and amplitude of phasic, rhythmic contractions, and an increase in basal lumen pressure. The maximal motility index achieved in each dog ranged between 51.3 and 68.6 mm Hg (average, 58.9±3.7) and occurred between 4-15 minutes after injection of L-NAME (average, 8±2 minutes). The motility index declined to a plateau between 9-24 minutes (average, 17±2 min) after injection of L-NAME (average, 39.8±2.6 mm Hg). The motility index then gradually declined toward a baseline for the duration of the experiment. However, the motility index was still significantly greater that control 50 minute after injection of L-NAME. As the motility index approached a maximum, jejunal blood flow began to increase to a level that was significantly greater than control blood flow. The maximum increase in blood flow typically occurred as the motility index declined from a maximum to a steady state level. This secondary increase in blood flow associated with the decline of motility from a maximum to steady state levels was observed in every dog. Blood flow then decreased below control levels and motility decreased toward baseline between 20 and 50 minutes after injection of L-NAME.

As stated previously, the maximum increase in jejunal blood flow occurred as the motility index began to decline from a maximum to a plateau. Because the maximum increase in the

motility index was achieved at different times after injection of L-NAME in each dog, the maximum changes in jejunal blood flow and oxygen uptake are not completely represented when our data are expressed at a function of time after the injection of L-NAME. Therefore, we analyzed the maximum changes in jejunal blood flow and oxygen uptake after injection of L-NAME in each dog (Table 3). The maximum vasoconstriction produced by L-NAME was observed between 1 and 4 minutes after injection. Jejunal blood flow was decreased by 44±4% and vascular resistance was increased by 93±13% relative to control. Conversely, the maximum increase in jejunal blood flow occurred 10-18 minutes after injection of L-NAME once motility had reached a steady state. At this time, jejunal blood flow was increased by 35±5% (from 76±4 to 101±5 ml/min/100g) and vascular resistance was not significantly different from control levels. During the increase in blood flow, oxygen uptake was increased by 23±6% relative to control. As the motility produced by L-NAME slowly decayed toward control values, jejunal blood flow and oxygen uptake decreased, whereas vascular resistance increased.

The effect of L-NAME (10 mg/Kg iv) on the reactive hyperemic response after release of arterial and venous occlusion for 30 seconds is shown in Figure 13. The resting jejunal blood flow after injection of L-NAME prior to vascular occlusion did not significantly differ from control (63±5 ml/min/100g during control vs. 59±4 ml/min/100g after L-NAME). L-NAME did not significantly affect the peak reactive hyperemic response (105±11 during control vs. 103±14 ml/min/100g after L-NAME) or the percent change in blood flow (71±12% during control vs. 72±18% after L-NAME) produced after 30 seconds of arterial and venous occlusion.

*Series 3.* The jejunal vascular and metabolic responses to arterial infusion of (L)- or (D)-arginine (12, 24, 58, 116, 297 mg/min.) in 4 dogs is summarized in Table 4. At low infusion rates (12-116 mg/min), neither (L)- or (D)- arginine significantly altered jejunal blood flow, vascular resistance, or oxygen uptake. However, both (L)- and (D)-arginine significantly increased

jejunal blood flow and oxygen uptake and decreased vascular resistance when infused at a rate of 297 mg/minute. Jejunal oxygen extraction, arterial pressure and luminal pressure were not significantly altered by (L)- or (D)- arginine at any infusion rate. Equal rates of infusion of the normal saline vehicle did not significantly alter blood flow, perfusion pressure, vascular resistance, oxygen uptake, or the motility index.

*Series 4.* The effect of local arterial infusion of L-arginine into the single jejunal artery 20 minutes after injection of L-NAME is shown in Figure 14. Continuous infusion of L-arginine (97 mg/min) for 20 minutes did not significantly alter mean arterial pressure. However, the L-NAME-induced increase in intestinal motility was almost completely abolished 20 minutes after arterial infusion of L-arginine. Jejunal blood flow initially increased upon infusion of L-arginine, however the hyperemia could not be maintained despite continuous infusion of L-arginine. The increase in blood flow produced by L-arginine was transcient lasting for no more than 5 minutes. Jejunal blood flow then decreased as motility decreased during the 20 minute infusion period with L-arginine.

The effect of arterial infusion of either L-arginine or D-arginine on the L-NAME-induced changes in mean arterial pressure, jejunal blood flow and vascular resistance, oxygen uptake, and motility index, followed by intra-arterial infusion of either L-arginine or D-arginine are shown in Tables 5 and 6. L-NAME produced a similar pattern and magnitude of increase in blood pressure and jejunal motility index, and a biphasic change in jejunal blood flow and vascular resistance before administration of either L- or D-arginine. These responses were similar to those in series 2. Neither arginine affected the L-NAME-induce increase in blood pressure. However, the increased motility index produced by L-NAME was significantly inhibited by L-arginine, but not by D-arginine. L-arginine also significantly increased jejunal blood flow for 5 minutes. The flow then decreased, and at 20 minutes after the start of L-arginine infusion, the blood flow was

significantly lower than those before either L-NAME or L-arginine. The action of L-arginine on jejunal vascular resistance was exactly opposite of that on blood flow. In contrast, D-arginine did not significantly alter blood flow and vascular resistance.

Figure 15 demonstrates the effect of systemic administration of L-NAME (10 mg/kg iv) on the relaxation of 3 separate mesenteric arterial rings produced by ACh and GTN taken from 3 different dogs. Each ring was preconstricted with 0.6 uM NE prior to testing the vasodilating agents. When compared to the control responses obtained from arterial rings that were not pretreated with L-NAME (ring A), the relaxation produced by ACh was less in the arterial rings that were harvested from dogs 20 minutes after administration of 10 mg/Kg L-NAME (Rings B and C). However, intravenous administration of L-NAME had no effect on the relaxation produced by nitroglycerin when compared to arterial rings that were not exposed to L-NAME. Incubation of the SMA rings exposed to L-NAME with 300 uM L-arginine reversed the inhibitory effect of L-NAME on the relaxation produced by ACh. In contrast, incubation of the SMA rings exposed to L-NAME with 300 uM D-arginine did not reverse the inhibitory effect of L-NAME on the relaxation produced by.

*Series 5.* The effect of L-NAME, followed by incubation of SMA rings with either L-arginine or D-arginine on the endothelium-dependent relaxation of canine SMA rings produced by ACh, substance P, and bradykinin are shown in Figure 16. The arterial rings from dogs treated with L-NAME (10 mg/Kg iv) responded to ACh, substance P, and bradykinin with less relaxation compared to those obtained from dogs that were not treated with L-NAME (control). ACh, substance P, and bradykinin relaxed arterial rings that were not treated with L-NAME by 89$\pm$2 %, 82$\pm$2%, and 84$\pm$4%, respectively. In contrast, ACh, substance P, and bradykinin relaxed mesenteric arterial rings treated with L-NAME (10 mg/Kg iv) by 58$\pm$3%, 36$\pm$4%, and 43$\pm$3%, respectively. Incubation of the mesenteric arterial rings that were exposed to intravenous L-

NAME with 300 uM L-arginine enhanced the relaxation produced by ACh, substance P, and bradykinin. After incubation with L-arginine, ACh, substance P, and bradykinin relaxed the preconstricted SMA rings by 86±2%, 62±6%, and 73±4%, respectively. The relaxation produced by ACh and bradykinin did not significantly differ from control responses. However, incubation with L-arginine did not completely reverse the inhibitory effect of L-NAME on the relaxation produced by substance P. Incubation of the L-NAME treated rings with D-arginine did not potentiate the relaxation produced by ACh, substance P, or bradykinin. As shown in Figure 17, the relaxation produced by the endothelium-independent vasodilating agents nitroglycerin or adenosine was not significantly affected by intravenous administration of L-NAME, or after incubation of the L-NAME treated arterial rings with either L-arginine or D-arginine.

## Discussion

Previous studies have suggested that nitric oxide may play a physiologic role in the regulation of mean arterial blood pressure (57,156) intestinal blood flow (57,59,60,133) and motility (11,30,32,122,179). However, no study to date has investigated the effect of nitric oxide synthesis inhibition on intestinal blood flow, oxygen uptake, and motility in vivo. The primary objective of this study was to determine the effect of L-arginine, the physiologic precursor to nitric oxide synthesis (143), and the L-arginine analogue L-NAME, an inhibitor of nitric oxide synthesis (133), on jejunal blood flow and intestinal motility in vivo. In this study, we found that L-NAME produced a sustained, dose-related increase in intestinal motility (Figure 10). We also demonstrate that systemic administration of L-NAME (10 mg/kg iv) produced a rapid decrease in jejunal blood flow and increase in jejunal vascular resistance (Figure 12 and Table 3). The jejunal vasoconstriction was concomitant with the hypertension produced immediately after the injection of L-NAME. Shortly after the increase in blood pressure and jejunal vasoconstriction of the

jejunal vascular bed produced by L-NAME had stabilized, there was a marked increase in intestinal motility (Figure 12). The motility produced an increase both in the basal lumen pressure as well as an increase in the frequency and amplitude of phasic, rhythmic contractions of the in situ jejunal segment. The increase in mean arterial pressure and motility produced by L-NAME (10 mg/kg) lasted for more than 50 minutes. There was also a significant increase in jejunal blood flow as motility reached a maximum and then declined to a steady state value between 10 and 18 minutes after injection of L-NAME. Jejunal vascular resistance returned to control during the increase in jejunal blood flow since mean arterial blood pressure remained elevated. Jejunal blood flow then decreased and vascular resistance increased as intestinal motility slowly waned toward baseline.

There is substantial evidence that nitric oxide, or a closely related nitroso compound that releases nitric oxide, is responsible for the relaxation of canine (11,30,32,181) and human (122) intestinal smooth muscle in vitro. The nerve-mediated relaxation of intestinal smooth muscle produced by electric field stimulation is mimmicked by exogenous nitric oxide, nitroglycerin, and sodium nitroprusside (11,37,122,181). The relaxation produced by electric field stimulation or exogenous nitric oxide is significantly attenuated in the presence of oxyhemoglobin (11,32,181). Moreover, incubation of canine lower esophageal sphincter (32), duodenum (81), ileocolonic circular smooth muscle (11), and the proximal colon (30,179), and the human ileal circular smooth muscle (122) with L-NMMA, L-NAME, or LNOARG has been shown to attenuate or even abolish the relaxation produced by electric field stimulation. The inhibitory effect of the L-arginine analogues can be reversed after incubation with excess L-arginine but not D-arginine (32,122,181). Similar results have been reported in the rat anococcygeus muscle (62,82,117) and in preparations circular smooth muscle from the guinea pig ileum (171). These studies are in agreement with our data indicating that inhibition of nitric oxide synthesis stimulates intestinal motility in vivo.

To our knowledge, this is the first study to demonstrate that systemic administration of L-NAME, a inhibitor of nitric oxide synthesis, stimulates intestinal motility in vivo. The minimum dose of L-NAME necesssary to stimulate an increase in motility was 0.5 mg/kg. The maximum effect of L-NAME on intestinal motility was achieved at a intravenous doses ranging between 10 and 20 mg/kg. The increase in motility was accompanied by a significant increase in oxygen uptake. The motility produced after bolus injection of L-NAME (10 mg/kg iv) was rapidly reversed by arterial infusion of L-arginine, but not D-arginine (Tables 5 and 6, respectively). These results are consistent with the hypothesis that the motility produced by L-NAME results from the inhibition of endogenous nitric oxide synthesis. Furthermore, serosal application of nitroglycerin, which relaxes vascular and visceral smooth muscle through the intracellular formation of nitric oxide (1,8), abolishes the motility produced by L-NAME (Figure 11). Therefore, the L-NAME-induced increase in jejunal motility in our study appears to be a result of inhibition of the L-arginine- nitric oxide synthesis pathway. This suggests that nitric oxide acts to suppress intestinal motility vivo.

The hypertension and decrease in heart rate produced by bolus injection of L-NAME is in general agreement with recent reports in rabbits (156) and rats (57,59,60,196) using either L-NMMA or L-NAME to inhibit nitric oxide synthesis. For example, Gardiner et al. have reported that bolus injection of L-NMMA or L-NAME to conscious rats caused a long-lasting, dose-dependent increase in mean arterial blood pressure and also decreased heart rate (57-60). The hypertension and decrease in heart rate produced by L-NMMA or L-NAME is enantiomer-specific, and hence, D-NMMA and D-NAME are without effect (57-59,156,196). In addition, the effects of L-NMMA or L-NAME can be reversed by high doses of L-arginine, but not D-arginine (57-59,156,196). In anesthetized rabbits, the hypertension and bradycardia produced by L-NMMA (100 mg/kg) was reversed by L-arginine (300 mg/kg), but not by D-arginine, indomethacin,

prazocin or by vagotomy (156). These studies strongly suggest that the hypertension and bradycardia observed after administration of L-NAME is a direct result of impaired nitric oxide synthesis and/or release.

Jejunal blood flow showed a triphasic response to L-NAME; an initial decrease, followed by an increase above and around the resting levels for 25 minutes, followed by an eventual decrease below resting levels at 40-50 minutes postinfusion (Figure 12 and Table 3). As shown in Figure 12, the maximum increase in jejunal blood flow typically occurred as motility declined from a maximum to a plateau. The response of jejunal vascular resistance was exactly opposite to the response of blood flow. As intestinal motility began to wane towards baseline during 40-50 minutes, oxygen uptake returned toward control level and the vasoconstriction reappeared. The triphasic blood flow response produced by L-NAME in this study is most likely a result of the marked increase in motility produced by L-NAME. It is known that spontaneous as well as chemically-induced intestinal motility can influence intestinal blood flow. Rhythmic contractions of intestinal smooth muscle can be accompanied by no change, a decrease, or an increase in blood flow, depending on the strength and pattern of the contractions (20,112). The increase in blood flow during muscle contractions usually is accompanied by an increase in oxygen uptake. As discussed above, the reversal of the L-NAME-induced vasoconstriction was accompanied by an increase in oxygen uptake and motility. Hence, the marked increase in motility produced by L-NAME could influence the L-NAME induced vasoconstriction and local oxygen uptake. Therefore, the direct action of L-NAME in the canine jejunum is most likely vasoconstriction.

Currently published studies investigating the effect of nitric oxide synthesis inhibition on intestinal blood flow are controversial. Utilizing an isolated rat mesenteric vascular bed constantly perfused with Kreb's solution, Moore et al. (133) have reported that the addition of L-NOARG or L-NMMA to the perfusate resulted in a rapid increase in mesenteric perfusion pressure.

However, the vasoconstriction produced by L-NOARG or L-NMMA was not maintained even in the continued presence of either inhibitor of nitric oxide synthesis (133). Nonetheless, the vasodilation produced by arterial infusion of ACh was significantly inhibited under these conditions (133), suggesting that nitric oxide synthesis is effectively inhibited. The reason why the increase in perfusion pressure was maintained in the rat mesenteric bed was not addressed by these investigators. It is possible that intestinal motility produced by inhibition of nitric oxide synthesis inhibition may have indirectly affected vascular resistance in this study. In contrast, Gardiner et al. has reported that bolus injection of L-NAME (10 mg/kg iv) to conscious rats produced an immediate, long-lasting vasoconstriction of the superior mesenteric artery that remained for at least an hour after injection (59). The reason for the discrepancy on the effect of L-NAME on blood flow and vascular resistance observed in our study compared to that reported by Gardiner et al (59) are not clear. It may reflect a difference in the effect of nitric oxide synthesis inhibition in various regions of the gastrointestinal tract. While we continuously determined venous outflow from an isolated jejunal segment by direct measurement in one minute intervals, Gardiner et al. (57-59) measured the total superior mesenteric arterial blood flow using a doppler flow probe, which represents the total blood flow distribution to the entire small intestine and proximal colon. Therefore, the transcient increase in jejunal blood flow observed after the onset of intestinal motility in our study may indicate a local change and not reflect the changes in blood flow to different sections of the gastrointestinal tract perfused by the superior mesenteric artery at any given time. Indeed, Humphries (85) has reported a significant decrease in blood flow to the stomach, duodenum, and colon, whereas blood flow to the ileum was not significantly altered 20 minutes after intravenous infusion of L-NOARG to anesthetized rabbits. Another possible explanation for the difference in mesenteric blood flow observed after L-NAME could result from a difference in the time of onset in motility in different regions of the

gastrointestinal tract perfused by the superior mesenteric artery. This is supported by our finding that due to the variation in the onset of motility observed in each dog, the transcient increase in jejunal blood flow after the onset of motility is not completely represented when the data are expressed as a function of time after injection of L-NAME. Further studies are necessary to determine the effect of nitric oxide synthesis inhibition on motility in other areas of the gastrointestinal tract.

As shown in Figure 14, in dogs pretreated with L-NAME (10 mg/Kg iv) arterial infusion of L-arginine produced an initial increase in blood flow that was transient lasting for only 3-6 minutes. Jejunal blood flow then slowly declined for the remainder of the 20 minute infusion period to a level which was not significantly different from the blood flow response observed after intravenous bolus of L-NAME at 40 minutes in series 2 (Figure 12). The inability of L-arginine to reverse the vasoconstrictor response to L-NAME in our study is not understood. However, it does not appear to result from an insufficient amount of L-arginine, since L-arginine readily reversed the motility produced by L-NAME. Furthermore, the inhibitory effect of L-NAME on the dilator responses to ACh, substance P, and bradykinin were reversed after incubation with 300 uM L-arginine for 15 minutes. One possibility that may account for the inability of L-arginine to reverse the effect of L-NAME may result from changes in the distribution of blood flow throughout the gut wall after the onset of motility. Phasic contractions of the intestinal smooth muscle have been shown to increase jejunal blood flow primarily to the muscularis serosal regions of the intestine (20,42). Therefore, the L-arginine infused after the onset of motility may be preferentially distrubuted to the muscularis layer of the intestine thereby shunting the L-arginine infused from the mucosal and submucosal layers of the intestine. However, studies utilizing microspheres to measure the total distribution of blood flow to individual layers of the intestinal segment would be required to answer this proposition.

To determine if nitric oxide mediates the reactive hyperemia produced by 30 seconds of arterial and venous occlusion, the effect of L-NAME on the reactive hyperemic response was determined. The peak hyperemic response after release of the vascular occlusion 10 minutes after bolus injection of L-NAME was not significantly different from the control response (Figure 13). This suggests that nitric oxide does not play a role in the reactive hyperemic response to brief periods of vascular occlusion in the canine jejunum. Similar results have been reported in isolated arterioles of the rat cremasteric muscle (197). Wolin et al. reported that in the presence of indomethacin, suffusion of L-NMMA over the isolated arteriole inhibited the vasodilation produced by ACh by more than 80% without altering the reactive hyperemic response to arteriole occlusion for periods of 15 and 120 seconds (197). These authors suggested that the reactive hyperemia to brief periods of arteriole occlusion is mediated by the local release of prostaglandins and possibly by an increase in hydrogen peroxide concentration within the endothelial cell as a result of the reoxygenation that occurs after release of the occlusion (197).

Arterial infusion of (L)-arginine, the precursor to nitric oxide synthesis (126,141,168), significantly increased jejunal blood flow and decreased jejunal vascular resistance. However, the vasodilation produced by L-arginine was observed only when infused at a rate of 290 mg/min. Furthermore, arterial infusion of D-arginine produced a similar effect to that produced by L-arginine. Therefore, the vasodilatory effect of L-arginine does not appear to be related to an increase in nitric oxide synthesis. The failure of L-arginine to directly increase jejunal blood flow is consistent with results obtained in conduit and in resistance vessels (40,146), and endothelial cells in culture (64,141), which suggest that under normal conditions there is sufficient endogenous L-arginine to saturate the enzyme responsible for nitric oxide synthesis.

The objective of the fifth series of experiments was to determine if the above actions of L-NAME were due to inhibition of endogenous nitric oxide. L- and D-arginine have been

regularly used for this purpose. As shown in Figures 16 and 17, L-NAME (10 mg/kg iv) effectively inhibited nitric oxide synthesis, as determined by an attenuation of the relaxation produced by ACh, substance P, and bradykinin in superior mesenteric arteries extracted 20 minutes after administration of L-NAME. The inhibitory effect of L-NAME was reversed by L-arginine, but not D-arginine. In contrast, the relaxation of the mesenteric artery produced by either nitroglycerin or adenosine were not significantly affected by bolus administration of L-NAME, L-arginine, or D-arginine (Figures 15 and 17). Results similar to these have been reported in the rabbit aorta extracted 10 minutes after systemic injection of L-NMMA (100 mg/kg iv) in anesthetized rabbits (156). L-NMMA significantly inhibited the release of nitric oxide from the endothelium and the relaxation produced by ACh in rabbit thoracic aorta in vitro without altering the response produced by nitroglycerin (156). The inhibitory effect of L-NMMA was reversed by infusing L-arginine through the aortic segments (156). Therefore, the inhibition of nitric oxide synthesis from mesenteric arterial rings produced by L-NAME in this study provides indirect evidence to indicate that the actions of L-NAME of blood pressure, jejunal blood flow and motility were due to inhibition of endogenous nitric oxide. This thesis is supported by the findings shown in Figure 11. Application of nitroglycerin, a source of nitric oxide which does not involve nitric oxide synthase, promptly abolished the L-NAME-induced increase in jejunal motility.

In conclusion, the present study demonstrates that inhibition of endogenous nitric oxide synthesis results in an increase in systemic arterial blood pressure,and jejunal motility and vascular resistance. The marked increase in motility can abolish or reverse the vasoconstriction. Therefore, endogenous nitric oxide may play a role in regulating motility and blood flow in resting canine jejunum.

Summary

We have shown that bolus intravenous injection of L-NAME, an inhibitor of nitric oxide synthesis, significantly increased mean arterial pressure and decreased jejunal vascular resistance in anesthetized dogs. L-NAME also produced a marked increase in intestinal motility, that was accompanied by a significant increase in local blood flow and decrease in local vascular resistance. As motility waned toward baseline levels the L-NAME-induced vasoconstriction reappeared. Topical application of nitroglycerin, a source of nitric oxide which does not involve the nitric oxide synthase enzyme system, to the serosal surface of the isolated jejunal segement abolished the increase in motility produced by L-NAME and produced a significant increase in jejunal blood flow. Local arterial infusion of L-arginine, the precursor to nitric oxide, or D-arginine at rates ranging between 12 and 116 mg/min had no effect on jejunal vascular resistance, oxygen uptake, or motility. When infused at a rate of 97 mg/min for 20 minutes, L-arginine reversed the motility, but not the vasoconstriction produced by L-NAME. Arterial infusion of D-arginine at the same rate did not affect the L-NAME-induced motility. The changes in intestinal motility produced by nitric oxide synthesis inhibition may alter jejunal blood flow indirectly. The effects of L-NAME appear to result from the inhibition of endogenous nitric oxide synthesis since intravenous administration of L-NAME (10 mg/Kg) specifically inhibited the nitric oxide-mediated relaxation of the superior mesenteric artery produced by ACh, substance P, and bradykinin in vitro. In conclusion, results from this study suggest that endogenous nitric oxide appears to play a role in the regulation of systemic arterial blood pressure, intestinal blood flow and motility in anesthetized dogs.

Table 1. Effect of cumulative doses of L-NAME on mean arterial blood pressure, jejunal blood flow, vascular resistance, and oxygen uptake.

| | CONTROL | 1 mg/kg | 3 mg/kg | 5 mg/kg | 10 mg/kg | 20 mg/kg |
|---|---|---|---|---|---|---|
| MAP | 123±5 | 131±4* | 139±7* | 137±4* | 137±4* | 136±6* |
| BF | 56±6 | 41±4* | 43±5* | 38±2* | 58±10 | 49±10 |
| R | 2.4±0.4 | 3.5±0.4* | 3.5±0.4* | 3.8±0.4* | 2.9±0.5 | 3.4±0.8 |
| $VO_2$ | 2.4±0.2 | 2.4±0.2 | 2.7±0.2 | 2.6±0.2 | 3.1±0.2* | 3.1±0.3* |
| MI | 1±0.3 | 7±2 | 11±2 | 18±5 | 27±5* | 30±5* |

The effect of systemic administration of L-NAME (1-20 mg/kg iv) on mean arterial blood pressure (MAP), jejunal blood flow (BF), vascular resistance (R), oxygen uptake ($VO_2$), and jejunal motility index (MI). Values are reported as means ± SE obtained during steady state at each dose of L-NAME in 8 separate dogs. * indicates a significant difference from steady state values taken before injection of L-NAME (control). $P<0.05$.

Table 2. Effect of nitroglycerin on the L-NAME-induced changes in mean arterial blood pressure, jejunal blood flow, vascular resistance, oxygen uptake, and motility index.

| | MAP | BF | R | $VO_2$ | MI |
|---|---|---|---|---|---|
| PRIOR TO GTN | 140±4 | 46±6 | 3.5±0.5 | 3.0±0.2 | 32±8 |
| AFTER GTN | 139±4 | 59±7* | 2.5±0.3* | 3.6±0.3* | 3±2* |

The effect of nitroglycerin (GTN; 0.4 ug/ml) on the L-NAME-induced changes in mean arterial blood pressure (MAP), jejunal blood flow (BF), vascular resistance (R), oxygen uptake($VO_2$), and motility index (MI). 3 mls of GTN was topically applied to the serosal surface of the jejunum once the effect of intravenous injection of L-NAME (20 mg/kg) had reached a steady state. Values are means ± SE from 5 separate dogs (n=5). * indicates a significant difference compared to steady state values prior to GTN. $P<0.05$.

Table 3. Effect of L-NAME(10 mg/kg iv) on mean arterial blood pressure (MAP), jejunal blood flow (BF), vascular resistance (R), and motility index (MI).

| TIME (MIN) | MAP | BF | R | $VO_2$ | MI |
|---|---|---|---|---|---|
| 0 MIN | 124±5 | 75±4 | 1.7±0.1 | 3.0±0.2 | 0 |
| 1-4 MIN | 152±5* | 43±4* | 3.2±0.1* | 2.4±0.2* | 23±5* |
| 10-18 MIN | 145±5* | 101±5*# | 1.5±0.1# | 3.7±0.3*# | 47±6* |
| 40 MIN | 143±6* | 61±8*#@ | 2.6±0.4*@ | 3.2±0.2* | 24±5*@ |
| 50 MIN | 148±5* | 49±6*@ | 3.2±0.7*@ | 2.7±0.2@ | 14±4*@ |

Values reported are means ± SE (n=6) taken at the maximum decrease (between 1-4 min) and the maximum increase (between 10-18 min) in BF after injection of L-NAME (1 mg/kg iv). * indicates a significant difference compared to control (i.e. before L-NAME), # indicates a significant difference compared to the maximum constrictor effect produced between 1-4 minutes after injection of L-NAME, and @ indicates a significant difference from maximum increase in blood flow after L-NAME. $P<0.05$.

Table 4. Dose-response of arterial infusion of L-arginine or D-arginine on jejunal blood flow, vascular resistance, and oxygen uptake.

L-ARGININE

| | 0 mg/min | 12 mg/min | 24 mg/min | 56 mg/min | 116 mg/min | 297 mg/min |
|---|---|---|---|---|---|---|
| BF | 54±7 | 53±5 | 52±5 | 53±5 | 64±6 | 86±7* |
| R | 2.3±0.3 | 2.1±0.4 | 2.1±0.4 | 2.1±0.3 | 1.7±0.3 | 1.2±0.2* |
| $VO_2$ | 1.9±0.1 | 2.0±0.2 | 1.9±0.1 | 2.0±0.1 | 2.6±0.1 | 3.3±0.2* |

D-ARGININE

| | 0 mg/min | 12 mg/min | 24 mg/min | 56 mg/min | 116 mg/min | 297 mg/min |
|---|---|---|---|---|---|---|
| BF | 64±12 | 62±12 | 62±12 | 73±16 | 74±16 | 86±7* |
| R | 2.0±0.2 | 1.9±0.4 | 1.9±0.4 | 1.7±0.4 | 1.6±0.3 | 1.1±0.2* |
| $VO_2$ | 2.0±0.1 | 2.1±0.2 | 2.0±0.2 | 2.3±0.3 | 2.5±0.1 | 3.4±0.2* |

The cumulative dose-response to arterial infusion of L-arginine or D-arginine (12-297 mg/min) on jejunal blood flow (BF), vascular resistance (R), and oxygen uptake ($VO_2$) under conditions of natural blood flow. Arterial infusion of normal saline vehicle was without effect at all infusion rates. Values are mean ± SE taken after 3 minutes of arterial infusion at each dose obtained in 4 separate dogs (n=4). * indicates a significant difference compared with steady state values (control) prior to infusion of L-arginine or D-arginine. $P<0.05$.

Table 5. Effect of L-arginine on the L-NAME-induced changes in mean arterial blood pressure, jejunal blood flow, vascular resistance, oxygen uptake, and motility index.

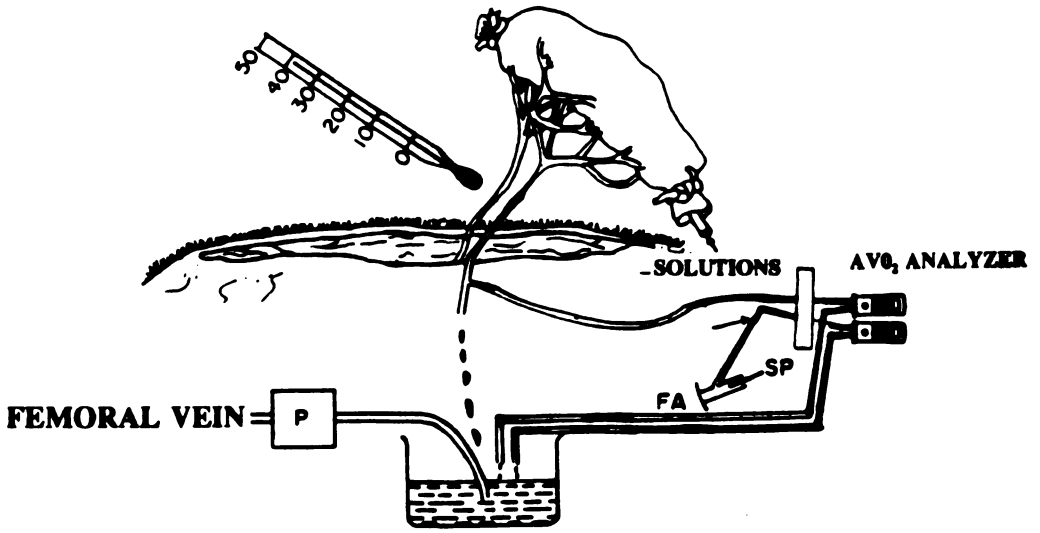
| | | | | | L-ARGININE | | |
|---|---|---|---|---|---|---|---|
| | 0 MIN. | 1 MIN. | 10 MIN. | 20 MIN. | 25 MIN. | 30 MIN. | 40 MIN. |
| MAP | 110±4 | 128±3* | 127±4* | 123±4* | 120±7* | 120±7* | 128±8* |
| BF | 56±6 | 47±8* | 58±6 | 70±5* | 89±4*# | 72±7 | 47±6# |
| R | 2.1±0.2 | 3.2±0.6* | 2.3±0.3 | 1.8±0.1 | 1.4±0.1*# | 2.0±0.3 | 2.9±0.4*# |
| $VO_2$ | 2.6±0.2 | 2.4±0.2 | 3.2±0.3 | 3.1±0.3 | 3.1±0.4* | 3.0±0.3* | 2.7±0.2 |
| MI | 1±1 | 4±2 | 49±3* | 40±4* | 19±5*# | 14±5*# | 9±6# |

The effect of L-arginine (97 mg/min) on the changes in mean arterial blood pressure (MAP), vascular resistance (R), oxygen uptake ($VO_2$), and motility index (MI) produced by L-NAME (10 mg/kg iv). Twenty minutes after systemic injection of L-NAME, L-arginine was continuously infused into the single jejunal artery for 20 minutes. Values are means ± SE obtained from 6 separate dogs. * indicates a significant difference from control (before L-NAME), and # indicates a significant difference from 20 minutes after injection of L-NAME. $P<0.05$.

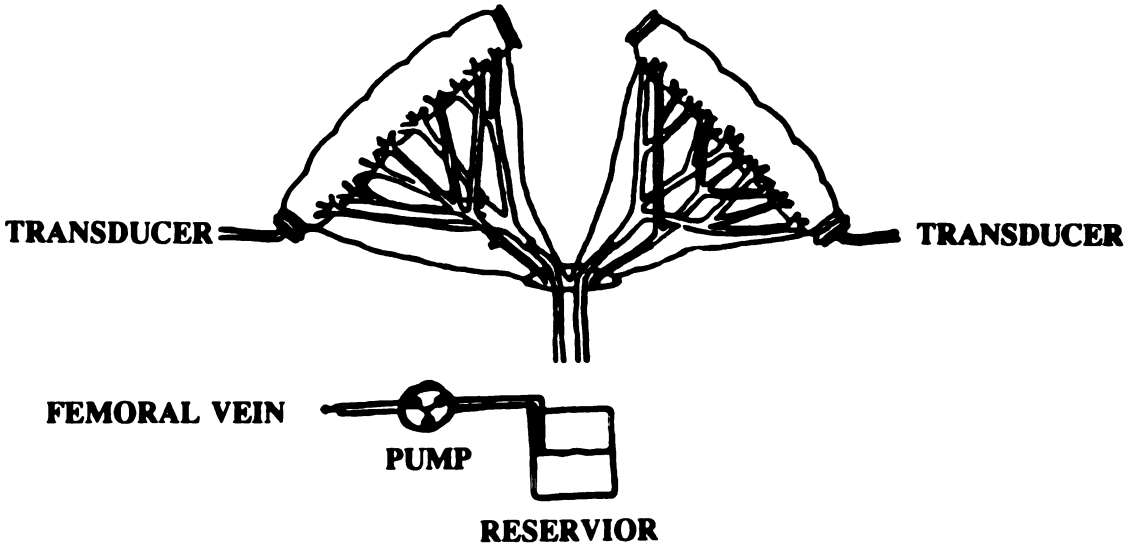
Table 6. Effect of D-arginine on the L-NAME-induced changes in mean arterial blood pressure, jejunal blood flow, vascular resistance, oxygen uptake, and motility index.

| | | | | | D-ARGININE | | |
|---|---|---|---|---|---|---|---|
| | 0 MIN. | 1 MIN. | 10 MIN. | 20 MIN. | 25 MIN. | 30 MIN. | 40 MIN. |
| MAP | 103±6 | 116±7* | 122±8* | 122±8* | 118±8* | 118±8* | 118±7* |
| BF | 49±5 | 39±4* | 49±5 | 61±6* | 62±8* | 62±9* | 62±10* |
| R | 2.2±0.2 | 3.5±0.7* | 2.7±0.4 | 2.1±0.3 | 2.0±0.3 | 2.0±0.4 | 2.0±0.5 |
| $VO_2$ | 1.8±0.2 | 1.8±0.3 | 2.0±0.1 | 2.3±0.2* | 2.3±0.2* | 2.3±0.4* | 2.5±0.2* |
| MI | 1±1 | 2±1 | 30±5* | 53±7* | 57±4* | 55±7* | 35±7* |

The effect of D-arginine (97 mg/min) on the changes in mean arterial blood pressure (MAP), vascular resistance (R), oxygen uptake ($VO_2$), and motility index (MI) produced by L-NAME (10 mg/kg iv). Twenty minutes after systemic injection of L-NAME, D-arginine was continuously infused into the single jejunal artery for 20 minutes. Values are means ± SE obtained from 6 separate dogs. * indicates a significant difference from control (before L-NAME), and # indicates a significant difference from 20 minutes after injection of L-NAME. $P<0.05$.




SINGLE IN SITU JEJUNAL SEGMENT PREPARATION




DOUBLE IN SITU JEJUNAL SEGMENT PREPARATION

Figure 9. Schematic of the surgical preparation utilizing either a single or double in situ jejunal segment.

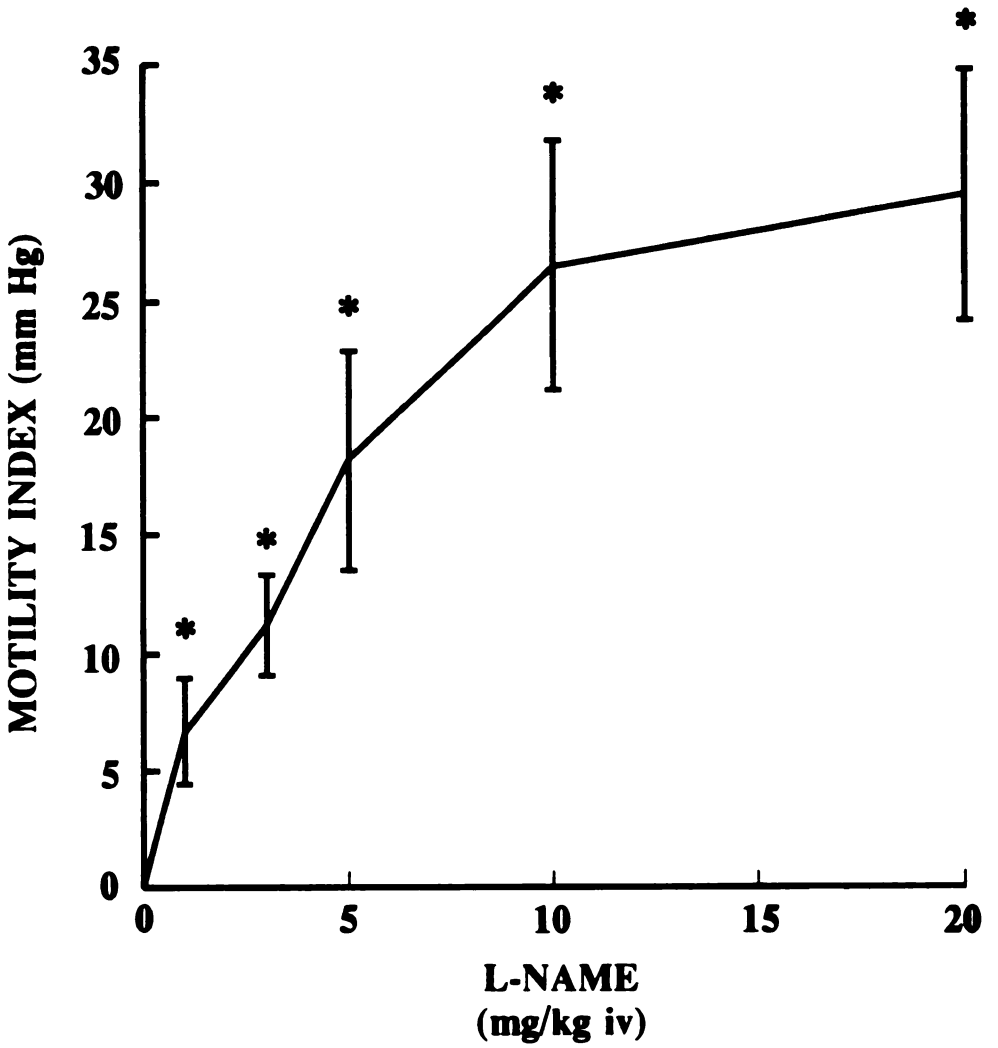



Figure 10. Dose-response of L-NAME (1-20 mg/kg iv) on the jejunal motility index. Values shown are mean ± SE (n=8) at steady state values at each dose of L-NAME. * indicates statistical difference from control values taken prior to administration of L-NAME. $P<0.05$.

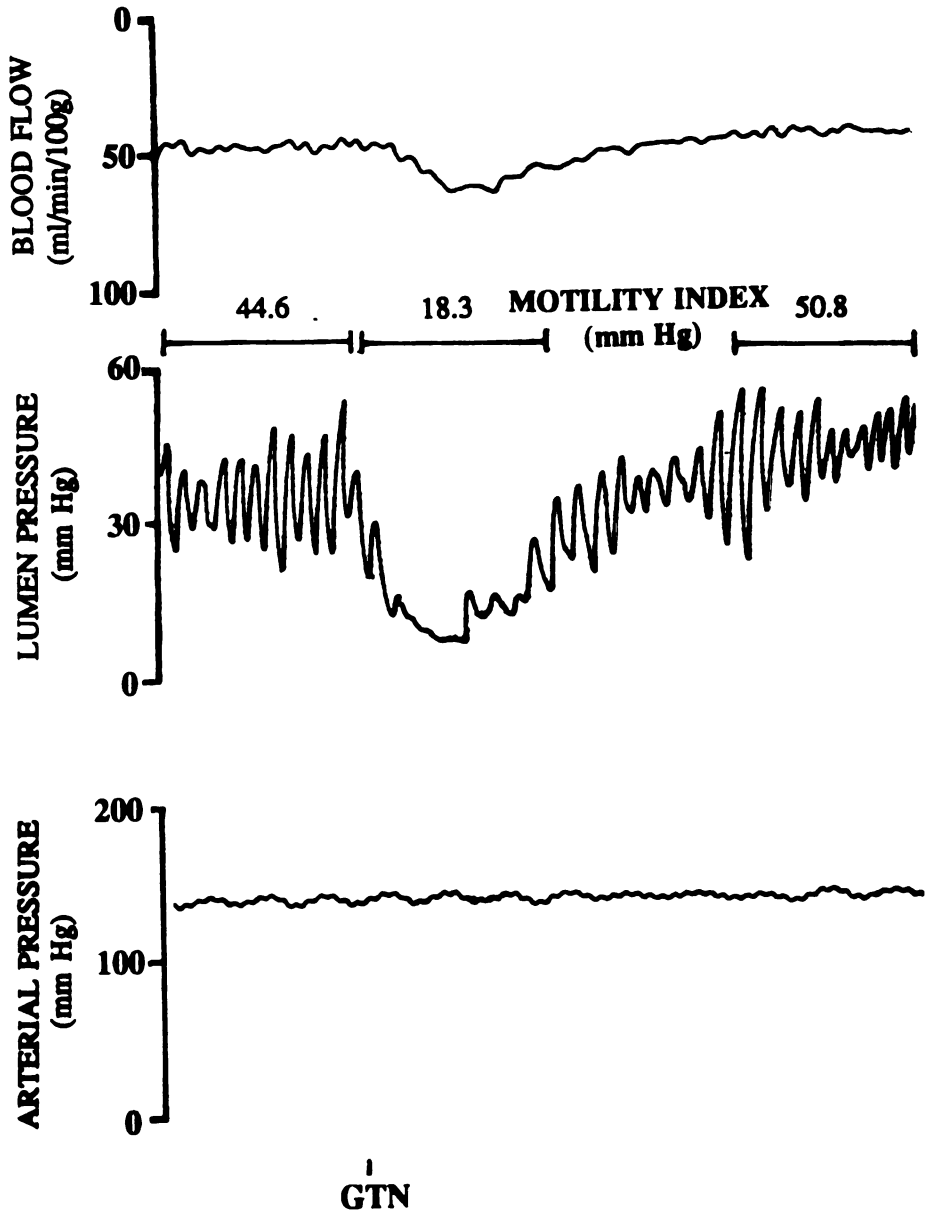



Figure 11. Effect of nitroglycerin on jejunal blood flow, motility index and mean arterial pressure. Topical application of 3ml nitroglycerin (0.4 mg/ml) to the serosal surface of the jejunal segment produced a transcient increase in blood flow and decrease in the lumen pressure, but had no effect on arterial pressure. Lines beneath values of motility index represent a time interval of 1 minute.

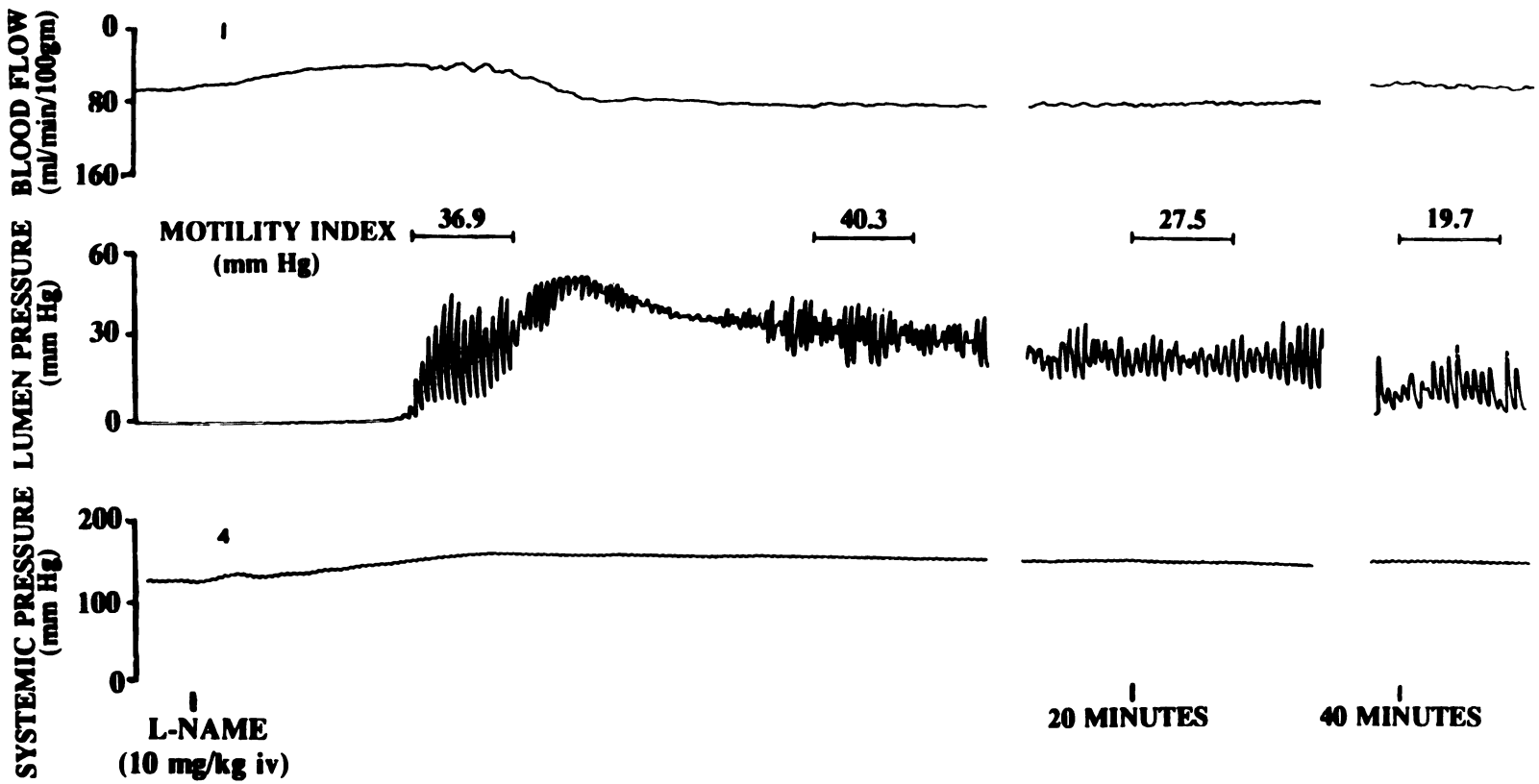



Figure 12. Experimental recording of the effect of L-NAME (10 mg/kg) on jejunal blood flow, motility index, and mean arterial blood pressure. L-NAME significantly increased mean arterial pressure and decreased jejunal blood flow. This was followed by a marked increase in the motility index. Note the increase in jejunal blood flow as motility increases to a maximum and then begins to decline. Lines beneath values of motility index represent a time interval of 1 minute.

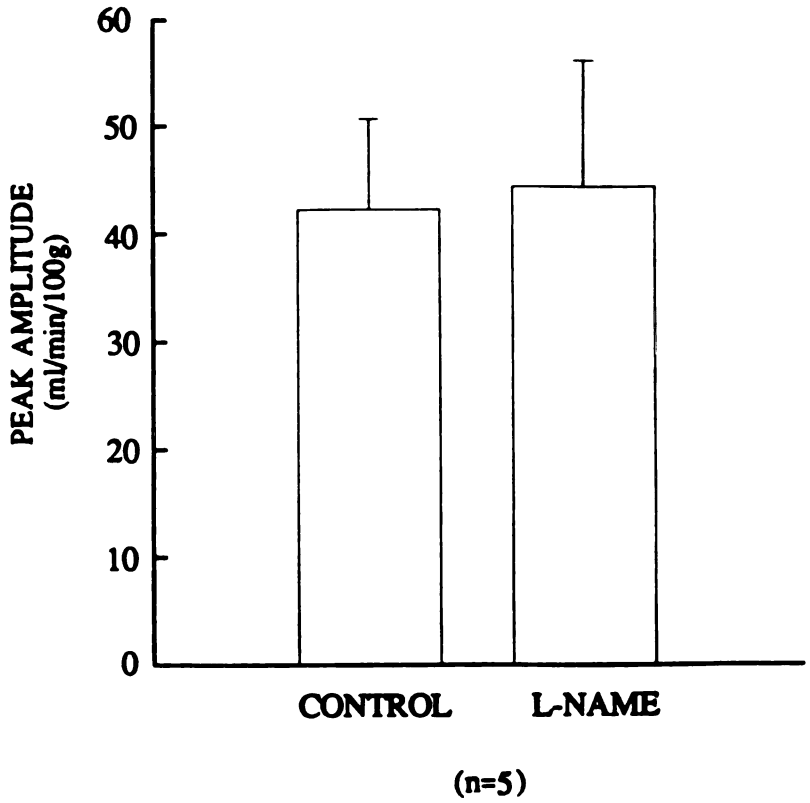



Figure 13. Effect of L-NAME (10 mg/kg iv) on the peak reactive hyperemic response after release of arterial and venous occlusion for 30 seconds. Values reported as means ± SE obtained in 5 separate dogs. The reactive hyperemic response before administration of L-NAME and 10 minutes after injection of L-NAME did not statistically differ. $P>0.05$.

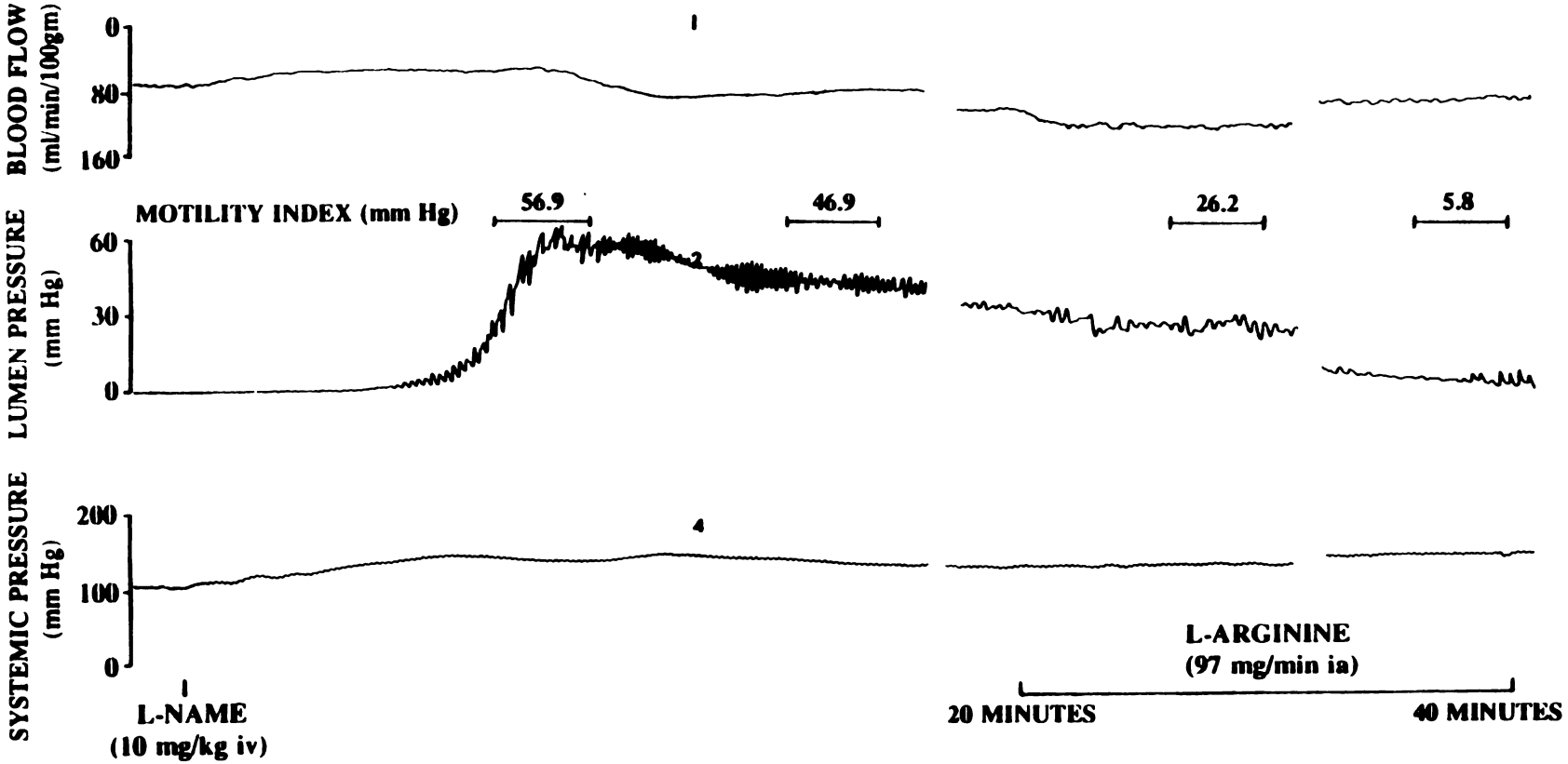



Figure 14. Experimental recording demonstrating the typical effect of arterial infusion of L-arginine (97 mg/min) on the L-NAME-induced changes in jejunal blood flow, motility index, and mean arterial blood pressure. Intestinal motility was nearly abolished after arterial infusion of L-arginine for 20 minutes. Lines beneath motility index values represent a time interval of 1 minute.

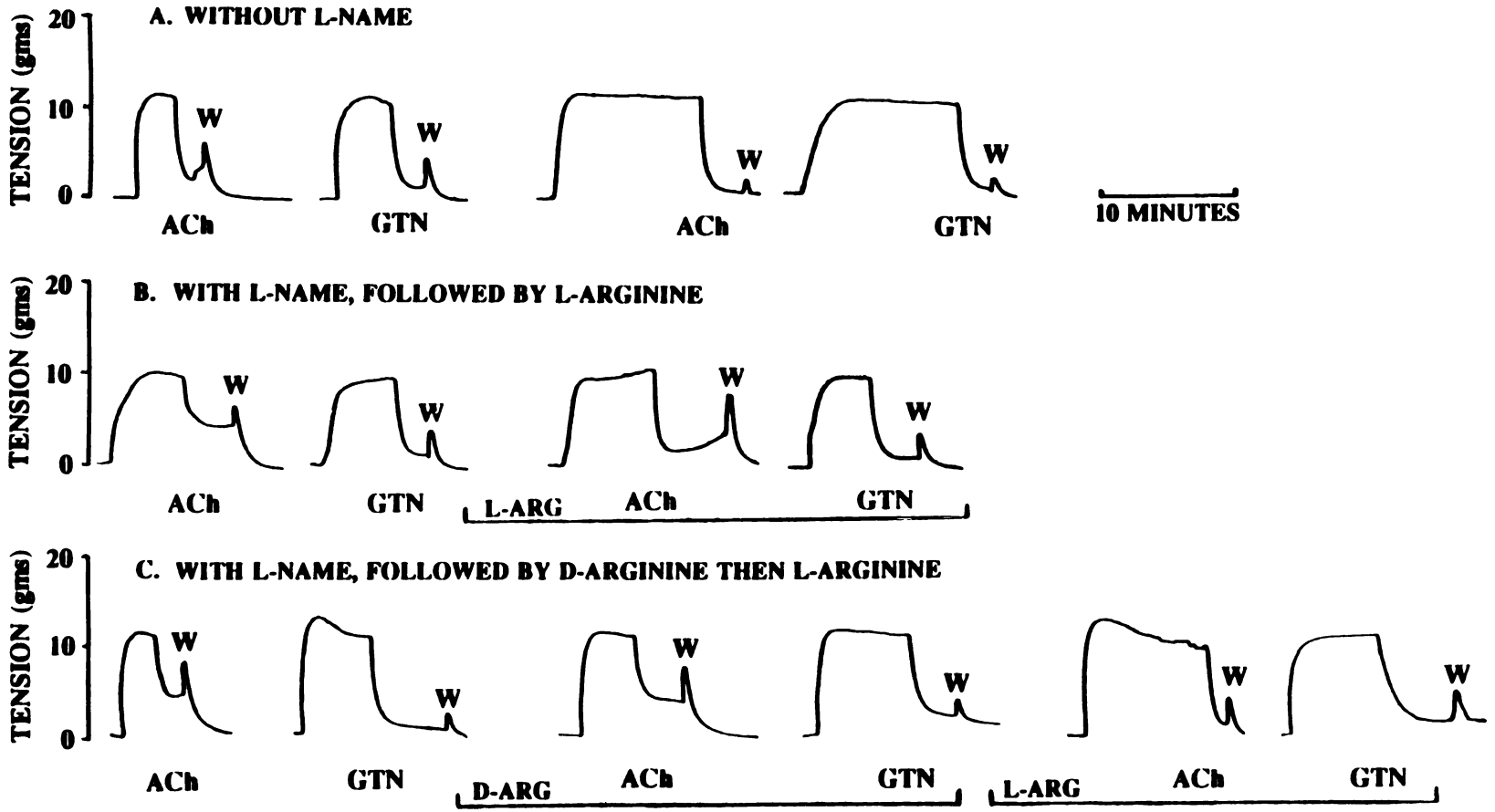



Figure 15. Experimental recording demonstrating the effect of L-NAME (10 mg/kg iv) on the relaxant effect of acetylcholine (ACh; 1 uM) or nitroglycerin (GTN; 0.2 uM) in superior mesenteric arterial rings obtained from an untreated dog (A), or two separate dogs minutes after administration of L-NAME (B and C). L-NAME selectively inhibited the relaxation produced by ACh, but did not inhibit the relaxation produce by nitroglycerin. The inhibitory effect of L-NAME on the relaxation produced by ACh was reversed after incubation with L-arginine, but not with D-arginine. W indicates the washout of Kreb's media bathing the arterial rings.

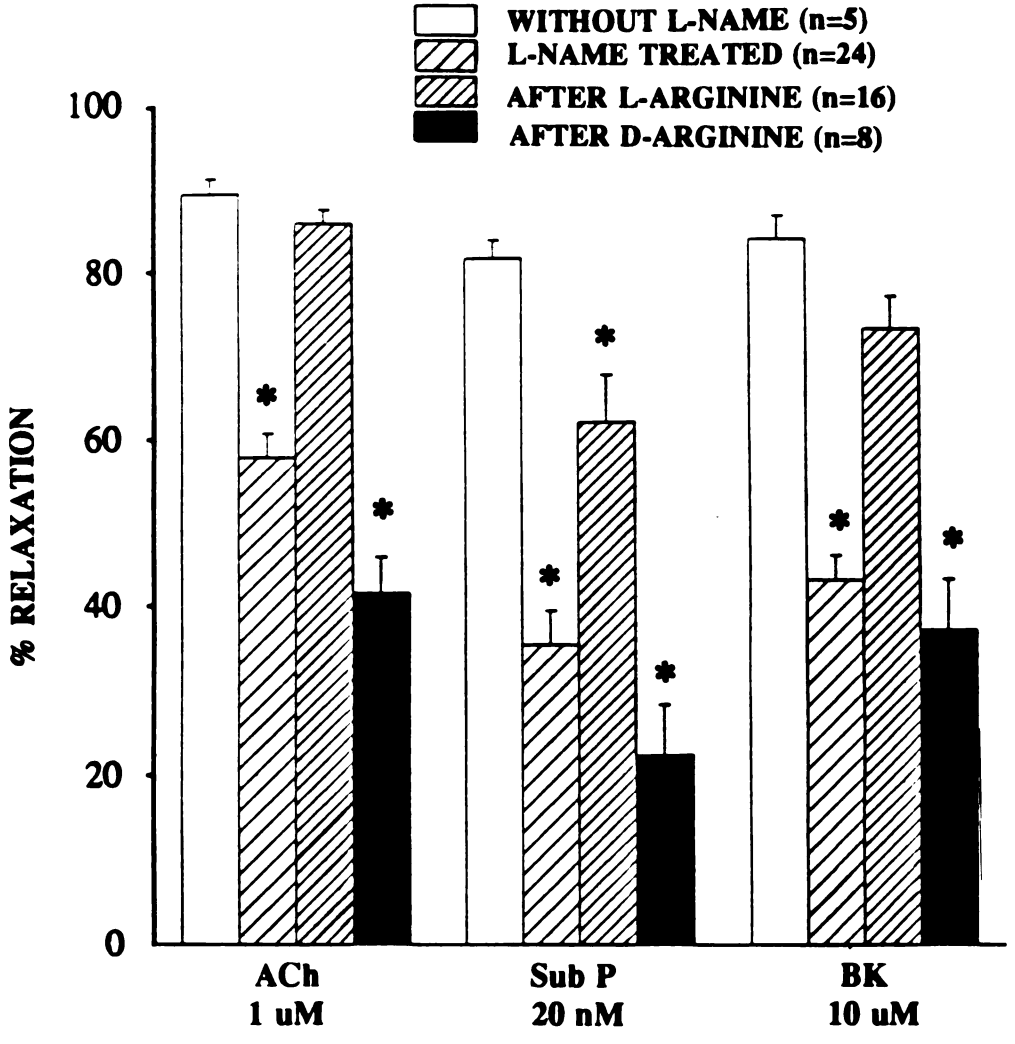



Figure 16. Effect of L-NAME (10 mg/kg iv) on the relaxation of canine mesenteric arterial rings produced by acetylcholine (ACh; 1 uM), substance P (sub P; 0.2 uM), and bradykinin (BK; 10 uM) in vitro. Vascular rings were preconstricted with 0.6 uM norepinephrine and the relaxant response to each agent was tested before and after incubation with either 300 uM L-arginine or 300 uM D-arginine. Control responses to ACh, sub P, and BK were obtained from 5 dogs that were not treated with L-NAME. Values are means $\pm$ SE and expressed as percent relaxation of the active tension produced by NE. * indicates a significant difference from control responses (rings that were not exposed to L-NAME). $P<0.05$.

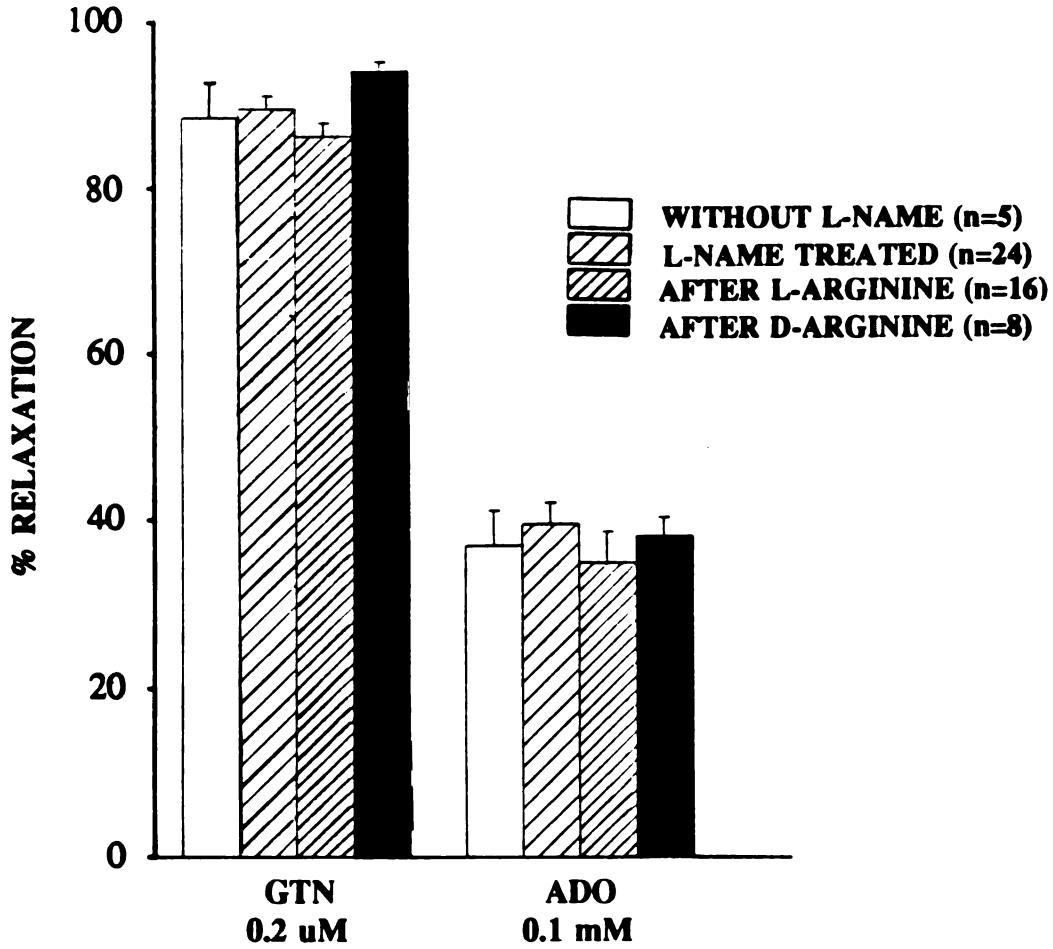



Figure 17. Effect of L-NAME (10 mg/kg iv) on the relaxation of canine mesenteric arterial rings produced by nitroglycerin (GTN; 0.2 uM) or adenosine (ADO; 0.1 mM) in vitro. Vascular rings were preconstricted with 0.6 uM norepinephrine and the relaxant response to each agent was tested before and after incubation with either 300 uM L-arginine or 300 uM D-arginine. Values are means $\pm$ SE and expressed as percent relaxation of the active tension produced by NE. Control responses to ACh, sub P, and BK were obtained from 5 dogs that were not treated with L-NAME.

# The Role of Nitric Oxide in the Vasodilation Produced by Substance P in vivo

## Introduction

Using the L-arginine analogue $N^G$-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthesis (157), we have shown that inhibition of nitric oxide synthesis produced an increase in mean systemic arterial pressure and intestinal motility, and an initial increase in jejunal vascular resistance. Also systemic administration of L-NAME (10 mg/kg iv) significantly attenuated the relaxation of superior mesenteric arterial rings produced by acetylcholine (ACh), substance P, and bradykinin in vitro. These results suggest that nitric oxide plays physiologic role of nitric oxide in the regulation of jejunal blood flow and motility in vivo. However, we could not determine whether the L-NAME-induced effects was a result of local inhibition of nitric oxide within the jejunum.

Nitric oxide has also been proposed to mediate the endothelium-dependent dilation in the mesenteric vascular bed produced by various endogenous vasodilator substances such as ACh (133). However, the experimental evidence is so far based almost exclusively on studies of large conduit arteries (i.e. the superior mesenteric artery) in vitro and in isolated mesenteric vascular beds perfused with Kreb's solution (12,102,133). New evidence has suggested that nitric oxide functions to regulate the basal tone of resistance vessels in the blood-perfused rabbit hindlimb, but nitric oxide does not mediate the dilation produced by ACh or substance P in vivo (134). Whether inhibition of nitric oxide synthesis attenuates the vasodilation produced by endothelium-dependent vasodilator agents in the blood-perfused mesenteric vascular bed has not yet been determined.

In this study, we further investigated the effect of L-NAME on jejunal vascular resistance

and motility in vivo. In addition, we also investigated the effect of L-NAME on the dilator response to substance P. Experiments were designed to answer the following questions: 1) Does local arterial infusion of L-NAME into an isolated mesenteric vascular bed increase vascular resistance and motility ?, 2) Is the motility produced by L-NAME a result of enhanced cholinergic activity ? and 3) Does systemic administration of L-NAME inhibit the vasodilation produce by substance P in vivo?

## Methods

Fourteen mongrel dogs (15.4-23.4 kgs) of either sex were anesthetized with pentobarbital sodium (30 mg/kg iv), and a jejunal segment (23.3-33.3 g, average 27.9$\pm$1.5 g) was isolated as previously described in chapter 5. After administration of heparin (500 Units/ml), the single vein draining and the single artery perfusing the jejunal segment were cannulated. The jejunal segment was perfused with aortic blood using an extracorporeal shunt placed between the jejunal artery and the left femoral artery. Once a steady state in mean arterial blood pressure and jejunal blood flow was achieved under free flow condition, the segment was then perfused at a constant rate with aortic blood using a Masterflex pump (Cole-Parmer Instruments, Chicago, IL) placed into the extracorporeal shunt. Jejunal perfusion pressure was monitored via a catheter in the arterial perfusion line attached to a pressure transducer (Statham P236b). In each dog, pump flow rate was adjusted until the jejunal perfusion pressure was equal to systemic arterial blood pressure and then maintained constant throughout the experiment. Two series of experiments were performed. *Series 1.* The purpose of this series of experiments (n=5) was to determine the effect of local arterial infusion of L-NAME on jejunal vascular resistance and motility. Under conditions of constant blood flow, L-NAME was infused directly into the single artery perfusing the segment at a rate of 3.1 mg/min. for 6 minutes, since preliminary studies showed that this was sufficient

to produce a longlasting increase in resistance and motility. Mean arterial blood pressure, jejunal perfusion pressure, and lumen pressure were continuously monitored throughout the experiment. After control perfusion pressure and lumen pressure were determined under conditions of constant blood flow (66 $\pm$ 11 ml/min/100g), L-NAME (8 mg/ml) was locally infused into the single artery perfusing the jejunal segment upstream from the Masterflex pump at a rate of 0.39 ml/min for 6 minutes. The total amount of L-NAME infused in a given dog ranged between 19-25 milligrams. Systemic administration of L-NAME at this dose (0.5-0.1 mg/kg iv) was previously shown to have no affect on jejunal blood flow or motility. Systemic arterial blood pressure and heart rate were continuously monitored to confirm that nitric oxide was not inhibited systemically. In addition, we diverted the venous effluent that was collected during infusion of L-NAME and then discarded the blood (average 50 mls) to be certain that L-NAME was not distributed systemically. Once the effect of L-NAME on jejunal perfusion pressure and intraluminal pressure had reached a steady state (15-25 minutes), nitroglycerin (80 ug/ml; 0.1 mM) and atropine (200 ug/ml; 0.3 uM) were separately injected (0.2 ml bolus) intra-arterially upstream from the Masterflex pump. The decrease in perfusion pressure and motility produced by nitroglycerin and atropine were determined. After the transcient effect of bolus injection of nitroglycerin or atropine were determined, L-arginine (300 mg/ml) was locally infused into the single artery perfusing the segment at a rate of 0.51 ml/min). The duration of L-arginine infusion was between 7 and 10 minutes.

*Series 2.* The objective of this series of experiments was to determine the effect of nitric oxide synthesis inhibition on the vasodilation produced by substance P (n=9). The surgical preparation was the same as that used in series 1. After perfusion pressure and lumen pressure had been determined under constant flow conditions (61$\pm$9 ml/min/100g), dose response curves to bolus injections of ACh, substance P, and nitroglycerin were obtained. In preliminary experiments we

had determined that ACh (1nM-10 uM), substance P (0.1-100 nM), and nitroglycerin (1nM-10uM) produced a dose-dependent decrease in jejunal vascular resistance without affecting mean arterial blood pressure. These doses were then used in the definitive experiments described here. Each vasodilator was injected upstream from the perfusion pump in an sequential order from lowest to highest dose. The vasodilation produced by 100 nM substance P was very large and slowly returned to control over a period of 6-9 minutes. Therefore, substance P was always the last vasodilator agent to be tested. The order that ACh and nitroglycerin were tested was randomly selected in each experiment. Normal saline, which served as the vehicle for each vasodilator agent, was also injected and found to have no effect on jejunal perfusion pressure. After the control responses to ACh, substance P, and nitroglycerin were obtained, L-NAME (10 mg/kg iv) was injected into the femoral vein and systemic arterial pressure, jejunal perfusion pressure, and lumen pressure were continuously measured. In our preliminary studies, we found that we could not accurately determine the effect of L-NAME on the vasodilator response to ACh, substance P, or nitroglycerin because of the motility produced by L-NAME markedly affected jejunal perfusion pressure. Therefore, once the effects of L-NAME on jejunal perfusion pressure and motility had reached a steady state (between 5-9 minutes), atropine was locally infused into the jejunal segment upstream from the Masterflex pump with a Harvard infusion pump set at a flow rate ranging between 20-50 ug/min. This concentration of atropine was found to effectively inhibit the phasic contractions of intestinal smooth muscle and increase in luminal tone produced by L-NAME. When perfusion pressure and lumen pressure had reached a steady state after systemic injection of L-NAME and during atropine infusion, the vasodilator action of ACh, substance P, and nitroglycerin were then repeated and compared to the vasodilation obtained prior to injection of L-NAME and arterial infusion of atropine. The increase in systemic arterial blood pressure and the decrease in heart rate produced by L-NAME was thus used to indicate that nitric oxide

synthesis remained effectively inhibited during atropine infusion.

Heart rate was derived from the continuous recording of pulse pressure. From steady-state intraluminal pressure tracings, motor activity was quantitated. The motility index was calculated by dividing the sum of the heights of all the pressure wave peaks by the number of all the pressure peak waves during a 1 minute period. Jejunal vascular resistance was calculated by dividing steady state jejunal perfusion pressure by jejunal blood flow. The maximum vasodilation produced by each agent at each concentration was calculated as the percent change in vascular resistance, i.e. the difference of the maximum decrease in resistance and resting resistance divided by the resting resistance.

*Preparation of chemicals.* Atropine sulphate, L-arginine hydrochloride, the L-arginine analogue L-NAME, acetylcholine hydrochloride, and substance P were obtained from Sigma Chemical Co. (St. Louis, MO). Nitroglycerin (Nitrostat, 0.4mg tablets, Parke-Davis, Morris Plains, NJ) was purchased from a local apothecary. All drugs were dissolved in normal saline and serially diluted to final concentrations the day of the experiment.

*Statistics.* Values in figures and tables are expressed as mean $\pm$ SE and represent paired of unpaired data, where n indicates the number of dogs. When multiple comparisons with a common control were made, the difference between the mean of each group was evaluated by analysis of variance. If the analysis of variance showed a significant difference among the means, then statistical differences between individual groups were determined using the least significant difference (LSD) test. Statistical significance for paired and unpaired observations was determined using a paired or unpaired two tailed Student's T test.

Results

Original tracings of the parameters recorded continuously with the present technique are shown in Figures 18-20. They illustrate the vascular and motility responses to L-NAME after local infusion (3.1 mg/min for 6 min) or systemic administration (10 mg/kg iv). Arterial infusion of L-NAME increased jejunal perfusion pressure and, therefore, increased vascular resistance (Figure 18). L-NAME also stimulated a marked increase in motility as indicated by the increase in basal lumen pressure and the rhythmic contractions of intestinal smooth muscle. The maximum increase in perfusion pressure produced by L-NAME often occurred at the onset of intestinal motility between 2-4 minutes after arterial infusion of L-NAME. The increase in jejunal vascular resistance and motility persisted for up to an hour after injection of L-NAME. Local infusion of L-NAME did not affect systemic arterial blood pressure or heart rate.

The effect of nitroglycerin or atropine on the vasoconstriction and motility produced by L-NAME are shown in Figure 19A. Bolus injection of nitroglycerin produced a vasodilation and abolished the motility produced after inhibition of nitric oxide synthesis. Similarly, injection of atropine produced a vasodilation and blocked the motility produced by L-NAME. The effect of nitroglycerin was transcient and lasted for about 45 seconds, whereas the effect of atropine was longer in duration, lasting from 3-6 minutes. Continuous arterial infusion of L-arginine reversed the motility produced by L-NAME, but did not significantly alter the vasconstriction produced by L-NAME (Figure 19B). The effect of nitroglycerin, atropine, and L-arginine on jejunal vascular resistance and the motility index after local infusion of L-NAME are summarized in Table 7.

Systemic administration of L-NAME (10 mg/kg) significantly increased mean arterial pressure by an average of 18 $\pm$ 5 mm Hg and significantly decreased heart rate by 43 $\pm$ 20 beats per minutes. L-NAME significantly increased the jejunal perfusion pressure by 61 $\pm$ 13 mm Hg, indicating that vascular resistance was increased by 48 $\pm$ 15% (significantly different from control,

$p<0.05$.). The vasoconstriction and motility produced by systemic injection of L-NAME produced a substantial increase in local perfusion pressure, and therefore, increased jejunal vascular resistance (Figure 20). The vasoconstrictor effect of L-NAME was rapid in onset (between 1-2 minutes) and was shortly followed by a significant increase in motor activity, as indicated by the marked increase in the motility index. The vasoconstriction and motility produced by local arterial infusion of L-NAME (Figure 18) was similar to that produced after systemic injection of L-NAME. However, local infusion of L-NAME did not affect systemic arterial blood pressure or heart rate. Continuous arterial infusion of atropine almost completely abolished the motility produced after systemic injection of L-NAME. The inhibitory effect of continuous infusion of atropine on the phasic contractions of intestinal smooth muscle was observed for more than 40 minutes. Jejunal perfusion pressure decreased slightly upon infusion of atropine and then gradually returned to levels similar to that observed after systemic administration L-NAME over a period of 5-7 minutes. Local arterial infusion of atropine into the isolated jejunal segment did not significantly alter the hypertension or the decrease in heart rate produced by L-NAME. Table 8 summarizes the effect of systemic administration of L-NAME (10 mg/kg iv) prior to and during infusion of atropine on mean arterial blood pressure, heart rate, jejunal perfusion pressure, vascular resistance and the motility index.

The effect of L-NAME during arterial infusion of atropine on the relaxation produced by ACh, substance P and nitroglycerin are shown in Figures 21, 22, and 23, respectively. Prior to administration of L-NAME (control), ACh, substance P, and nitroglycerin each produced a dose-dependent vasodilation in the mesenteric vascular bed. The vasodilation produced by ACh was significantly inhibited after injection of L-NAME and during atropine infusion. However, the relaxation produced by substance P and nitroglycerin were not significantly inhibited after L-NAME and during atropine.

Discussion

In the present study, we demonstrate that the L-arginine analogue L-NAME, an inhibitor of nitric oxide synthesis (157), increased jejunal vascular resistance and stimulated intestinal motility under conditions of constant blood flow. The vasoconstriction and motility produced by L-NAME was identical regardless of the route of administration, i.e. local arterial infusion or systemic intravenous injection. However, local arterial infusion of L-NAME did not affect systemic blood pressure or heart rate. As shown in Figure 18, arterial infusion of L-NAME (3.1 mg/min) markedly increased jejunal perfusion pressure, and therefore vascular resistance, without affecting systemic arterial pressure. L-NAME also produced a marked increase in motility, as shown by the increase in phasic, rhythmic contractions of intestinal smooth muscle and increase in basal luminal pressure. This data indicates that the vasoconstriction and motility produced by L-NAME results from local inhibition of nitric oxide synthesis within the isolated jejunal segment and is in agreement with the proposal that the action of nitric oxide is very close to its site of synthesis.

The vasoconstriction produced by local infusion of L-NAME in this study is consistent with results reported previously in peripheral vascular beds of cats (37) and humans (185,186) after infusion of L-NMMA in vivo. Arterial infusion L-NMMA significantly increased the resistance of the skeletal muscle vascular bed by approximately 100% within 5 minutes after beginning infusion of L-NMMA (37,185,186). The constrictor effect of L-NMMA was long-lasting and dissappeared gradually over a period of an hour (37,185). In addition, L-NMMA was shown to effectively attenuate the vasodilation produced by exogenously infused ACh (37,185,186) but not to nitroglycerin (185,186). These observations suggest that the formation of nitric oxide from L-arginine plays an important role in regulating the tone of resistance vessels

in the skeletal muscle bed and the vasodilation produced by ACh in vivo.

Bolus injection of nitroglycerin, which has been shown to relax vascular and nonvascular smooth muscle through the release of nitric oxide (1), reversed the vasoconstriction and motility produced by L-NAME (Figure 19A and Table 7). The vasodilation and relaxation of intestinal smooth muscle produced by nitroglycerin was transcient and lasted for no more than 1 minute after injection. These results provide further evidence that the motility and vasoconstriction produced by L-NAME is a result of nitric oxide synthesis inhibition and that L-NAME does not affect the action produced by injection of an exogenous source of nitric oxide.

Like nitroglycerin, bolus injection of atropine effectively reversed the motility after inhibition of nitric oxide synthesis produced by local arterial infusion of L-NAME. Injection of atropine was also associated with a decrease in jejunal perfusion pressure and vascular resistance. However, the decrease in perfusion pressure produced by atropine was less than that observed after bolus injection of nitroglycerin and perfusion pressure quickly returned to values comparable to that seen after L-NAME infusion as phasic intestinal smooth muscle contractions reappeared. Similarly, continuous arterial infusion of atropine also produced a slight decrease in perfusion pressure (Figure 20). However, the decrease in perfusion pressure observed during continuous infusion of atropine remained as did the attenuation in intestinal motility. This suggests that the motility produced by nitric oxide synthesis inhibition indirectly increases the vascular resistance of the isolated jejunal segment. Our data indicates that the motility produced by nitric oxide synthesis inhibition results from enhanced cholinergic responses, and suggests that endogenous nitric oxide plays a physiological role in suppressing cholinergic-mediated intestinal motility in vivo. Results similar to our have been recently reported in anesthetized rats (17). Calignano et al (17) have demonstrated that systemic administration of atropine (4 mg/kg iv) effectively attenuated the frequency and amplitude of the phasic contractions produced by injection of L-

NAME (10 mg/kg iv).

Endogenous nitric oxide has been proposed to mediate the nerve-mediated hyperpolarization and relaxation of intestinal smooth muscle of dogs, guinea pigs and humans in vitro (11,122,171,179,181). This relaxation of intestinal smooth muscle produced by nerve stimulation is blocked by nitric oxide synthesis inhibitors (32,122,181), and is similar to the relaxation produced by exogenous nitric oxide or organic nitrates (32,179,181). These observations suggest that the jejunal motility produced by L-NAME in our study results from preventing the direct action of nitric oxide on the intestinal smooth muscle. Therefore, it would seem that the suppression of motility produced by endogenous nitric oxide results from a direct action of nitric oxide on the intestinal smooth muscle. However, we cannot rule out the possibility that nitric oxide acts presynaptically to suppress the cholinergic-mediated motility from our data.

Unlike the sustained inhibitory action of L-arginine on the L-NAME-induced increase in jejunal motility, L-arginine did not significantly alter the vasoconstriction produced by L-NAME (Figure 19B). This data is similar to that of our previous study where continuous arterial infusion of L-arginine for 20 minutes reversed the L-NAME-induced motility but did not reverse the vasoconstriction produced by systemic administration of L-NAME. It thus appears that the effect of L-arginine on L-NAME-induced vasoconstriction is different from its effect on the increase in jejunal motility. The inability of L-arginine to reverse the vasconstriction produced by L-NAME is not clear. Gardiner et al. (57-60) have reported that the action of L-arginine on the L-NMMA-induced vasoconstriction is complete in the kidney, but the reversal of vasoconstriction in the superior mesenteric, internal carotid and hindlimb vascular beds is transient and incomplete. Therefore, the inhibitory action L-arginine on the vasoconstriction produced by L-NMMA or L-NAME might differ among various organs. Furthermore, nitric oxide synthase has been localized

within endothelial cells as well as enteric nerves (15). A difference in the nitric oxide synthase enzyme subtype of different tissues may also explain the differential effect of L-arginine on the L-NAME-induced increase in jejunal vascular resistance and motility.

L-NMMA and L-NOARG have been shown to attenuate the dilation produced by ACh in the isolated mesenteric vascular bed perfused with Kreb's solution (133). Although ACh is typically the "gold standard" used to evaluate the role of nitric oxide on vascular smooth muscle relaxation, we were unable to use ACh for this purpose because we utilized a continuous infusion of atropine to supress the intestinal motility produced by L-NAME. Therefore, we used substance P to determine if systemic administration of L-NAME (10 mg/kg iv) inhibited the nitric oxide-induced relaxation in the jejunal vascular bed. We chose to use substance P for several reasons. First, substance P has been shown to enhance the release of nitric oxide from rabbit aortic endothelial cells in vitro, and L-NMMA inhibits the relaxation and nitric oxide release produced by substance P (155). Moreover, we have shown that the relaxation of the superior mesenteric arterial rings produced by substance P was inhibited by after incubation of the arterial rings with either L-NAME or methylene blue. In addition, we have demonstrated that systemic administration of L-NAME (10 mg/kg iv) effectively inhibits the relaxation of mesenteric arterial rings produced by substance P in vitro. Secondly, substance P is a potent vasodilator when infused into the mesenteric vascular bed of dogs or guinea pigs (56,149,189), and relaxation produced by substance P appears to result from its direct action on the vascular smooth muscle since tetrodotoxin and several neurotransmitter receptor antagonists do not influence the vasodilation produced by substance P (59,189). Thirdly, immunohistochemistry has shown an abundance of substance P-containing neurons in the vagus (119) and in perivascular nerves (54,118,145) of several mammals, including dog, guinea pig and human. Therefore, it is conceivable that release of substance P from these sources could stimulate the release of nitric

oxide from vascular endothelial cells. Lastly, substance P has been proposed to play a role in postprandial regulation of blood flow in the ileum (149).

As shown in Figures 22 and 23, the relaxation produced by substance P and nitroglycerin were not significantly affected by systemic administration of L-NAME. The lack of effect of L-NAME on the vasodilation produced by substance P is not clear. However, it is not likely to result from an insufficient amount of L-NAME necessary for inhibition of nitric oxide synthesis since we have shown beforehand that the vasoconstriction and motility produced at this concentration of L-NAME was maximum and not enhanced by higher concentrations of L-NAME. Similar results have been reported after injection of L-NAME or L-MMMA in conscious rats (59,60). Moreover, this concentration of L-NAME (10 mg/kg iv) is associated with a significant decrease in nitric oxide release from endothelial cells of the mesenteric artery, as indicated by the impaired responses to ACh, substance P, and bradykinin in vitro (see Figure 16). Consequently, the dilation of the mesenteric vascular bed produced by substance P under conditions of constant blood flow in vivo does not appear to be mediated by nitric oxide.

Using microsperes to measure blood flow to the rabbit hindlimb, Mugge et al. (134) have recently reported results similar the results of this study. These authors found that systemic administration of either L-NMMA or L-NOARG decreased hindlimb blood flow by about 46% and increased vascular resistance by about 23% (134). Furthermore, L-NMMA and L-NOARG effectively inhibited the relaxation of femoral arterial rings produced by ACh or substance P in vitro, but did not block the dilation produced by ACh or substance P in vivo (134). Preliminary experiments comparing the mechanism of ACh-induced relaxation between guinea pig mesenteric artery (>650 uM diameter) and mesenteric arterioles (250 uM diameter) in vitro also suggests that the mechanism of relaxation elicited by ACh in different size arteries may vary. Hwa and Chatterjee have demonstrated that incubation of the superior mesenteric artery with L-NMMA,

methylene blue, or oxyhemoglobin reduced the relaxation produced by ACh, but had no effect on the relaxation of mesenteric arterioles in vitro (86,87). These results are in agreement with our data and suggests that the dilation of mesenteric resistance vessels produced by substance P is mediated by a mechanism that is independent of nitric oxide synthesis.

In conclusion, the present study demonstrates that local inhibition of endogenous nitric oxide synthesis within the jejunum results in an increase in vascular resistance and intestinal motility. The increase in motility is reversed by arterial infusion of L-arginine or bolus injection of nitroglycerin, which is a source of nitric oxide that is not dependent on nitric oxide synthase (1). Furthermore, arterial infusion of atropine markedly attenuates the motility produced after inhibition of endogenous nitric oxide. Thus, endogenous nitric oxide may function to suppress motility mediated by cholinergic nerves in vivo. These findings support our previous results and suggest that endogenous nitric oxide plays a role in the regulation of intestinal blood flow and motility in vivo. However, inhibition of endogenous nitric oxide after systemic adminstration of L-NAME did not affect the vasodilation produced by substance P. This suggests that the mechanism of relaxation produced by substance P in situ is different from the nitric oxide-dependent relaxation produced by substance P in vivo.

Summary

We have shown that local arterial infusion or systemic intravenous administration of the L-arginine analogue L-NAME results in a marked increase in jejunal vascular resistance and intestinal motility in vivo. These observations suggest that endogenous nitric oxide plays an important role in the regulation of jejunal blood flow and intestinal motility in vivo. The effects of L-NAME on jejunal vascular resistance and motility are readily reversed by nitroglycerin, an organic nitrate that has been shown to act through the release of nitric oxide (1). In contrast, L-arginine effectively reversed the motility produced by L-NAME, but did not reverse the vasoconstriction produced by arterial infusion of L-NAME. The motility produced by nitric oxide synthesis inhibition is mediated by muscarinic, cholinergic receptors since atropine abolished the motility produced by L-NAME. These results are consistent with the concept that the basal tone of vascular and nonvascular smooth muscle in the intestine are regulated by the release of endogenous nitric oxide in vivo. However, systemic administration of L-NAME (10 mg/kg iv) did not significantly affect the vasodilator response to substance P in vivo. In contrast, we have previously demonstrated that systemic administration of L-NAME (10 mg/kg iv) effectively attenuated the relaxation of mesenteric arterial rings produced by substance P in vitro. This suggests that the mechanism of relaxation in response to substance P may be different in resistance vessels and large arteries, and the response of resistance vessels may involve mechanisms other than the synthesis and release of nitric oxide.

Table 7. Effect of nitroglycerin, atropine, and L-arginine on the L-NAME-induced changes in jejunal perfusion pressure and motility index.

| | PERFUSION PRESSURE | % CHANGE IN PERFUSION PRESSURE | MOTILITY INDEX |
|---|---|---|---|
| CONTROL | 104±9 | | 0 |
| L-NAME | 178±20* | +71±4%* | 43±3* |
| L-NAME | 134±13* | | 36±7* |
| GTN | 77±12# | -41±9%# | 3±2# |
| L-NAME | 122±15* | | 38±10* |
| ATROPINE | 99±21# | -23±11%# | 3±2# |
| L-NAME | 132±17* | | 33±8* |
| L-ARGININE | 120±14 | -10±7% | 9±6# |

Effect of arterial infusion of L-NAME (3.1 mg/min), followed by nitroglycerin, atropine, or L-arginine on jejunal perfusion pressure and the motility index in an isolated jejunal segment under conditions of constant blood flow. Once steady state values were obtained after L-NAME infusion, the effect of bolus injection (0.2 ml) of nitroglycerin (16 ug) and atropine (40 ug) were determined. The effect of arterial infusion of L-arginine (150 mg/min) on the L-NAME induced changes was then evaluated. Values are mean ± SE obtained from 5 dogs. *indicates a significant difference from control (steady state values obtained prior to infusion of L-NAME), and # indicates a significant difference from preceeding steady state values obtained after infusion of L-NAME. $P<0.05$.

Table 8. Effect of L-NAME and arterial infusion of atropine on mean arterial blood pressure, jejunal perfusion pressure, and motility index.

| | MAP | HEART RATE | JEJUNAL RESISTANCE | PERFUSION PRESSURE | MOTILITY INDEX |
|---|---|---|---|---|---|
| CONTROL | 139±4 | 171±13 | 1.8±0.3 | 143±11 | 1±1 |
| L-NAME | 158±6* | 126±14* | 2.6±0.5* | 221±9* | 42±6* |
| L-NAME + ATROPINE | 158±7* | 122±16* | 2.4±0.5* | 188±13*# | 7±1 |

The effect of systemic administration of L-NAME (10 mg/kg iv) on mean arterial blood pressure (MAP), heart rate, jejunal vascular resistance, perfusion pressure, and motility index under conditions of constant blood flow. After the onset of motility, atropine (range, 20-50 ug/min) was continuously infused into the single artery perfusing the jejunal segment. Values are mean ± SE obtained from 9 separate dogs. * indicates a significant difference from control (steady state values obtained before injection of L-NAME), and # indicates a significant difference from steady state values after injection of L-NAME. $P<0.05$.

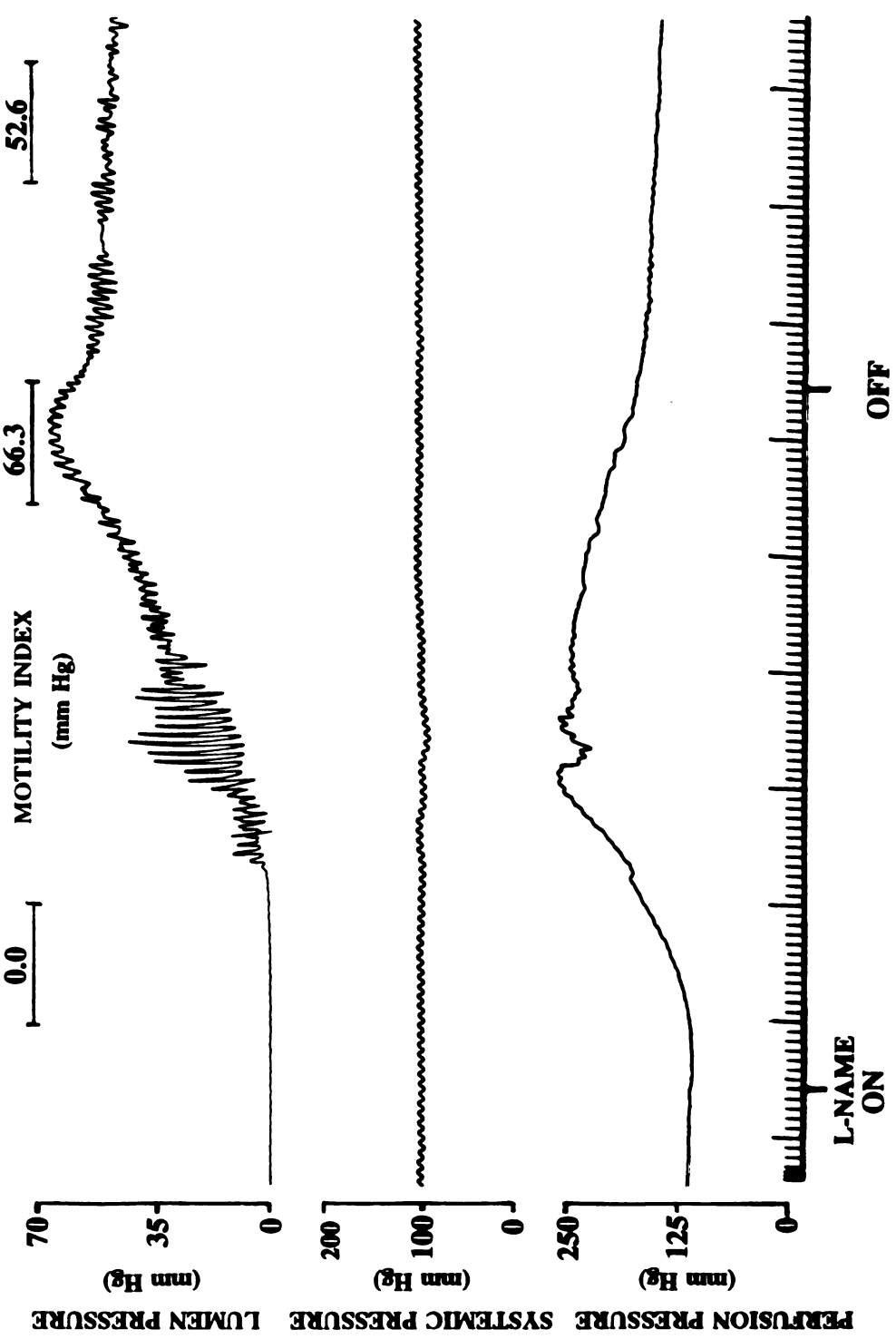



Figure 18. Experimental recording of the effect of local infusion of L-NAME on jejunal perfusion pressure, lumen pressure and systemic arterial pressure. L-NAME was infused for 6 minutes directly into the artery of the isolated jejunum at a rate of 3.1 mg/ml. Lines beneath values of motility index represent a time interval of 1 minute.

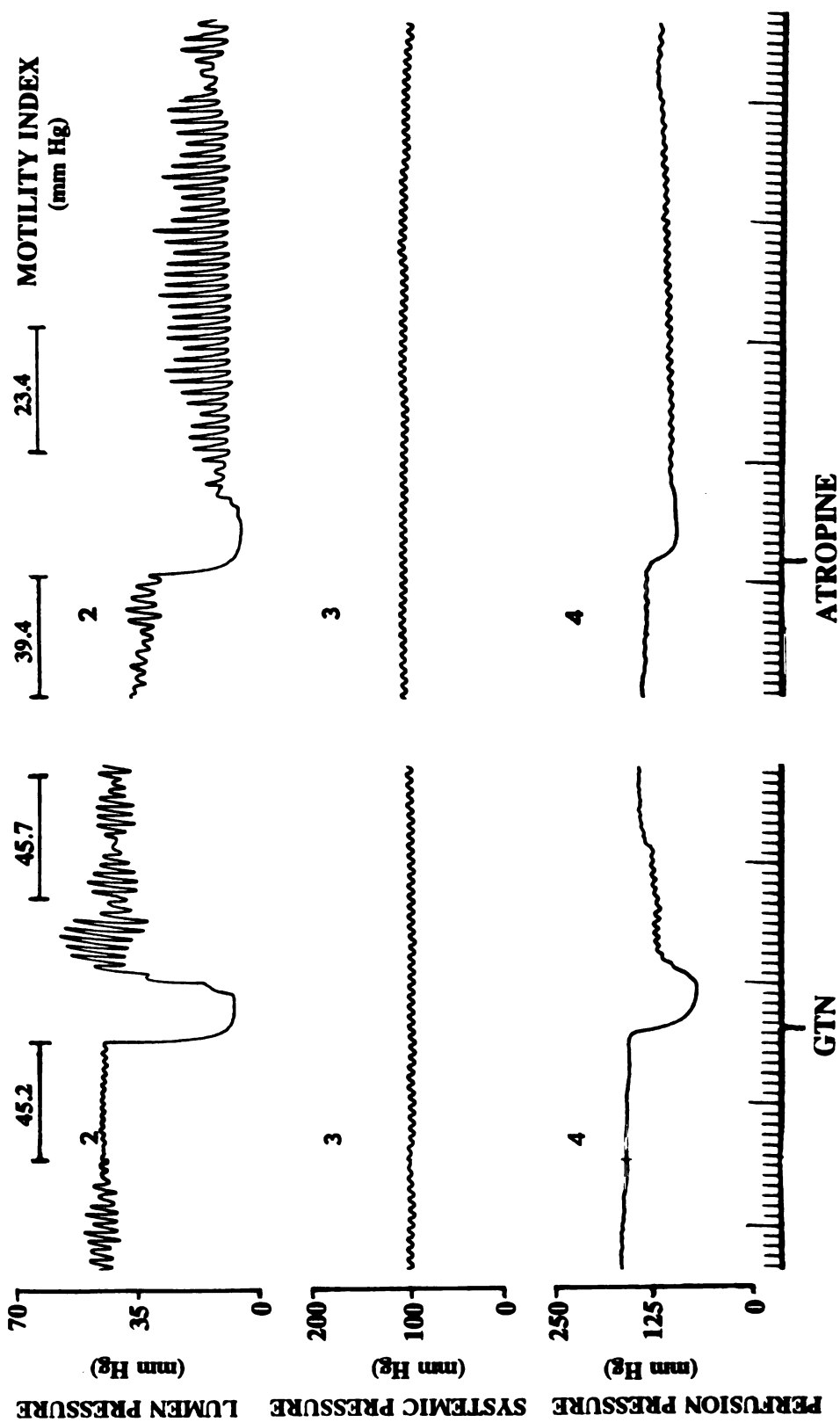



Figure 19A. Experimental recording of the effect of nitroglycerin and atropine on the changes in jejunal perfusion pressure, lumen pressure, and systemic arterial blood pressure produced by local infusion of L-NAME. Bolus injection of nitroglycerin or atropine decreased the perfusion pressure and reversed the motility produced by L-NAME. Lines beneath values of motility index represent a time interval of 1 minute.

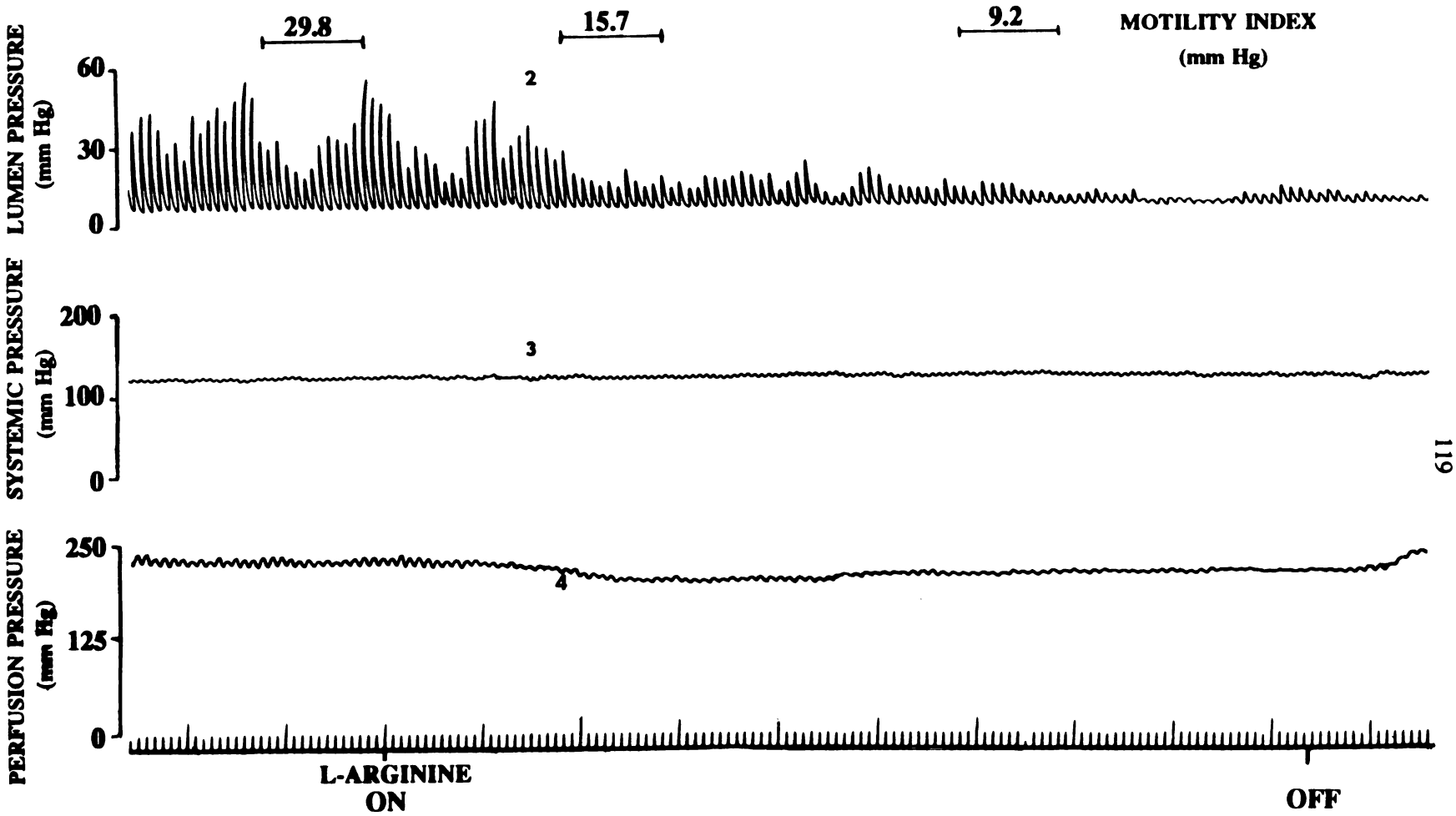





Figure 19B. Experimental recording of the effect of L-arginine on the changes in jejunal perfusion pressure, lumen pressure, and systemic arterial blood pressure produced by local infusion of L-NAME. L-arginine was continuosly infused at a rate of 60 mg/min. Lines beneath values of motility index respresent a time interval of 1 minute.

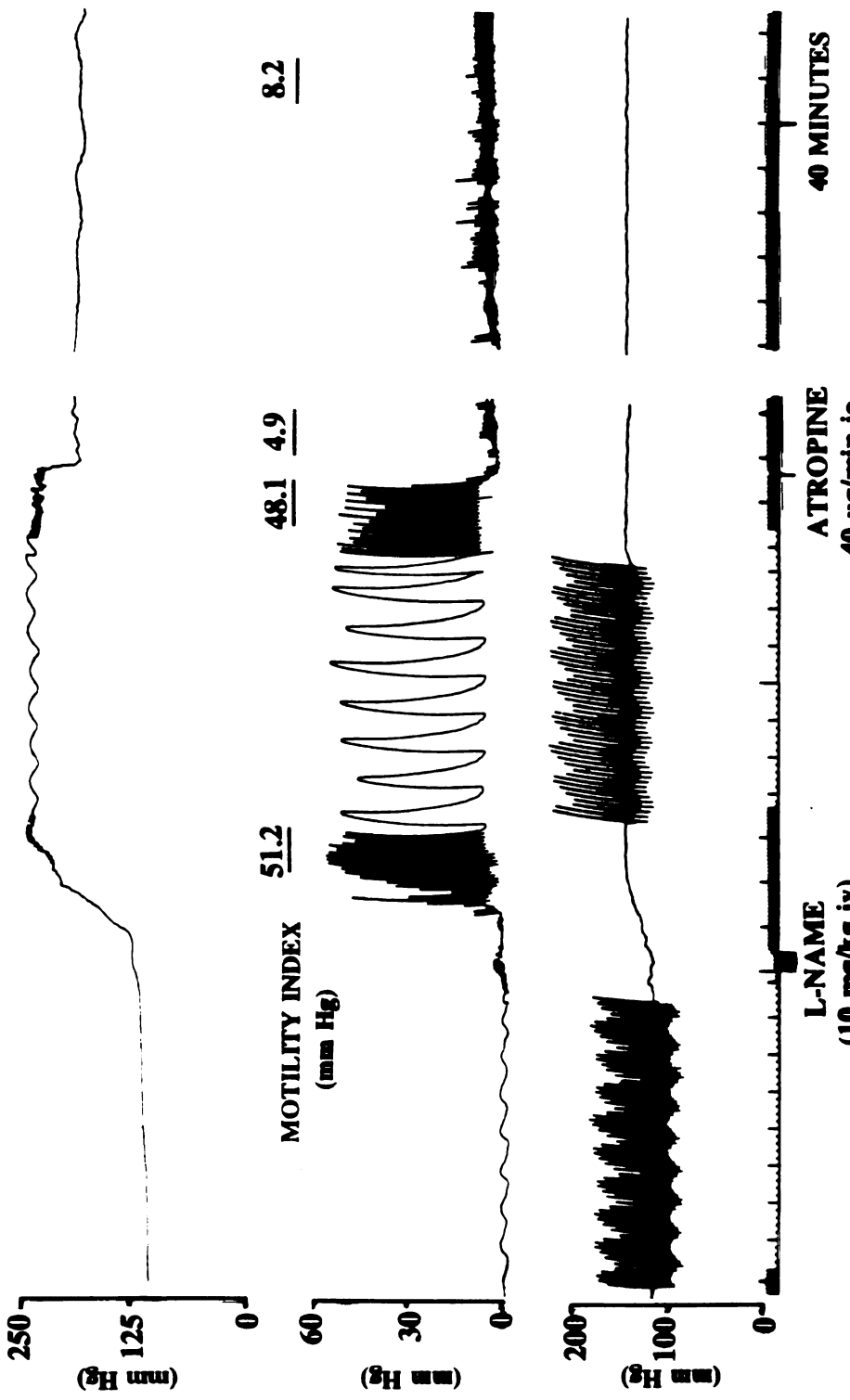



Figure 20. Experimental recording of the effect of arterial infusion of atropine (40 ug/min) on the L-NAME-induced changes in jejunal perfusion pressure, lumen pressure, and systemic arterial pressure. Atropine was continuously infused once the motility produced by L-NAME (10 mg/kg iv) appeared. Lines beneath values of motility index respresent a time interval of 1 minute.

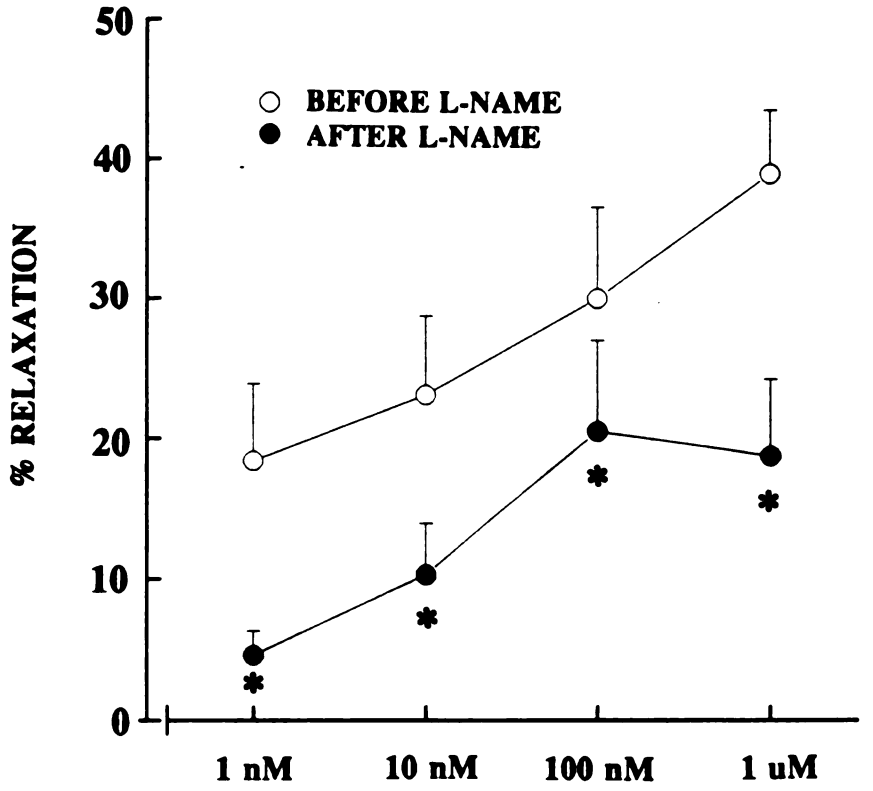



Figure 21. Effect of L-NAME plus atropine on the dilator response to ACh in vivo. The vasodilation produced by bolus injection of ACh (0.2 ml) under conditions of constant blood flow was attenuated by L-NAME plus atropine.

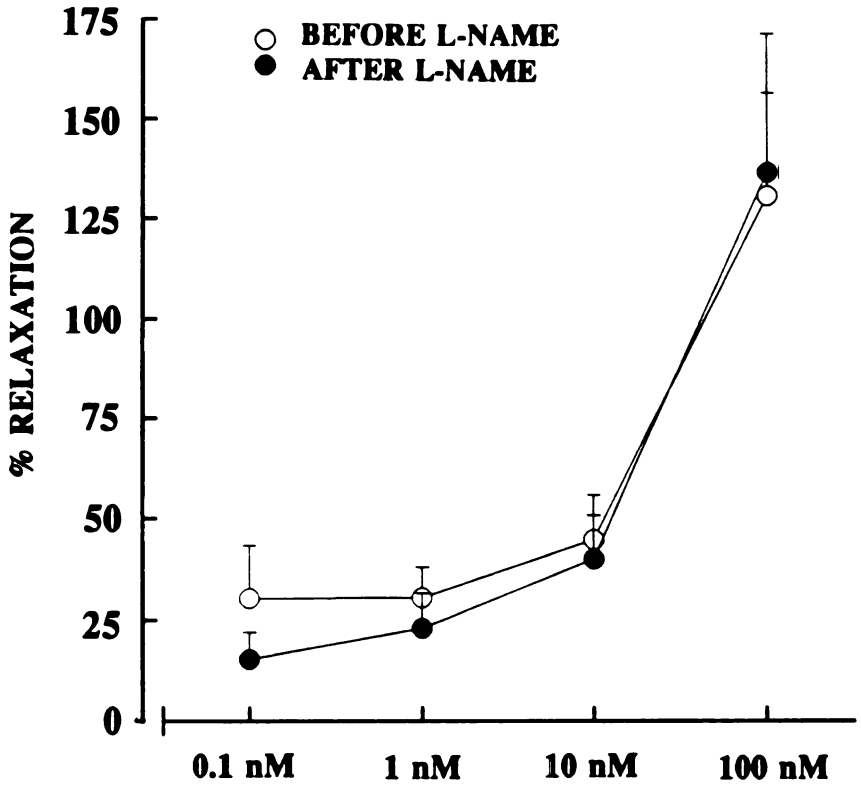



Figure 22. Effect of L-NAME plus atropine on the dilator response to substance P in vivo. The vasodilation produced by bolus injection of substance P (0.2 ml) under conditions of constant blood flow was not affected by L-NAME plus atropine.

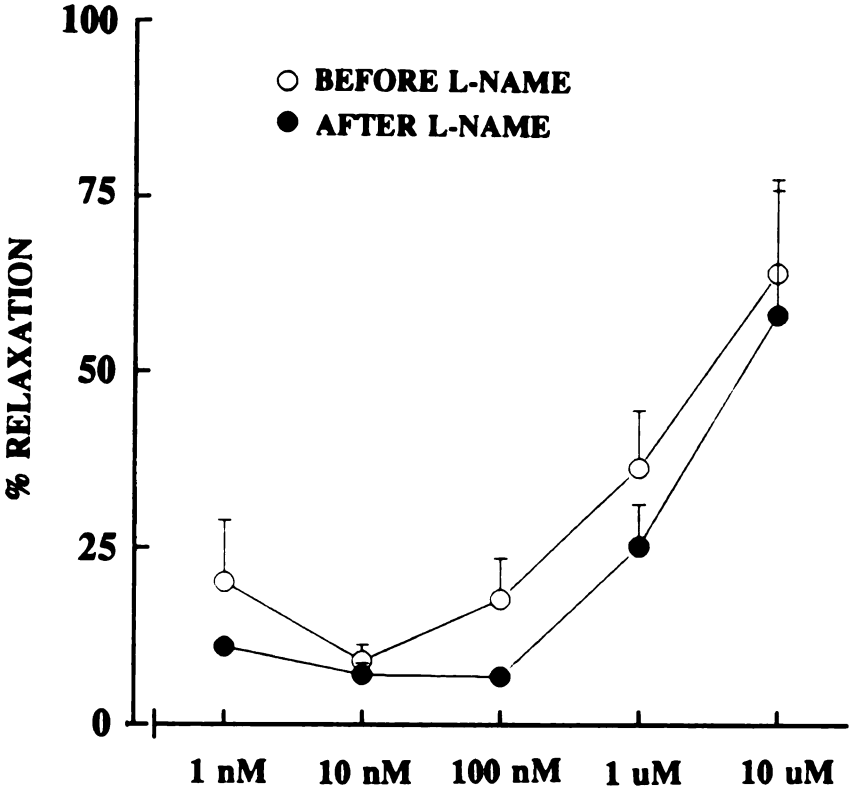



Figure 23. Effect of L-NAME plus atropine on the dilator response to nitroglycerin in vivo. The vasodilation produced by bolus injection of nitroglycerin (0.2 ml) under conditions of constant blood flow was not affected by L-NAME plus atropine.

# The Effect of Methylene Blue on Jejunal Blood Flow and Oxygen Uptake at Rest and Postprandially

## Introduction

Intestinal blood flow and oxygen uptake increase after ingestion of a meal (21,25). The food-induced increase in jejunal blood flow and oxygen uptake is localized primarily to the mucosal region in direct contact with the digested food (24). The stimulus for the postprandial intestinal hyperemia has been shown to be the products of digested food (21,26), however, the mechanism of action has not yet been clearly determined. Evidence to date indicates that the mechanisms involved are complex with many factors, such as enteric nerves (159), histamine (27), oxidative metabolism (165), substance P (149), bradykinin (75,170), and prostanoids (55) thought to play a role.

Recently, endothelial-derived nitric oxide has been proposed to mediate vascular smooth muscle relaxant response to several endogenous vasodilator agents, including ACh, substance P, and bradykinin (142,155,156). Nitric oxide relaxes vascular smooth muscle through the activation of soluble guanylate cyclase (1), which results in the accumulation of 3'-5' cyclic guanosine monophosphate (cyclic GMP) within the vascular smooth muscle (94,126,135). Organic nitrates, such as nitroglycerin and nitroprusside, also relax vascular smooth muscle through the activation of soluble guanylate cyclase and accumulation of cyclic GMP within vascular smooth muscle (110,111). Methylene blue, an inhibitor of soluble guanylate cyclase (67,126), prevents the accumulation of cyclic GMP and attenuates the relaxation elicited by vasodilating agents that stimulate the release of endogenous nitric oxide from the endothelium, exogenous nitric oxide (88,107), and organic nitrates (110,126). Methylene blue has been used to inhibit the relaxation

produced by ACh and exogenous nitric oxide in isolated canine mesenteric arteries (107). Similarly, Falcone and Bolhen (38) have reported that suffusion of methylene blue over the mucosal surface of isolated arterioles of rat small intestine produced a vasoconstriction of the arterioles and attenuated the arteriolar dilation produced by ACh, but did not affect the relaxation produced by adenosine. These studies suggest that endogenous nitric oxide may function to regulate mesenteric vascular smooth muscle tone in vivo. Therefore, we reasoned that nitric oxide may function as a mediator of the intestinal hyperemia produced by placement of digested nutrients into the lumen of the jejunum. The purpose of this study was to determine if inhibition of soluble guanylate cyclase by intraluminal placement of methylene blue altered jejunal blood flow and oxygen uptake under resting conditions and postprandially.

## Methods

18 mongrel, heartworm negative dogs (16.4-28.6 kgs) of either sex were fasted for 24 hours and anesthetized with pentobarbital sodium (30 mg/kg). A schematic of the surgical preparation is shown in Figure 9. In this study, 3 series of experiments were performed utilizing the surgical technique as previously described in chapter 4.

*Series 1.* The objective of this series of experiments was to determine the effect of intraluminal placement of methylene blue (0.3 mM 1 mg/ml of normal saline or food), an inhibitor of soluble guanylate cyclase, on jejunal blood flow oxygen uptake and motility at rest and postprandially (n=7). This concentration of methylene blue was chosen based on previous studies that have shown that topical application of $10^{-4}$M methylene blue to arterial smooth muscle selectively inhibits the relaxation elicited by ACh (38,96). The venous blood flow from the single vein draining the segment was continuously monitored by an extracorporeal flow transducer (BL

2048-E04, biotronex laboratories, Silver Spring, MD) placed in the venous outflow line and connected to an electromagnetic flowmeter (BL 610, Biotronex Laboratories). In addition, venous outflow was also measured used a stop watch and guaduated cylinder. After the preparation had stabilized for a minimum of 30 minutes after surgery, 15 mls of normal saline was placed into the lumen of the single isolated jejunal segment for 15 minute periods until blood flow reached a steady state, at which time the luminal contents were changed to digested food. The change in blood flow due to food was determined as the difference in steady state flows during luminal placement of normal saline and digested food. After determining the response to food, normal saline was again placed into the lumen for 15 minutes. Methylene blue was then added to the normal saline and digested food and the entire protocol was repeated. The effect of methylene blue on jejunal blood flow was determined as the change in blood flow during luminal placement of normal saline alone and normal saline plus methylene blue. The effect of methylene blue on the postprandial intestinal hyperemia was determined as the change in steady state blood flow during intraluminal placement of normal saline plus methylene blue and digested food plus methylene blue. Prior to and after the exposure to the jejunal segment to solutions containing methylene blue, the reactive hyperemic response to occlusion of the single artery and vein for 30 seconds using a latex tipped hemostat was also determined.

*Series 2.* The objective of this series of experiments was the same as that for series 1, except that two adjacent jejunal segments (Figure 9) were isolated in 6 separate dogs. The single vein draining each segment was cannulated and the venous outflows were measured with a graduated cylinder and stopwatch. The protocol of the series of experiments is shown in Figures 25 and 26 and its design is such that one segment could serve as a time control for the second segment. After a steady state in blood flow and oxygen extraction were achieved with normal saline in the lumen, digested food without methylene blue was placed into the lumen of each

jejunal segment for 15 minutes. At different 15-minute periods, the lumen of each segment contained normal saline, normal saline plus methylene blue, food, and food plus methylene blue. After each 15-min period, the lumen contents were withdrawn, and the lumens were gently and thoroughly rinsed with normal saline. The change in blood flow due to food was calculated as the difference in steady state blood flow 12-15 minutes after placement of normal saline into the lumens and during placement of food plus bile into the lumens of the jejunal segments. After determining the response to food in both segments, one segment was randomly selected to receive methylene blue (1 mg/ml solution = 0.3 mM) when added to normal saline or digested food. The jejunal blood flow, oxygen uptake and motor activity in response to luminal placement of digested food with and without methylene blue were then determined simultaneously in separate segments. The jejunal segment not treated with methylene blue thus served as a time control. In 4 of the 6 dogs, the solutions were reversed so that the test segment that was exposed to methylene blue received normal saline alone followed by digested food alone, whereas the control segment received normal saline plus methylene blue followed by digested food plus methylene blue. The change in blood flow due to food plus methylene blue was determined as the difference in steady state flows during intraluminal placement of normal saline plus methylene blue and digested food plus methylene blue. The animals were then euthanized with an overdose of pentobarbital and the segments removed and weighed for standardization of blood flow.

*Series 3.* Because methylene blue enhanced the resting jejunal blood flow in series 1 and 2, a third series of experiments were performed in 5 separate dogs. In this series of experiments, the single artery perfusing the jejunal segment and the single vein draining the segment were cannulated. The jejunum was perfused naturally with aortic blood by interposing an extracorporeal shunt between the left femoral artery and jejunal artery. After the vascular and metabolic responses to normal saline and food were determined, isoproterenol, an endothelium-

independent vasodilator (126), was infused into the artery at a rate of 0.5 ug/min via the extracorporeal shunt to increase jejunal blood flow to approximately the same extent as that observed when normal saline plus methylene blue is exposed to the mucosal surface. The vascular and metabolic responses to normal saline and food plus bile were then determined during isoproterenol infusion and compared with those obtained prior to isoproterenol infusion.

*Quantitation of motility.* Motor activity was quantitated from intraluminal pressure tracings in series 2. A motility index was calculated by dividing the sum of the heights of all the pressure waves during luminal placement of normal saline and digested food, with and without methylene blue, in 3 minute intervals for 15 minutes. This motility index was expressed in millimeters of mercury (mm Hg) and reflects the average strength of intestinal contractions (112,194).

*Preparation of food solutions.* The food solution used contained equal parts by weight of fat, carbohydrate, and protein. It was prepared by adding 30 g high-fat test diet, 15 g high-protein test diet, and 5 g high-carbohydrate test diet (United States Biochemical, Cleveland, OH) to 400 ml of 0.1 N $NaHCO_3$ containing 750 mg of a pancreatic enzyme preparation (Viocase, VioBin, Monticello, IL). The mixture was then mixed gently with a magnetic stirrer at room temperature for 5 h to permit digestion. Prior to each experiment, bile was extracted from the gallbladder and added to the food solution to yield a final concentration of 10%. In some experiments methylene blue (1 mg/ml solution) was added to normal saline and food plus bile. All solutions were kept at 37° C during the experiment.

*Statistics.* All values in text are reported as mean±SE and represent either paired or unpaired data. In each series of experiments, n indicates the number of observations in each dog. The specific statistical test used varied depending on the series of experiment. Where comparisons were made between two sample means, the data were analyzed using Student *t* test. Where multiple comparisons with a common control were made, the difference between the means of each experiment were evaluated by analysis of variance. If the analysis of variance showed a significant difference between the individual groups were determined using the least significant difference test. In situations were there was no common control, a Student *t* test for unpaired observations was used. Statistical significance was set at $P<0.05$.

Results

Systemic arterial pressure averaged 125 ± 3 mm Hg (n=18) and was not significantly altered by intraluminal placement of methylene blue, normal saline, digested food, or by intra-arterial infusion of isoproterenol into the single artery perfusing the isolated jejunal segment.

Figure 24 shows the effect of normal saline followed by digested food, with and without methylene blue (1 mg/ml) on jejunal blood flow and oxygen uptake in a single jejunal segment. Prior to luminal placement of methylene blue, digested food significantly increased jejunal blood flow and oxygen uptake when compared to the control values at 12-15 minutes (normal saline without methylene blue). The addition of methylene blue to normal saline significantly increased resting blood flow and oxygen uptake compared to normal saline alone at 12-15 minutes. The addition of methylene blue to the digested food solution did not affect the initial hyperemia or oxygen uptake during the first 3 minutes after luminal placement. However, blood flow and oxygen uptake between 4 and 15 minutes after luminal placement of food plus methylene blue did not statistically differ from values during 12-15 minutes after luminal placement of methylene

blue plus normal saline. Therefore, the postprandial intestinal hyperemia was not maintained in the presence of methylene blue.

To determine if the effect of methylene blue on jejunal blood flow and oxygen uptake was a time-dependent phenomemon, we preformed the same protocol as the first series utilizing two adjacent jejunal segments. The effects of methylene blue on jejunal blood flow and oxygen uptake at rest and during luminal placement of a digested meal in the double jejunal segment preparation are shown in Figures 25 and 26. Resting jejunal blood flow and oxygen uptake were comparable between the two segments, and luminal placement of digested food alone produced a significant increase in blood flow and oxygen uptake in both segments. Luminal placement of normal saline plus methylene blue produced a significant increase in resting blood flow and oxygen uptake when compared to the control values obtained at 12-15 minutes after luminal placement of normal saline in either the same or adjacent segment. There was a significant increase in blood flow and oxygen uptake during the first 3 minutes after luminal placement of food plus methylene blue. However, the increase in blood flow and oxygen uptake was not maintained and rapidly returned to values similar to those observed during luminal placement of normal saline plus methylene blue. Conversely, simultaneous placement of digested food without methylene blue into the adjacent segment significantly increased blood flow and oxygen uptake for the entire 15 minute period.

After determining the effect of methylene blue on blood flow and oxygen uptake at rest and postprandially in the double jejunal segment preparation, the luminal contents were switched so that the jejunal segment that was previously exposed to methylene blue received normal saline followed by digested food without methylene blue. The effect of methylene blue was simultaneously studied in the adjacent segment that had served as a control in 4 of the 6 dogs. After gently rinsing the methylene blue from the lumen, placement of normal saline alone into the

jejunal segment for 15 minutes did not affect the increase in blood flow or oxygen uptake. Jejunal blood flow and oxygen uptake during luminal placement of normal saline did not statistically differ from the values obtained during luminal placement of normal saline plus methylene blue. Luminal placement of digested food alone into the test segment after exposure to methylene blue produced results similar to those observed during luminal placement of digested food plus methylene blue. There was an significant increase in blood flow and oxygen uptake during the first 3 minutes after luminal placement of digested food alone. However, the food-induced hyperemia could not be maintained between 4-15 minutes after the mucosal surface was exposed to methylene blue. When compared to normal saline alone, the addition of methylene blue to normal saline significantly increased blood flow and oxygen uptake in the jejunal segment that had previously served as a time control. Again, placement of digested food plus methylene blue produced a significant increase in blood flow and oxygen uptake during the initial 3 minutes. However, the food-induced increase in blood flow and oxygen uptake was not maintained in the presence of methylene blue.

Figure 27 shows the effect of methylene blue on the food-induced change in blood flow and oxygen uptake. The data were obtained from the data shown in Figure 24. Digested food alone (Food-NS) produced a significant increase in both blood flow and oxygen uptake during the entire 15 minute period of luminal placement. The addition of methylene blue to food (Food+MB) did not significantly alter the level of the food-induced increase in blood flow or oxygen uptake during the initial 3 minute period. However, the food-induced increase in blood flow observed between 4-15 minutes was significantly attenuated by methylene blue. Although the mean change in oxygen uptake with food plus methylene blue was almost zero during the 4-15 minute periods, the change in oxygen uptake during food plus methlyene blue was greater than that observed with food alone in 3 dogs and less than that observed by food alone in 3 other dogs.

This led to a result that methylene blue did not significantly alter the increase in oxygen uptake produced by food. The effect of methylene blue on the food-induced change in blood flow and oxygen uptake in the second series of experiments utilizing double jejunal segments (as shown in Figures 25 and 26 shown) was similar to that shown in Figure 27 when analyzed with the same statistical method.

Figure 28 is an experimental recording illustrating the effect of luminal placement of normal saline plus methylene blue on the motor activity in the jejunum. There were sporatic increases in phasic, rhythmic contractions associated with luminal placement of methylene blue. The effects of luminal placement of normal saline and digested food, with and without methylene blue on the motor activity are summarized in Table 9. The resting motor activity was equally negligible in both jejunal segments when normal saline was present in the lumen. Motor activity was significantly increased for the 15 minute period of luminal placement of digested food alone in both segments. The motor activity produced by digested food was greatest during the first 3 minutes after luminal placement. Methylene blue did not significantly affect the motor activity produced by digested food. However, methylene blue did significantly increase the resting motor activity compared to luminal placement of normal saline alone at 12-15 minutes. The motor activity during luminal placement of normal saline plus methylene blue was similar to that produced by digested food alone.

Figure 29 shows the increase in blood flow and oxygen uptake observed when digested food is placed into the lumen of jejunal segment, prior to and during arterial infusion of isoproterenol (0.5 ug/min). Prior to infusion of isoproterenol, digested food produced a significant increase in jejunal blood flow and oxygen uptake for the entire period of luminal placement. Isoproterenol significantly increased resting blood flow, but did not affect oxygen uptake when normal saline was in the lumen. During infusion of isoproterenol, digested food significantly

increased blood flow and oxygen consumption for the entire 15 minute period compared to the control period of 12-15 minutes during luminal placement of normal saline.

Myers and Honig (137) have shown that initial resistance plays a significant role in determining the magnitude of the vascular response to a given stimulus. Thus, it is possible that the inhibition of the food-induced jejunal hyperemia observed during luminal placement of methylene blue in series 1 and 2 may have been due solely to a change in initial resistance, i.e., an increase in resting blood flow produced by normal saline plus methylene blue. This possibility was examined by analyzing the relation between initial resistance and the change in resistance produced by food alone or food plus methylene blue (Figure 30 A). Resistance was calculated as the quotient of mean arterial blood pressure and jejunal blood flow. For the purpose of analysis, the data in series 1 and series 2 were divided into two groups, i.e., the change in resistance produced by food alone during the 12-15 minute time period and the change in resistance produced by food plus methylene blue during the 12-15 minute time period. We chose to analyze the 12-15 minute time period after placement of food with or without methylene blue because this is the time period at which blood flow was at a steady state. The initial resistances for food alone or food plus methylene blue were obtained immediately prior to food placement when the lumen contained normal saline or normal saline plus methylene blue. The data were analyzed assuming that the regression line passed through the origin. In the case of food alone (Figure 30 A), there was a significant correlation between initial resistance and the magnitude of the vascular response to food ($P<0.05$), whereas there was a poor correlation between initial resistance and the magnitude of the vascular response to food plus methylene blue. However, the linear regression equation for the response to food plus methylene blue was significantly different from the linear regression equation for the response to food alone ($P<0.05$). That is, for a given initial resistance, the food alone elicits a significantly greater decrease in resistance than when

food plus methylene blue does.

To further test this relationship, the jejunal vascular response to food was examined before and during intra-arterial infusion of isoproterenol, a potent vasodilator (Figure 30 B). The infusion of isoproterenol significantly decreased the initial resistance from 2.30±0.22 to 1.40±0.25 PRU. In addition, the food-induced decrease in resistance decreased from -0.85±0.17 PRU before infusion to -0.38±0.16 PRU during infusion of isoproterenol. As in the previous series (Figure 30 A), there was a significant correlation between initial resistance and the change in resistance due to food before and during infusion of isoproterenol (Figure 30 B). The linear regression equation for food alone before and during isoproterenol was not significantly different from the regression line for food alone obtained in Figure 30 A. However, the regression line for food alone during arterial infusion of isoproterenol was significantly different from the regression line obtained for food with methylene blue (Figure 30 A). Thus, although initial resistance does play a role in determining the jejunal vascular response to food, the inhibition of the food-induced hyperemia after inhibition of guanylate cyclase with methylene blue is independent of the associated decrease in vascular resistance produced by normal saline plus methylene blue.

The effect of intraluminal placement of methylene blue on the reactive hyperemic response to arterial and venous occlusion for 30 seconds is shown in Figure 31. The resting blood flow prior to vascular occlusion did not significantly differ from control (70±8 ml/min/100g vs. 65±6 ml/min/100g after methylene blue). Luminal placement of methylene blue produced a slight but statistically insignificant decrease in the peak amplitude of the reactive hyperemia (148±13 during control vs. 136±15 ml/min/100g after exposure to methylene blue) and percent change in blood flow (78±12% during control vs. 72±13% after methylene blue) after 30 seconds of arterial and venous occlusion. Since methylene blue has been shown to inhibit the reactive hyperemia in skeletal muscle (197), we also measured the total area of the hyperemia from the tracing of jejunal

blood flow to take into account any change in the duration of the hyperemia using an integration program on the Hewlett-Packard (HP41CV) microcomputer. Using this method we found that methylene blue produced a slight, but statistically insignificant decrease in the toatal area of the hyperemic response. The area of the hyperemia before methylene blue was 143±31 $mm^2$ compared to 108±26 $mm^2$ after methylene blue.

Discussion

Recent studies have identified nitric oxide as the substance released from vascular endothelial cells responsible for the relaxation produced by ACh (142,155). Removal of the endothelial cells from the canine superior mesenteric artery (107) or from isolated mesenteric vascular beds of rats and rabbits (49,192) has been shown to abolish the relaxation produced by ACh, but does not alter the relaxation produced by nitric oxide or nitroprusside. Methylene blue, an inhibitor of soluble guanylate cyclase (126), attenuates the relaxation induced by both ACh and nitric oxide (38,107,150), and inhibits the vasodilation produced by graded increases in flow (150). These studies suggest that nitric oxide may play a role in the regulation of intestinal blood flow at rest. Whether or not nitric oxide release plays a role in modulating intestinal blood flow during the course of normal digestion has not yet been determined. Therefore, we examined the effects of methylene blue on the jejunal and metabolic responses to a physiologic food stimulus.

Methylene blue per se significantly increased resting blood flow from 55.7±4.5 ml/min/100 gm to 67.4±7.8 ml/min/100gm, but had no effect on the food-induced hyperemia during the initial 3 minutes after luminal placement. However, the food-induced hyperemia was significantly attenuated for the duration of the 15 minute period (Figures 24, 25 and 27). This effect of methylene blue on the food-induced hyperemia was observed in both single and double jejunal segment preparations. As shown in Figures 24 and 26, the increase in resting blood flow

produced by methylene blue was also associated with a significant increase in oxygen uptake compared to normal saline alone (control). However, methylene blue did not signficantly alter the food-induced increase in oxygen uptake (Figure 27). The discrepency between the result of blood flow and oxygen uptake might be due to the methylene blue being absorbed into the venous blood which altered the accuracy of the oxygen extraction difference analyzer. Because of the finding that methylene blue actually stained the tubing and venous cuvette, the effect of methylene blue on oxygen uptake could not be interpreted with much confidence.

Myers and Honig (137) have shown that, in skeletal muscle, initial resistance plays a significant role in determining the magnitude of a vascular response to a given stimulus. Hence, it could be argued that the increase in resting blood flow and oxygen uptake produced by methylene blue accounts for the inhibition of the hyperemic response to food. However, this does not appear to be the case for at least two reasons. First, there was a significant increase in both blood flow and oxygen uptake during the first three minutes after luminal placement of digested food plus methylene blue. This increase in blood flow and oxygen uptake is not likely to result from mechanical distension produced by instillation of the solution into the segment since equal volumes of normal saline with or without methylene blue does not significantly increase blood flow or oxygen uptake during the initial 3 minutes after luminal placement. To further analyze the hyperemic effect of food under conditions of decreased initial vascular resistance by infusing isoproterenol into the single artery perfusing the segment. Like methylene blue, isoproterenol decreased the initial vascular resistance. However, isoproterenol did not significantly inhibit the food-induced hyperemia (Figure 29). Furthermore, as shown in Figures 30 A and 30 B, the linear regression equation of the methylene treated jejunal segments was significantly different from the linear regression equation of either food alone or the vascular response to food during infusion of isoproterenol. Therefore, the inhibition of the food-induced hyperemia elicited by methylene

blue occurs independent of the decrease in resting jejunal vascular resistance produced by methylene blue.

From our results, it appears that the effect of methylene blue is localized to the jejunal segment in direct contact with methylene blue and that the effects of methylene blue remain after methylene blue was washed out of the lumen. As shown in Figure 25 and 26, luminal placement of normal saline plus methylene blue into jejunal segment did not alter resting jejunal blood flow or oyxgen uptake or the hyperemic response to food without methylene blue in the adjacent segment. In addition, resting blood flow and oxygen uptake remain elevated even after the methylene blue was thoroughly rinsed from the lumen with normal saline. Also, the hyperemic effect of luminal placement of food alone into the segment that was previously exposed to methylene blue was not maintained after the initial 3 minutes.

We have previously shown that methylene blue constricts isolated superior mesenteric arterial rings in vitro, and specifically inhibits the relaxation produced by ACh, substance P, and bradykinin in vitro (Figures 3 and 4). The inhibition produced by methylene blue was specific for relaxation mediated through the release of nitric oxide and did not affect the relaxation produced by adenosine. Similar results have been reported in the isolated mesenteric vascular bed of rats (38,150). Arterial infusion of methylene blue (1 mg/100 ml) has been shown to increase the mean mesenteric vascular resistance (150) and suffusion of methylene blue over the mucosal surface decreased the diameter of the isolated mesenteric arterioles (38). These results are consistent with the hypothesis that the mesenteric arterioles are under a constant dilation in response to the basal release of nitric oxide. Therefore, our results indicating that methylene blue increased resting blood flow was certainly unexpected.

However, we found that the increase in resting active tension of preconstricted superior mesenteric arteries produced by methylene blue in our in vitro study was also observe in arterial

preparations where the endothelium had been removed. Similar results have been reported in rabbit aorta and can be blocked if the whole rabbit is pretreated with reserpine prior to extraction of the aorta (126). These finding suggest that the constrictive effect produced by methylene blue in vitro results from an increase in norepinephrine release from perivascular nerve terminals rather than by inhibition of nitric oxide activation of methylene blue. Thus, the vasoconstrictive effect of methylene blue reported in isolated rat mesenteric vascular beds in situ may also result from an enhanced release of norepinephrine.

Substantial evidence has accumulated from in vitro studies using isolated strips of intestinal smooth muscle indicating that increased levels of cyclic GMP account for the nonadrenergic noncholinergic relaxation produced by electric field stimulation (29,140,183). Furthermore, 10 uM methylene blue has been shown to directly depolarize smooth muscle cells in the circular muscle layer of proximal colon, elicit a forceful tonic contracture, and a loss in the phasic contraction associated with slow wave formation (163). Therefore, the increase in blood flow and oxygen uptake induced by methylene blue may result from an increase in motor activity. As shown in Figure 28 and Table 9, methylene blue does produce a slight, but statistically significant increase in the mean motility index at rest. The increase in motor activity was primarily composed of spontaneous bursts of phasic rhythmic contractions which has been shown to increase intestinal flow (20,23,42). We have previously shown that inhibition of nitric oxide synthesis under free flow conditions also produced a secondary increase in jejunal blood flow that was associated with intestinal motility (Table 3). These results support the hypothesis that the increased motor activity produced by methylene blue is due to an inhibition of soluble guanylate cyclase activity.

However, Sobey et al. (176) has reported that continuous arterial infusion of methylene blue (10 mg/ml) into the femoral artery of anesthetized dogs increased femoral blood flow, but

had no effect on arterial blood pressure, thus resulting in a decrease in resting resistance. These authors concluded that the vasodilator effect of methylene blue was due to a local effect since systemic arterial pressure was not affected, and it also occurred after ganglionic bloackade using hexamethonium (176). This would suggest that the increase in resting jejunal blood flow produced by methylene blue in our study is not due to the increase in motility, but rather another mechanism that is common to the femoral artery as well. To date, our study and the report by Sobey et al. (176) are the only two studies that report an increase in resting organ blood flow after exposure to methylene blue. Other studies have reported that methylene blue either increased vascular resistance (38) or had no effect on resting vascular resistance (193). Further studies are necessary to clarify this effect of methylene blue.

Numberous studies indicate that the relaxant action of nitric oxide on vascular and nonvascular smooth muscle occurs through the activation of soluble guanylate cyclase activity (1,9,11,140,183). We have previously shown that systemic administration of L-NAME, an inhibitor of nitric oxide synthesis (142,156), produced a marked increase in motility. The motility was associated with an increase in rhythmic contractions that were accompanied by large increases in basal lumen tone. Therefore, one would reason that inhibition of soluble guanylate cyclase activity would produce motility responses similar to those produced by nitric oxide synthesis inhibition. Conversely, we found that luminal placement of methylene blue only slightly enhanced motor activity of an isolated jejunal segment (Table 9). Furthermore, the increase in motility produced by methylene blue was brief and occurred sporatically, whereas the motility response produced by nitric oxide synthesis inhibition was continuous and lasted for more than 40 minutes. The reason for this discrepancy may result of the difference in the route of administration of methylene blue and L-NAME, i.e. intraluminal vs. intravenous.

Since methylene blue has been shown to inhibit the reactive hyperemic response in

skeletal vascular beds (197), We also determined the effect of intraluminal methylene blue on the reactive hyperemic response to 30 seconds of vascular occlusion. As shown in Figure 31, methylene blue did not significantly affect the peak hyperemic response after release of the occlusion. This implies that nitric oxide does not mediate the hyperemic response to occlusion and is consistent with our finding that L-NAME did not inhibit the reactive hyperemia (Figure 13). Conversely, Wolin et al (197) have reported that suffusion of isolated rat cremasteric arterioles with methylene blue, but not L-NMMA, inhibited the reactive hyperemia produced by arteriole occlusion for 15-20 seconds. However, the investigators' interpretation was that methylene blue inhibited the reactive hyperemia by preventing an increase in guanylate cyclase activity produced by hydrogen peroxide accumulation during reperfusion (197) and that nitric oxide released from endothelial cells did not mediate the reactive hyperemia to vascular occlusion. The reason for the difference in the effect of methylene blue reported in our study and that reported by Wolin (197) may relate to the method of occlusion. These investigators (197) opted to occlude just the arteriole that was under study. In contrast, we examined the hyperemic response after occluding both the artery and vein thereby preventing vascular collapse and blood excavation downstream from the occlusion site. Under these conditions with such a brief occlusion period it is not likely that local tissue oxygen content would be reduced to a point where oxygen radical were formed upon reperfusion.

In this study, digested food without methylene blue produced a significant increase in jejunal blood flow and oxygen uptake for the entire duration of luminal placement. This hyperemic effect of food has been reported previously by Chou and his colleagues (21,26,27). Methylene blue did not inhibit the initial hyperemia produced by food, however, the increase in blood flow and oxygen uptake could not be maintained in the presence of methylene blue. These results suggests that different mechanisms may account for the initiation and the maintenance of

the food-induced hyperemia. One possible explanation could be that adenosine, which also plays a role in regulating the hyperemic response to food, is responsible for the initiation of the hyperemic response and other factors that depend on nitric oxide synthesis and release are responsible for maintaining the hyperemia. This is supported by the fact that methylene blue does not affect the relaxation produced by adenosine in isolated superior mesenteric arteries (Figures 3 and 4) or in isolated rat mesenteric arterioles in situ (38). The factor responsible for nitric oxide release in currently unclear. Substance P does not appear to be the stimulus for nitric oxide release during luminal placement of food since the relaxation produced by substance P is not affected by inhibition of nitric oxide synthesis (Figure 22). One possibility is that the initial hyperemia produced by food increases the shear stress on the luminal surface of the mesenteric vessels and resulting in an increase in nitric oxide release from the endothelial cells. Increases in pulsatile flow and shear stress have both been shown to endothelium-dependent (5,100,147,160) and are thought to enhance nitric oxide release (68,70). However, other possibilities may also explain the effects of methylene blue, such as changes in intestinal motor activity, and should not be ruled out. As has been shown, inhibition of nitric oxide synthesis produced a marked increase in intestinal motility (Figures 10, 12, 14, 18, 19A, and 19B). Future studies should investigate the effect of methylene blue on intestinal motility.

Summary

In this study we demonstrate that methylene blue, an inhibitor of soluble guanylate cyclase and smooth muscle relaxation mediated by nitric oxide, had different effects on jejunal blood flow and oxygen uptake at rest and postprandially. Methylene blue enhanced resting blood flow and oxygen uptake, and these increases may result from an increase in intestinal smooth muscle motor activity. Methylene blue did not affect the initial increase in blood flow and oxygen uptake produced by luminal placement of food, but abolished the maintenance phase of the postprandial intestinal hyperemia. These results suggest that nitric oxide may mediate the maintenance phase of the food-induced increase in blood flow and oxygen uptake. Methylene blue did not alter the reactive hyperemic in response to vascular occlusion. This studies also suggests that the jejunal reactive hyperemia and the initial hyperemic reponse to food are nitric oxide-independent which may be mediated through a common mechanism.

Table 9. Effect of luminal placement of normal saline and digested food, with and without methylene blue on intestinal motility.

| | SEGMENT A (CONTROL) | | | |
|---|---|---|---|---|
| | 0-3 MIN. | 4-7 MIN. | 8-11 MIN. | 12-15 MIN. |
| SALINE | 2.5±0.7 | 2.0±0.7 | 2.3±0.8 | 2.2±0.7 |
| FOOD | 6.5±1.9* | 5.0±1.8* | 5.2±1.4 | 6.2±2.4* |
| SALINE | 2.2±0.9 | 2.3±1.1 | 2.3±1.2 | 2.0±0.8 |
| FOOD | 9.0±1.9* | 6.3±2.8* | 5.2±1.0* | 6.5±1.2* |
| SALINE+MB | 1.8±1.1 | 1.7±1.0 | 4.0±1.1 | 3.7±1.3 |
| | SEGMENT B (TEST) | | | |
| | 0-3 MIN. | 4-7 MIN. | 8-11 MIN. | 12-15 MIN. |
| SALINE | 2.7±0.8 | 1.8±0.6 | 1.3±0.6 | 0.7±0.6 |
| FOOD | 9.2±3.2* | 2.8±0.9* | 2.9±1.2* | 6.2±2.4* |
| SALINE+MB | 1.5±0.7 | 7.0±3.8*# | 5.3±1.7*# | 1.8±1.1 |
| FOOD+MB | 13.4±5.0* | 10.4±6.3* | 9.0±4.1* | 7.8±3.4* |
| SALINE | 4.7±1.4* | 6.5±2.4*# | 8.3±2.0*# | 10.3±4.2*# |

Effect of normal saline (NS) and digested food, with and without methylene blue (MB;1 mg/ml solution) on intestinal motility. Motor activity was quantified using the motility index (the sum of the height of the pressure waves during each 3 minute period divided by 3 minutes). Values are means ± SE taken from 6 separate dogs and are expressed in mm Hg. *indicates a significant difference from preceeding normal saline at 12-15 min. in the same segment, and # indicates a significant difference from control segment during normal saline in the same time period. $P<0.05$.

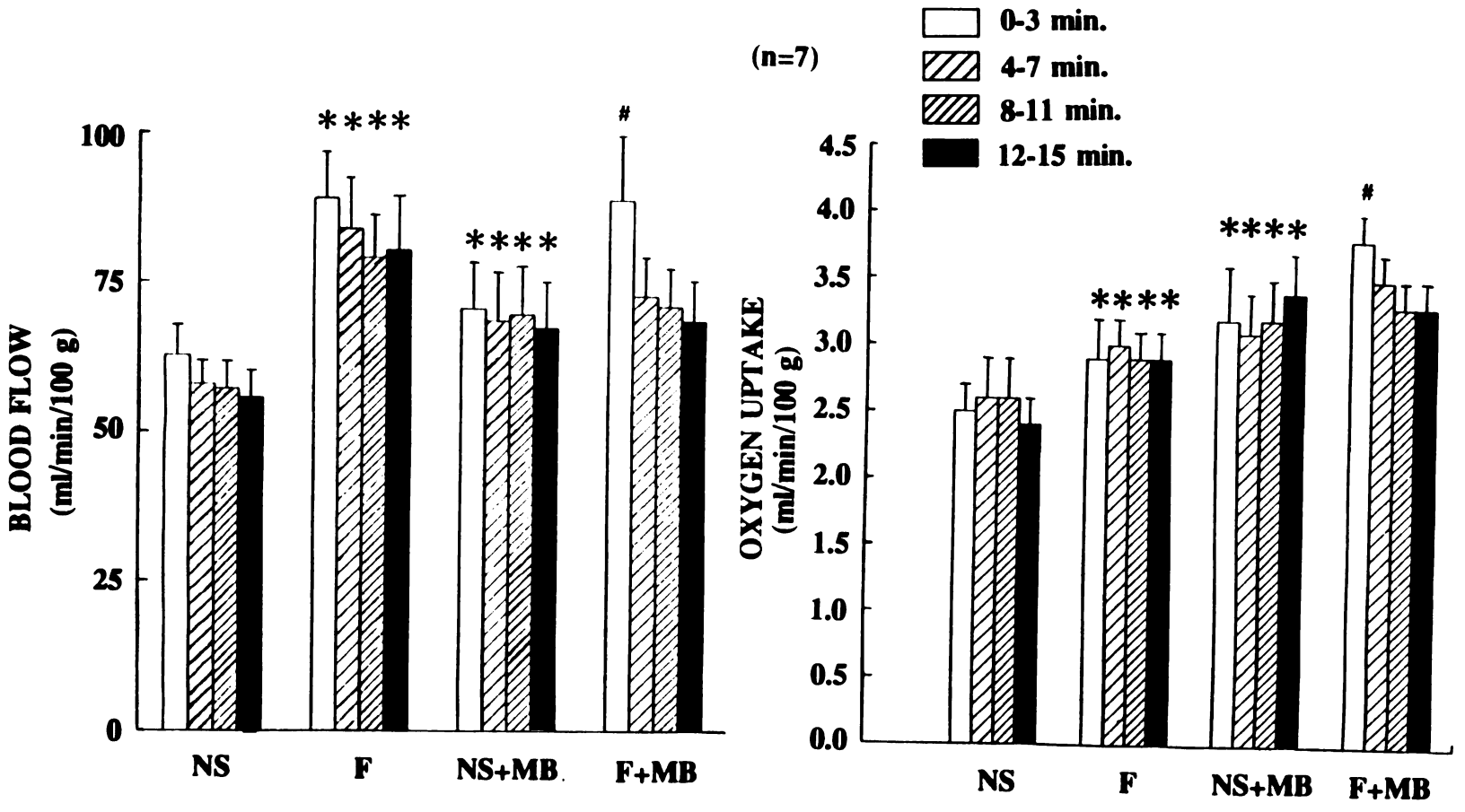



Figure 24. Jejunal blood flow and oxygen uptake during luminal placement of normal saline (NS) or food plus bile (F), with and without methylene blue (MB;1 mg/ml) in a single isolated jejunal segment. Values are means ± SE (n=7). * indicates a significant difference from NS at 12-15 min., and # indicates a significant difference from NS+MB at 12-15 min. $P < 0.05$.

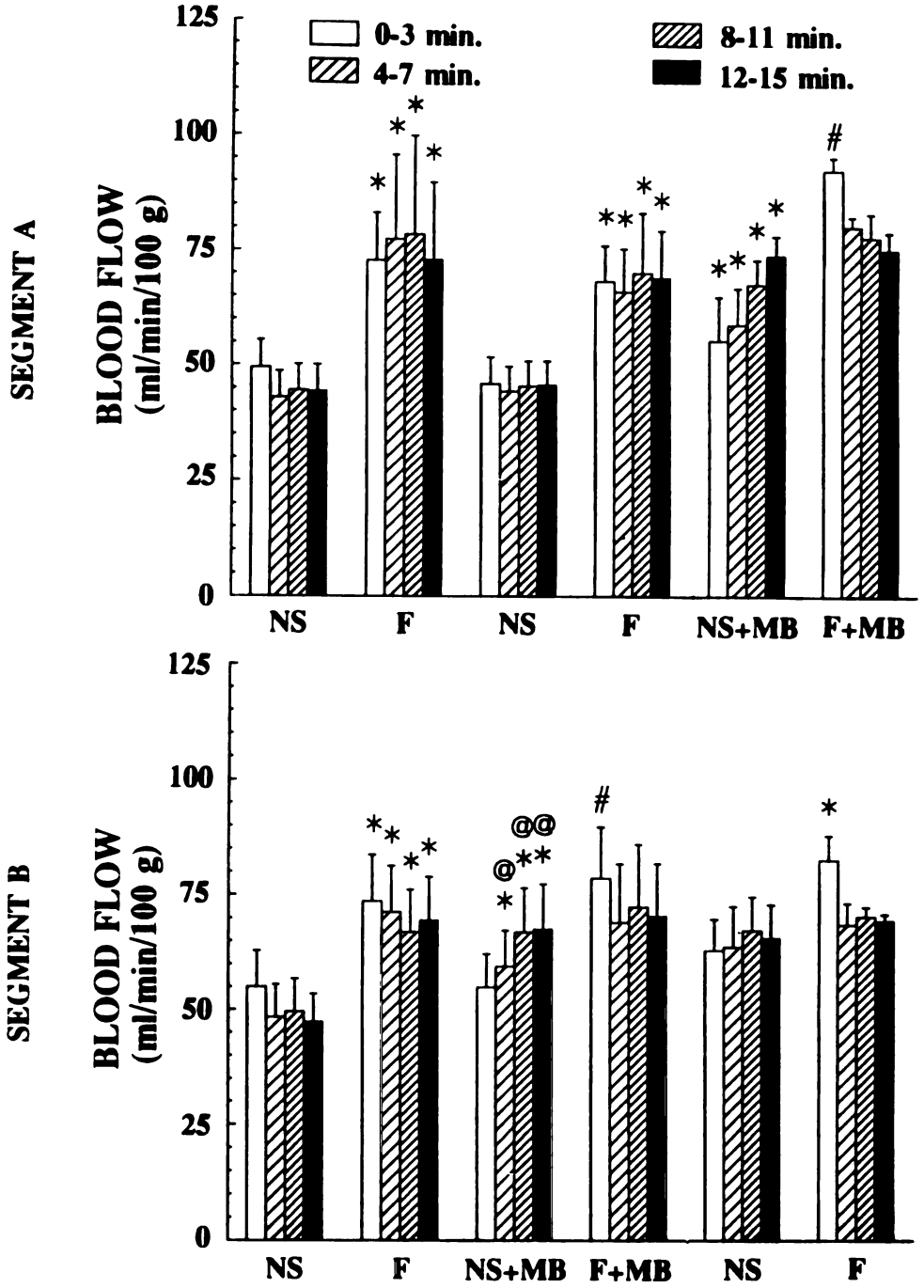



Figure 25. Jejunal blood flow during luminal placement of normal saline (NS) or food plus bile (F), with and without methylene blue (MB;1 mg/ml) in a two adjacent jejunal segments. Values are means ± SE (n=6). * indicates a significant difference from NS at 12-15 min., # indicates a significant difference from NS+MB at 12-15 min., and @ indicates a significant difference from the adjacent control segment in the same time period. $P < 0.05$.

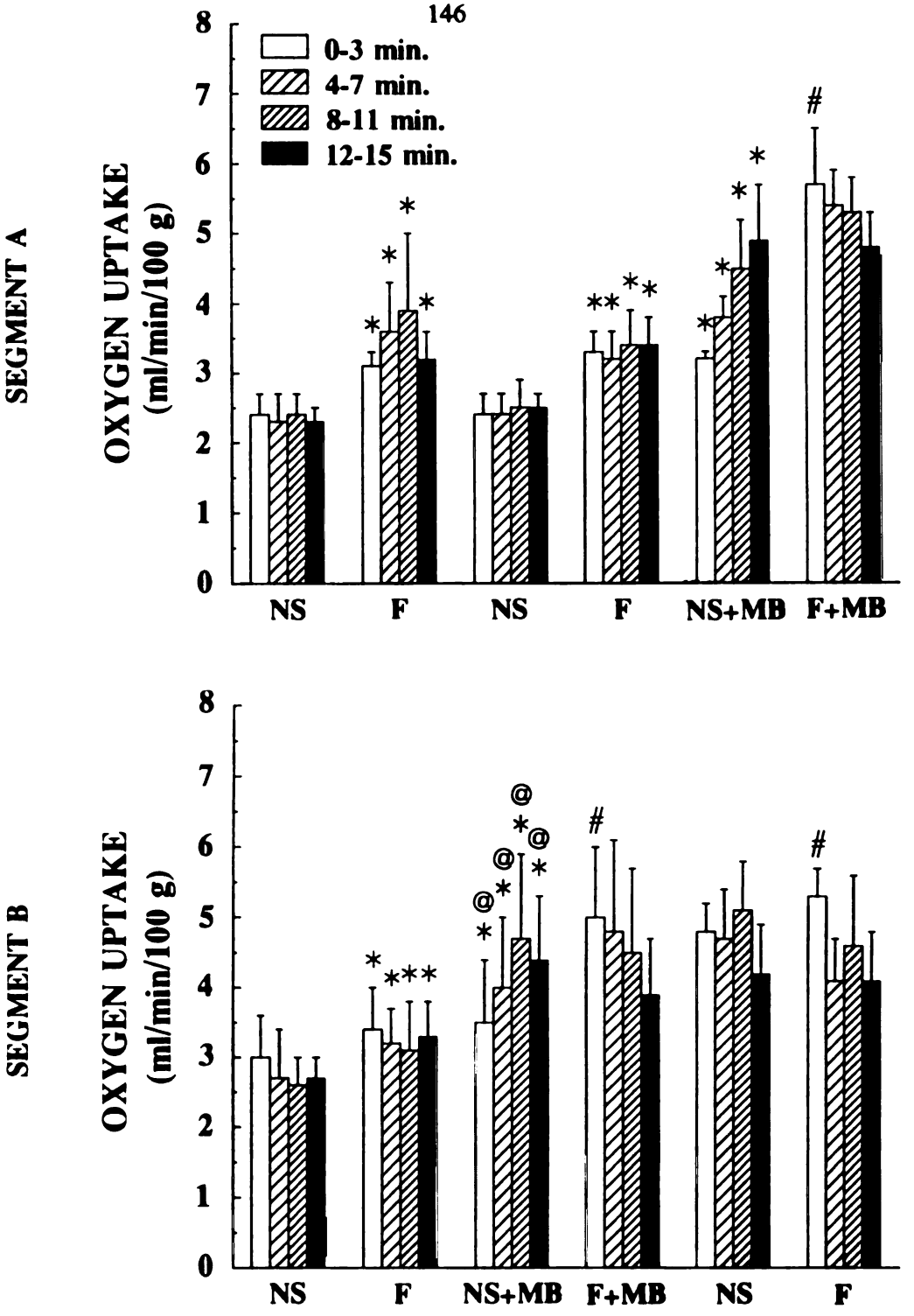



Figure 26. Jejunal oxygen uptake during luminal placement of normal saline (NS) or food plus bile (F), with and without methylene blue (MB;1 mg/ml) in adjacent jejunal segments. Values are means ± SE (n=6). * indicates a significant difference from NS at 12-15 min., # indicates a significant difference from NS+MB at 12-15 min., and @ indicates a significant difference from the adjacent control segment in the same time period. $P< 0.05$.

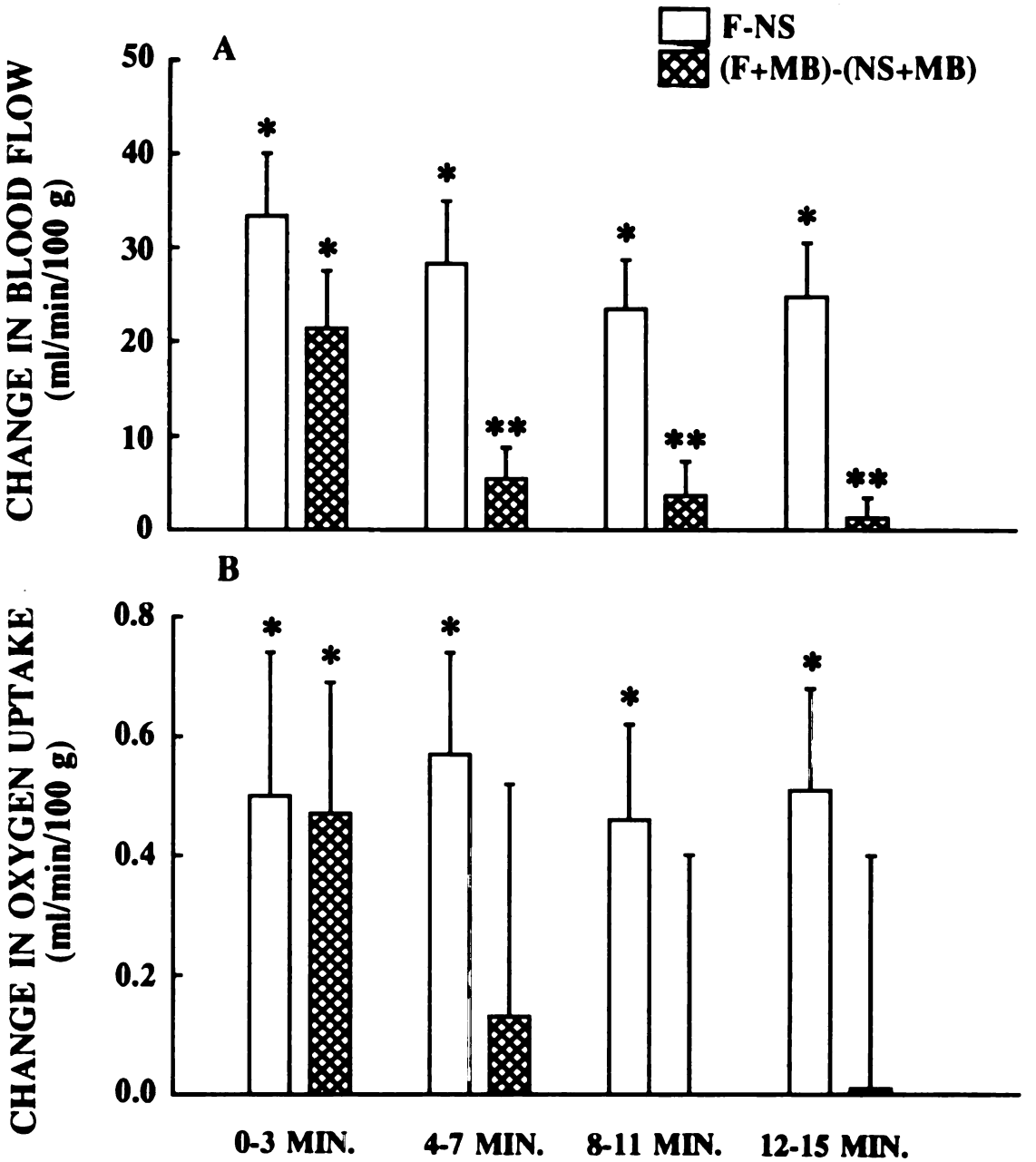



Figure 27. The change in blood flow and change in oxygen uptake produced by food (F) without methylene blue (MB) and food with MB. The data are obtained from the data shown in Figure 24. Each value of blood flow or oxygen uptake is the difference between food alone and normal saline (F - NS) or the difference between food plus MB and NS plus MB (F + MB) - (NS + MB). n = 7 for each value. * indicates a significant change; ** indicates a significant difference from respective F-NS ($P<0.05$).

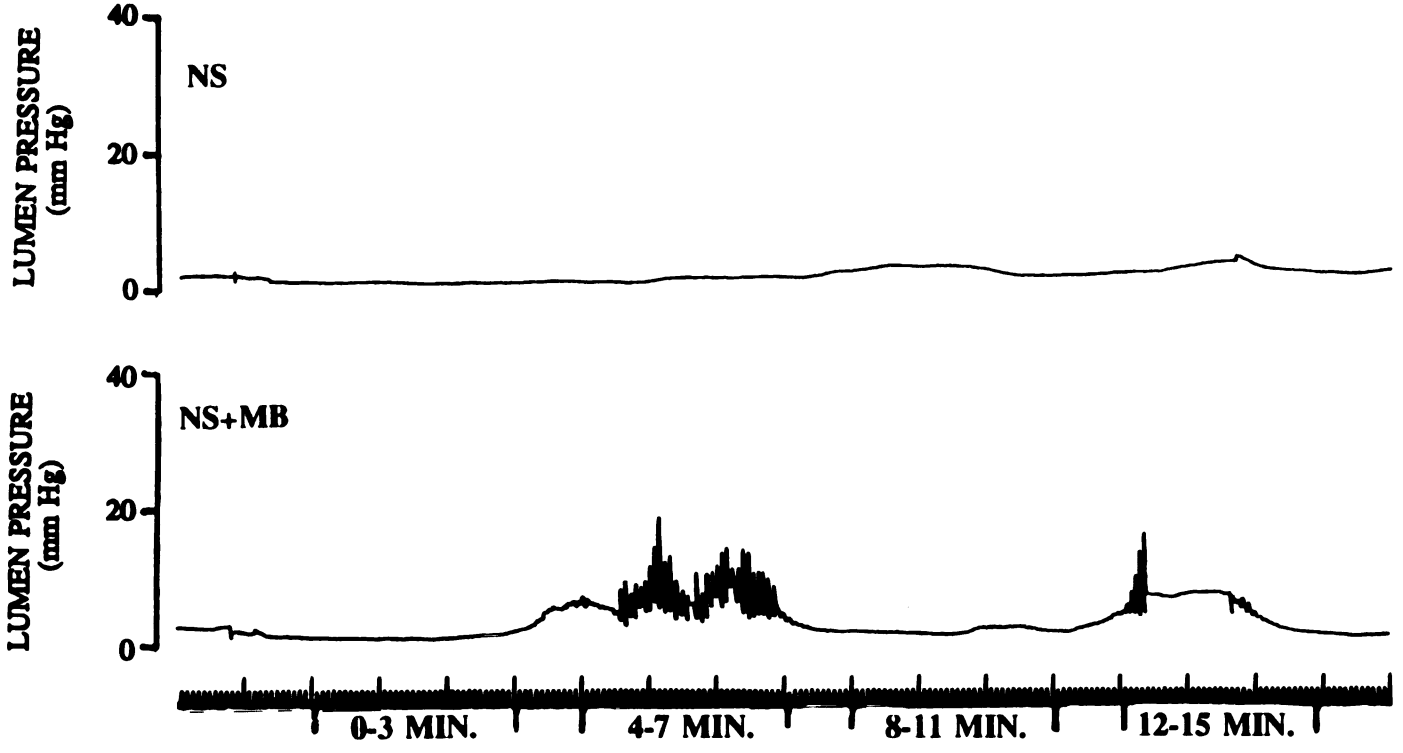



Figure 28. Experimental recording of the effect of luminal placement of methylene blue (MB;1mg/ml of normal saline) on lumen pressure in two adjacent segments. MB produced spontaneous bursts of motor activity that consisted primarily of phasic, rhythmic contractions.

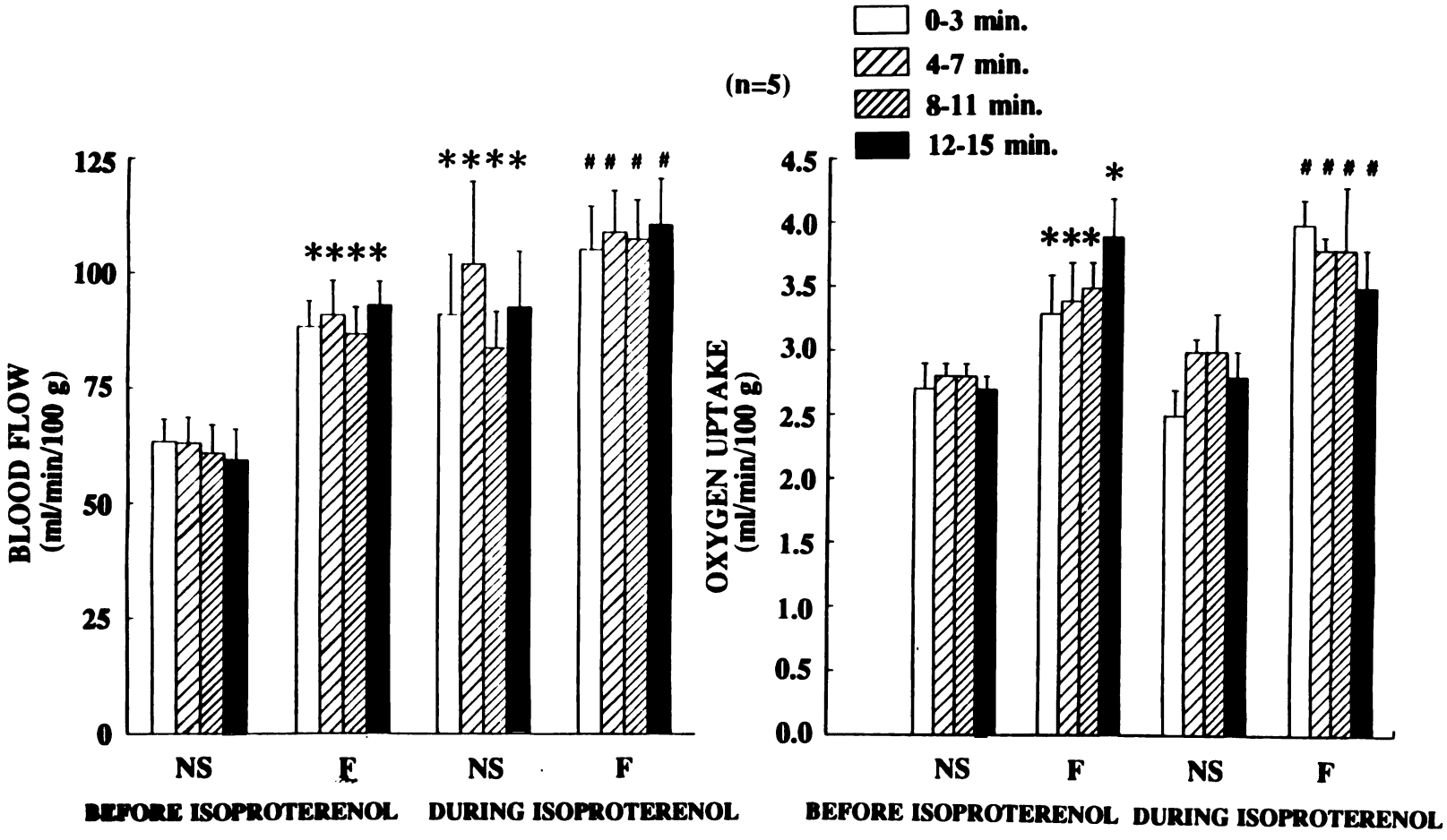



Figure 29. Jejunal blood flow and oxygen uptake during luminal placement of normal saline (NS) or food plus bile (F), before and during arterial infusion of isoproterenol (0.5 ug/min) in a single isolated jejunal segment. Isoproterenol increased resting blood flow to a similar degree as that observed by methylene blue. Food produced a significant increase in blood flow and oxygen uptake for the entire 15 period prior to and during infusion of isoproterenol. Values are means $\pm$ SE (n=7). * indicates a significant difference from NS at 12-15 min., and # indicates a significant difference from NS+MB at 12-15 min. $P < 0.05$.

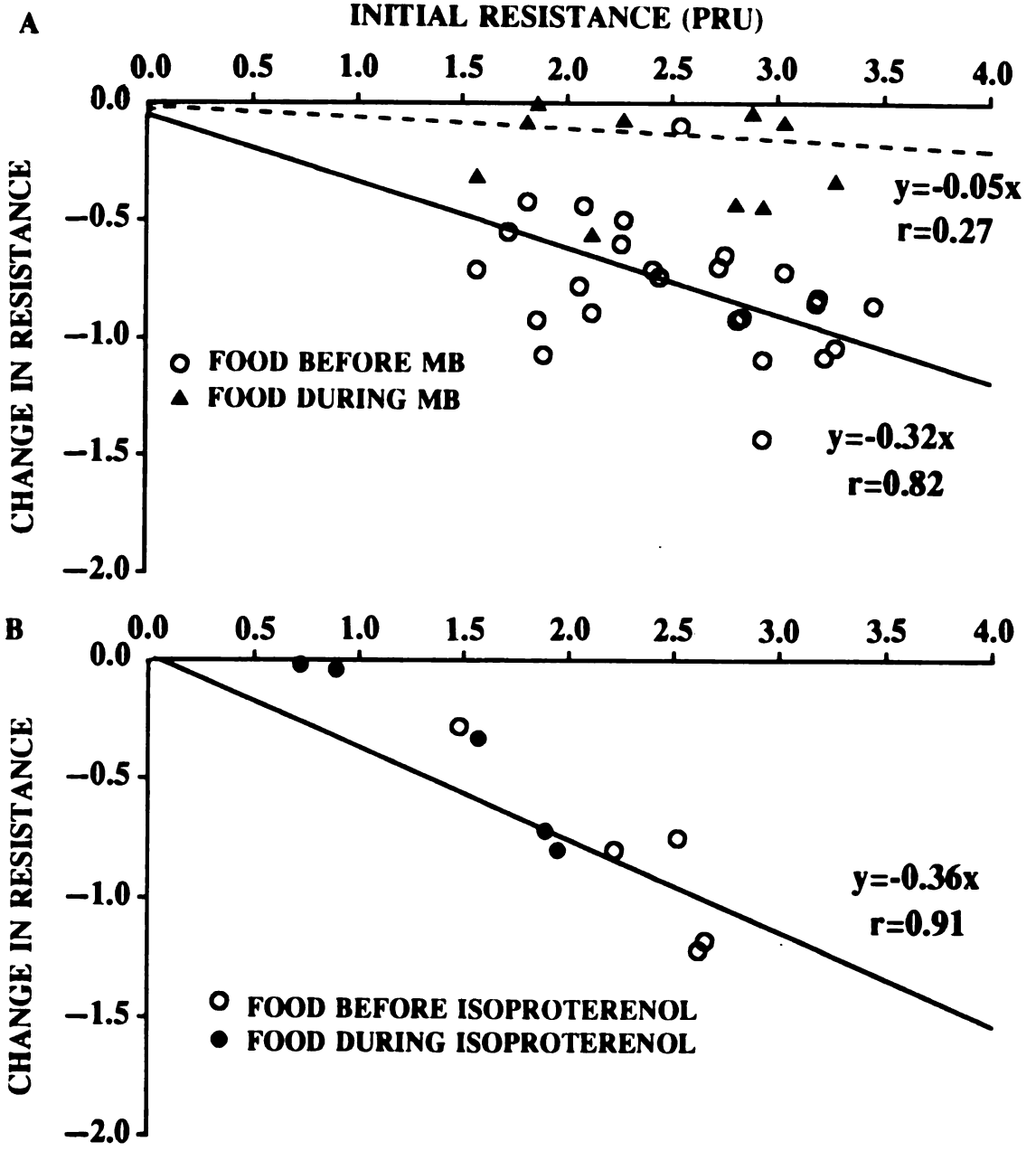



Figure 30. Relation between initial resistance and the change in resistance due to A) food alone (F) (open circles) or food plus methylene blue (F + MB) (closed triangles), and B) food alone before (open circles) and during (closed circles) isoproterenol. Initial resistances for F or F+MB were obtained immediately prior to food placement when the lumen still contained normal saline (NS) or NS+MB. Change in resistance is the difference in resistance between the value obtained during luminal placement of F or F+MB during the 12-15 minute time period and the respective initial resistance value. The slope of the regression line for F+MB (dashed line) is significantly ($P<0.05$) different from the regression line for food alone (A) or food before and during isoproterenol (B). PRU = peripheral resistance units.

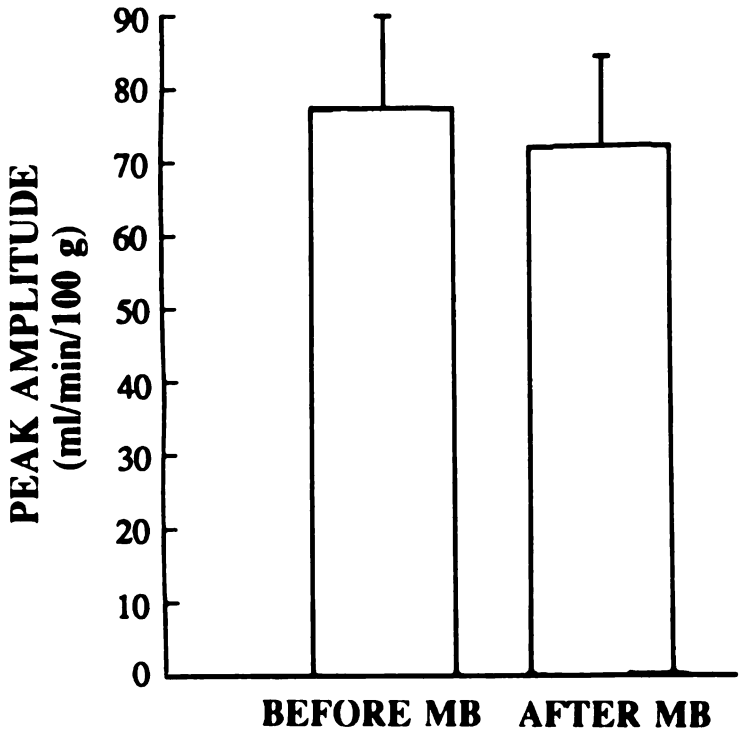



Figure 31. Peak reactive hyperemic response to arterial and venous occlusion for 30 seconds before and 15 minutes after luminal placement of methylene blue (1 mg/ml). The values are means $\pm$ SE (n=5). Methylene blue did not affect the peak reactive hyperemia.

## Summary and Conclusions

Several studies have suggested that nitric oxide plays a role in the regulation of mesenteric blood flow (38,57-60,133) and motility (11,17,30,32,163,164,179,181). However, this proposal is primarily based on studies utilizing isolated strips of mesenteric arterial or intestinal smooth muscle in vitro. A comprehensive study investigating the role of endogenous nitric oxide in the regulation of both intestinal blood flow and motility has not yet been done. In this dissertation, the L-arginine analogue L-nitro-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthesis, and methylene blue, an inhibitor of soluble guanylate cyclase, were used to investigate the role of nitric oxide on the regulation of jejunal blood flow and intestinal motility. Isolated rings of superior mesenteric arteries were also utilized to determine whether nitric oxide mediated the relaxation produced by acetylcholine, substance P, bradykinin, or adenosine in vitro. In addition, the effect of L-NAME on substance P-induced vasodilation under the conditions of constant blood flow in situ was evaluated.

The data allows for the following conclusions: 1) Nitric oxide mediates the endothelium-dependent relaxation of superior mesenteric arterial rings produced by ACh, substance P, and bradykinin in vitro. 2) Endogenous nitric oxide is a vasodilator of the jejunal vascular bed and appears to modulate jejunal vascular resistance in vivo. 3) Nitric oxide also acts to suppress intestinal motility under resting conditions. 4) The mechanism of vasodilation produced by substance P in jejunal resistance vessels in situ and the superior mesenteric artery in vitro are different. The relaxation of mesenteric arterial rings produced by substance P is nitric oxide-dependent, whereas the dilation of resistance arterioles elicited by bolus injection of substance P is nitric oxide-independent. 5) Nitric oxide may play a role in the regulation of the food-induced intestinal hyperemia, but does not mediate the reactive hyperemia produced by short periods of

vascular occlusion.

Systemic administration of L-NAME (0.5-20 mg/kg iv) produced a dose-dependent increase in mean arterial pressure and jejunal vascular resistance, and decrease in heart rate (Table 1). The maximum response of these variables was achieved at 10 mg/kg L-NAME (Table 1). This hemodynamic effect of L-NAME is similar to that already reported for L-NMMA and L-NOARG, other analogues of L-arginine that inhibit nitric oxide synthesis in rabbits (82,134), rats (57-60,133), and guinea pigs (3). The vasoconstriction in the mesenteric vascular bed produced by inhibition of endogenous nitric oxide synthesis is long lasting, and readily reversed by nitroglycerin when either applied topically (Figure 11 and Table 2) or injected into the single artery perfusing the isolated jejunal vascular bed (Figure 19A and Table 7). This would suggest that nitric oxide plays an important role in the regulation of systemic arterial blood pressure and intestinal blood flow in vivo.

Inhibition of endogenous nitric oxide synthesis also produced a marked increase in jejunal motility, associated with an increase in the basal lumen pressure, and an increase in the frequency and amplitude of phasic intestinal smooth muscle contractions (Figures 12 and 18). As shown in Figures 14 and 19A, the motility produced by L-NAME (10 mg/kg iv) was readily reversed by arterial infusion of the precursor to nitric oxide synthesis L-arginine (Figures 14 and 19B), but not by D-arginine. Nitroglycerin, a source of nitric oxide which does not involve nitric oxide synthase, promptly reversed the motility produced by L-NAME (Figure 11 and 19A). In addition, the motility produced after inhibition of nitric oxide synthesis was reversed by atropine (Figures 19A and 20). Calignano and Moncada (17) described similar results in rats using intraluminal manometers placed into the small intestine. These investigators reported that L-NAME produced an increase in frequency of phasic contractions in the small intestine that was attenuated after systemic administration of atropine (4 mg/kg). Therefore, endogenous nitric oxide most likely

functions to suppress intestinal motor activity, and inhibition of nitric oxide synthesis produces a cholinergic-mediated increase in motility at rest. These results are consistent with in vitro studies that have proposed that nitric oxide is a mediator of the nonadrenergic, noncholinergic relaxation of intestinal smooth muscle in vitro (11,17,30,32,164,171,189).

In contrast to the motility produced after administration of L-NAME, L-arginine did not effectively reverse the vasoconstriction produced by systemic administration of L-NAME (Figure 14) or local arterial infusion of L-NAME (Figure 19B). The inability of L-arginine to reverse the vasoconstriction produced by L-NAME is not clear. However, it does not appear to result from insufficient amounts of L-arginine, since L-arginine readily reversed the L-NAME-induced motility and the L-NAME- induced attenuation of the relaxation of mesenteric arterial rings to ACh, substance P, or bradykinin in vitro. It thus appears that the effect of L-arginine on the L-NAME-induced vasoconstriction is different from its effect on motility. The underlying mechanism for this difference is unclear. A possible explanation for the difference in the effect of L-arginine on the L-NAME-induced changes in motility and vasoconstriction may be due to changes in the blood flow distribution to the jejunum after motility appears. Phasic contractions of the small intestine have been shown to increase jejunal blood flow primarily to the muscularis serosal layers (20,42). Thus, the L-arginine infused after the onset of motility may be preferentially distributed to the muscularis layer and is thereby shunted away the from resistance vessels located in submucosa and mucosa. However, this seem unlikely since it has recently been demonstrated in Dr. Chou's lab that pretreatment with L-arginine prevented the motility, but did not prevent the vasoconstriction produced by L-NAME (data not shown). Gardiner et al. (57-60) have reported that the action of L-arginine on the vasoconstriction produced by L-NAME or L-NMMA is complete in the kidney, but the reversal of vasoconstriction in the superior mesenteric, internal carotid and hindlimb vascular beds is transcient and incomplete. The inhibitory action of L-

arginine on L-NAME or L-NMMA, therefore, might differ among different organs. Furthermore a difference in nitric oxide synthase enzyme subtype in endothelial cells and enteric nerves responsible for gut motility might be different. This difference may account for the differential action of L-arginine on the L-NAME-induced changes in jejunal blood flow and motility. Lastly, the inability of L-arginine to reverse the constrictive effect of L-NAME may result from L-NAME-induced effects that are not related to nitric oxide synthesis per se.

When the isolated jejunal segment was perfused naturally with aortic blood, a secondary increase in blood flow and decrease in vascular resistance was observed. The increase in blood flow and decrease in vascular resistance typically presented as motility declined from a maximum to a steady state in each dog. As motility began to wane toward basal levels the initial vasoconstriction produced by L-NAME reappeared. The decrease in vascular resistance as the L-NAME-induced motility decayed from maximum was similar to that observed under conditions of constant jejunal blood flow (Figure 18). However, when motility is prevented by continuous arterial infusion of atropine the secondary decrease in vascular resistance is negligible. Therefore, the motility produced after inhibition of nitric oxide synthesis appears to secondarily influence the vascular responses to blockade of nitric oxide synthesis.

Luminal placement of methylene blue significantly increased resting blood flow and decreased vascular resistance. In addition, methylene blue significantly increased motility, although not to the degree of that observed after inhibition of nitric oxide synthesis with L-NAME. The reason for the discrepancy in the amount of motility produced after methylene blue versus L-NAME most likely results from different routes of administration for methylene blue and L-NAME. L-NAME was given as a systemic injection into the femoral vein or infused directly into the artery perfusing the isolated jejunal segment, whereas methylene blue was administered intraluminally. Preliminary data that I did not present in this dissertation showed that arterial

infusion of methylene blue (2-4 mg/ml of normal saline) markedly enhanced motility, as indicated by an increase in the frequency of phasic contractions and tonic pressure of the jejunal segment. We chose not to administer methylene blue arterially because it tended to aggregate to the walls of the extracorporeal shunt and thereby limit perfusion of the isolated jejunal segment.

To prove that the L-NAME-induced vasoconstriction and motility produced after systemic administration of L-NAME did not result from baroreflexor stimulation by an increase in systemic arterial pressure, L-NAME was locally infused into the single artery perfusing the jejunal segment. The vasoconstriction and motility produced by arterial infusion of L-NAME (Figures 18, 19A and 19B) was similar to that produced when L-NAME was systemically administered (Figures 12 and 14). However, systemic arterial blood pressure was not affected when L-NAME was infused locally into the single artery perfusing the jejunal segment. Therefore, an increase in systemic blood pressure and subsequent change in the baroreflex cannot account for the effect of L-NAME seen within the jejunum. In addition, this study demonstrated that the L-NAME-induced vasoconstriction and motility resulted from the inhibition of endogenous nitric oxide within the jejunum.

Endothelial cell removal from isolated superior mesenteric arterial rings abolished the relaxation produced by acetylcholine (ACh), substance P, and bradykinin, but did not affect the relaxation produced by nitroglycerin or adenosine (Figures 1 and 2). In addition, incubation of the arterial rings with methylene blue, an inhibitor of soluble guanylate cyclase, markedly attenuated the relaxation produced by ACh, substance P, bradykinin, or nitroglycerin, whereas the relaxation produced by adenosine was not inhibited (Figures 3 and 4). Incubation of the arterial rings to L-NAME also inhibited the relaxation produced by ACh, substance P, or bradykinin (Figure 5). Systemic administration of L-NAME (Figure 15) also inhibited the endothelium-dependent relaxation produced by ACh, substance P, or bradykinin to a similar degree as that

observed after incubation of the arterial rings with L-NAME in vitro. Furthermore, the L-NAME induced inhibition of the relaxation produced by ACh, substance P, or bradykinin was reversed after incubation with L-arginine, but not D-arginine. In contrast, the endothelium-independent relaxation elicited by nitroglycerin or adenosine was not affected after inhibition of endogenous nitric oxide synthesis. These results indicate that the endothelium-dependent relaxation of mesenteric arteries produced by ACh, substance P, or bradykinin in vitro is mediated by nitric oxide. The relaxation produced by adenosine is not dependent on the endothelium and is not mediated by nitric oxide.

Although the in vitro results are consistent with the view that nitric oxide mediates the relaxation of mesenteric arteries produced by the substance P, L-NAME failed to inhibit the dilation of mesenteric resistance vessels produced by substance P in vivo (Figure 22). This result was not expected and the reason for the inability of L-NAME to block substance P-induced vasodilation in situ is not clear. Since systemic administration of L-NAME (10 mg/kg iv) was effective in inhibiting the relaxation produced by substance P in vitro as well as eliciting a long lasting vasoconstrictive effect, it would appear that this concentration of L-NAME would be sufficient to inhibit the dilation produced by substance P in situ. The in vitro and in vivo studies differ in regard to the size of the vessels (large artery vs. resistance vessels) and the medium in which vessels are exposed to substance P (Kreb's solution vs. whole blood). From these data, it appears that endogenous nitric oxide is released from the endothelial cells of the mesenteric vascular bed and modulates arteriolar tone under resting conditions. However, the vasodilation produced by substance P in situ appears to be mediated by a mechanism(s) that is independent of endogenous nitric oxide.

In summary, the results of this dissertation are consistent with the concept that endogenous nitric oxide regulates resting tone of jejunal resistance vessels and intestinal motility. L-NAME,

an inhibitor of nitric oxide synthesis, produced a marked increase in motility and vasoconstriction when administered either systemically or locally within the jejunum. Nitric oxide derived from the endothelium also mediated the relaxation of superior mesenteric arterial rings to acetylcholine, substance P, or bradykinin in vitro. Removal of the endothelial cells, or incubation with methylene blue or L-NAME inhibited the relaxation of the superior mesenteric artery produced by acetylcholine, substance P, and bradykinin in vitro. In contrast, L-NAME did not effect the dilator response to substance P in vivo. These findings suggest that intestinal motility, the basal tone of resistance vessels in the jejunal vascular bed, and the relaxation of large conduit arteries produced by acetylcholine, substance P, or bradykinin in vitro are mediated by endogenous nitric oxide. However, the mechanism responsible for the relaxation of resistance vessels produced by substance P is different, and the relaxation is independent of endogenous nitric oxide synthesis.

## Proposed Mechanisms Responsible for the Action of Nitric Oxide in the Jejunum

From the results of this dissertation, the site(s) of nitric oxide synthesis that is responsible for the regulation of motility and vasodilation in the jejunum are not completely clear. However, the endogenous nitric oxide that relaxes intestinal and vascular smooth muscle are most likely derived from different sources. The nitric oxide responsible for the inhibition of motility is probably derived from enteric nerves, whereas the nitric oxide that relaxes vascular smooth muscle seems to be located within the endothelial cells. The following indirect evidences support this hypothesis. First, the resistance arterioles that regulate vascular resistance are located within the submucosa. It thus seems implausible that the nitric oxide derived from endothelial cells of these arterioles could diffuse to the circular smooth muscle before degradation of nitric oxide were to occur, considering the extremely short halflife of nitric oxide in biologic solutions (between 3-7

seconds). Secondly, immunohistochemical studies utilizing antibodies raised against rat cerebellar nitric oxide synthase demonstrate that nitric oxide synthase is present in nerve cells located within the myenteric plexus as well as the endothelial cells of the mesenteric vascular bed. Thirdly, arterial infusion of L-arginine, the precursor to nitric oxide synthesis, or atropine effectively reversed the L-NAME-induced motility, but not the vasoconstriction produced after inhibition of nitric oxide synthesis. This also suggests that the source of nitric oxide responsible for modulating intestinal and vascular smooth muscle reactivity are different. Future studies should investigate the possibility that the sites of endogenous nitric oxide synthesis that regulate intestinal motility and vascular smooth muscle relaxation differ.

Figure 32 illustrates the current mechanism by which nitric oxide has been proposed to regulate intestinal motility. The mechanism of nitric oxide-induced relaxation of intestinal smooth muscle shares a great deal of similarity to the mechanism that has been proposed to occur within the endothelial cells and vascular smooth muscle (see Figure 33). Nitric oxide is synthesized from its precursor L-arginine by the enzyme nitric oxide synthase within neurones located in the myenteric plexus of the gastrointestinal tract. When stimulated, nitroxidergic neurones release nitric oxide (NO), or a short lived nitrosothiol (RNO) that degrades to nitric oxide, from nerve terminals. Nitric oxide can diffuse into intestinal smooth muscle cells, bind to the heme moiety of soluble guanylate cyclase and activate soluble guanylate cyclase. The activation of soluble guanylate cyclase enhances the conversion of guanosine triphosphate (GTP) to 3',5' cyclic guanosine monophosphate (cyclic GMP). The accumulation of cyclic GMP within the intestinal smooth muscle results in relaxation by a mechanism that is currently not clear, but may involve the dephosphorylation of myosin light chain kinase. Methylene blue (MB) and oxyhemoglobin (OxyHb), inhibitors of soluble guanylate cyclase, prevents the conversion of GTP to cyclic GMP and thereby prevents the relaxation of intestinal smooth muscle produced by nitric oxide. In

addition, oxyhemoglobin and superoxide anion ($O_2^-$) have been shown to bind and inactivate nitric oxide in transit.

Intestinal smooth muscle contraction occurs primarily through the activation of cholinergic nerves and the contraction can be blocked in the presence of a muscarinic antagonist such as atropine. The relaxation of intestinal smooth muscle produced by endogenous nitric oxide appears to reduce cholinergic-mediated intestinal smooth muscle contraction. This is supported by the finding that inhibition of nitric oxide synthesis with L-NAME elicits intestinal smooth muscle contractions that are inhibited in the presence of atropine. In this way, endogenous nitroxidergic nerves may mediate the receptive relaxation of smooth muscle that allow for the aboral movement of digested chyme within the gastrointestinal tract. From the data presented in this dissertation we cannot rule out the possibility that nitroxidergic nerves inhibit cholinergic nerve activity directly prior to the neuromuscular junction. However, numberous in vitro studies indicate that stimulation of nitroxidergic nerves within intestinal smooth muscle strips results in the relaxation of intestinal smooth muscle in the presence of muscarinic blockade (11,30,32,163,164,179,181). This would suggest that nitroxidergic nerves directly innervate intestinal smooth muscle rather than presynaptically modulate cholinergic nerve activity.

Figure 33 illustrates the hypothetical mechanism of vascular smooth muscle relaxation mediated by endothelium-derived nitric oxide in vitro. When bound to their respective receptors, acetylcholine (ACh), substance P (Sub P), and bradykinin (BK) stimulate the synthesis of nitric oxide (NO) within endothelial cells. Once synthesized, nitric oxide can diffuse into vascular smooth muscle cells and activate soluble guanylate cyclase. This results in cyclic GMP accumulation and relaxation of the vascular smooth muscle. Inhibition of nitric oxide synthase with L-NAME prevents the relaxation produced by acetylcholine, substance P, or bradykinin. Nitroglycerin, an exogenous nitrovasodilator that spontaneously releases nitric oxide, directly

activates soluble guanylate cyclase. Therefore, the relaxation of vascular smooth muscle produced by nitroglycerin is independent of the endothelium and is not affected by nitric oxide synthesis inhibitors. However, inhibition of soluble guanylate cyclase with methylene blue blocks the relaxation produced by endogenous nitric oxide as well as the relaxation produced by nitroglycerin.

From the present studies, it appears that nitric oxide is tonically released from enteric nerves and vascular endothelial cells since L-NAME-induced inhibition of endogenous nitric oxide synthesis produced a rapid increase in motility as well as vasoconstriction. At the present time, the physiologic stimuli responsible for nitric oxide synthesis and release from the jejunum remain speculative. However, it is plausible that there are several stimuli for nitric oxide synthesis within the small intestine, and that the stimuli reponsible for nitric oxide synthesis within endothelial cells and enteric nerves may differ. Intraluminal distension of the intestine may stimulate nitric oxide release from enteric nerves. In this way, when a bolus of food distends an intestinal segment, activation of nitroxidergic nerves would relax descending segments of intestine to allow for the aboral movement of food. Similarly, the stimuli responsible for the release of nitric oxide from vascular endothelial cells is not known. Several autocoids, including acetylcholine, substance P, and bradykinin have been proposed to stimulate the release of nitric oxide from endothelial cells. We were unable to determine if the vasodilation produced by exogenous acetylcholine was mediated by endogenous nitric oxide because it was necessary to suppress the L-NAME-induced motility with atropine. However, data from this dissertation indicate that the substance P-induced relaxation of mesenteric resistance vessels is not altered after inhibition of nitric oxide synthesis. One possible physiologic stimulus for nitric oxide synthesis and release appears to result from ingestion of a meal. This is supported by our data showing that the addition of methylene blue to digested food significantly inhibited the food-induced hyperemia. Our experiments also suggest

that if substance P plays a substantial role in regulating the postprandial intestinal hyperemia, it does not appear to be dependent on nitric oxide synthesis.

Mechanical factors such as pulsatile flow and shear stress of the vascular wall have been shown to stimulate the release of nitric oxide from resistance vessels in situ (69-70,128,160). In addition, other studies have shown that nitric oxide release is greatest in arterioles in which the hydraulic pressures and shear stress are greatest (69,70). If this is true, then the release of nitric oxide from the large arterioles in response to hydraulic pressure and shear stress may be of great importance in controlling blood flow distribution in the vasculature.

In conclusion, endogenous nitric oxide appears to play a role in the regulation of motility and blood flow in the small intestine. Nitric oxide may also be a mediator of the food-induced hyperemia, but does not appear to mediate in the reactive hyperemia produced by brief periods of vascular occlusion or the vasodilation produced by exogenous substance P in the vacular bed of the small intestine. The stimulus for nitric oxide release from enteric nerves and vascular endothelial cells is currently not clear.

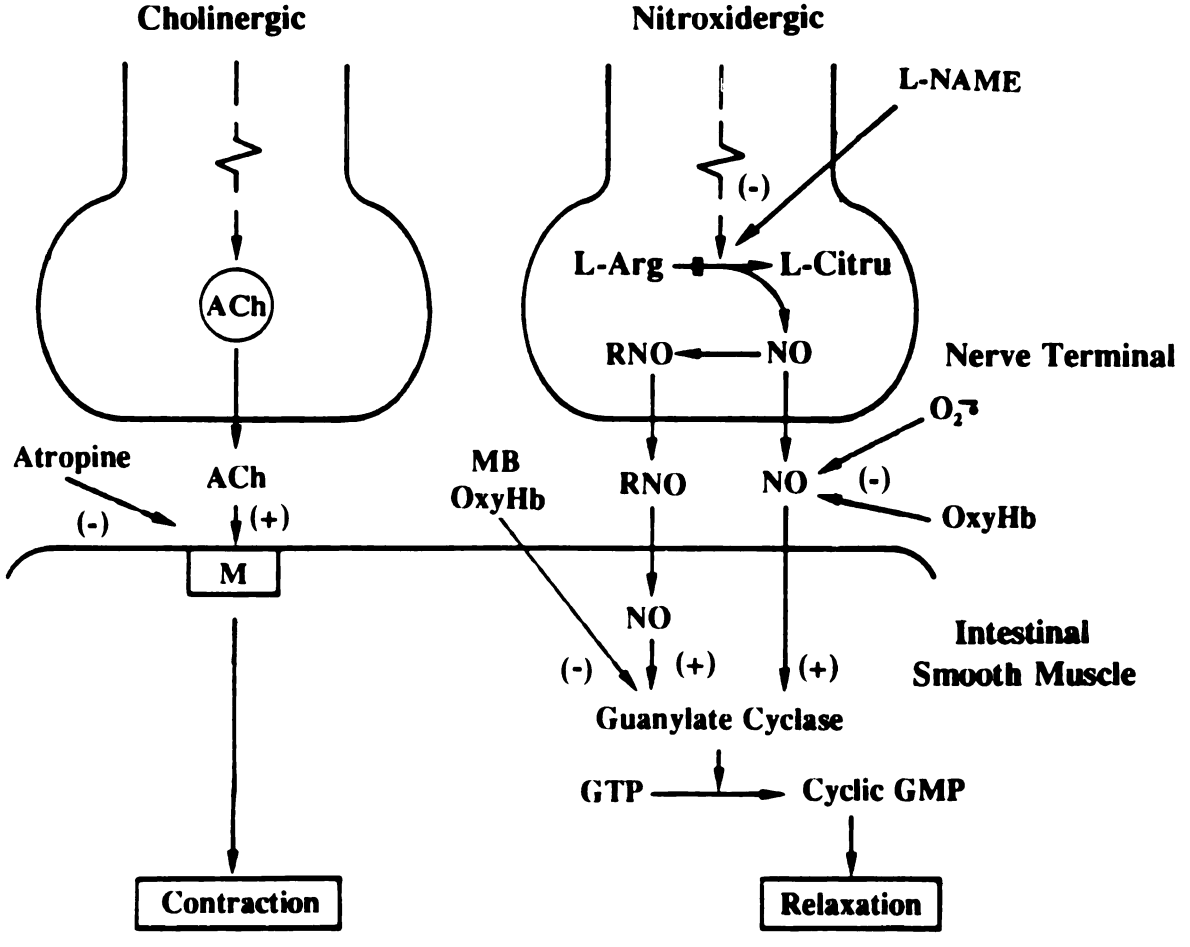



Figure 32. Hypothetical diagram of the reciprocal nitroxidergic and cholinergic innervation of intestinal smooth muscle. L-NAME, L-nitro-arginine methyl ester; L-Arg, L-arginine; L-Citru, L-Citrulline; NO, nitric oxide; RNO, nitrosothiol; GTP, guanosine triphosphate; MB, methylene blue; $O_2^-$, superoxide anion; OxyHb, oxyhemoglobin; ACh, acetylcholine; M, muscarinic receptor.

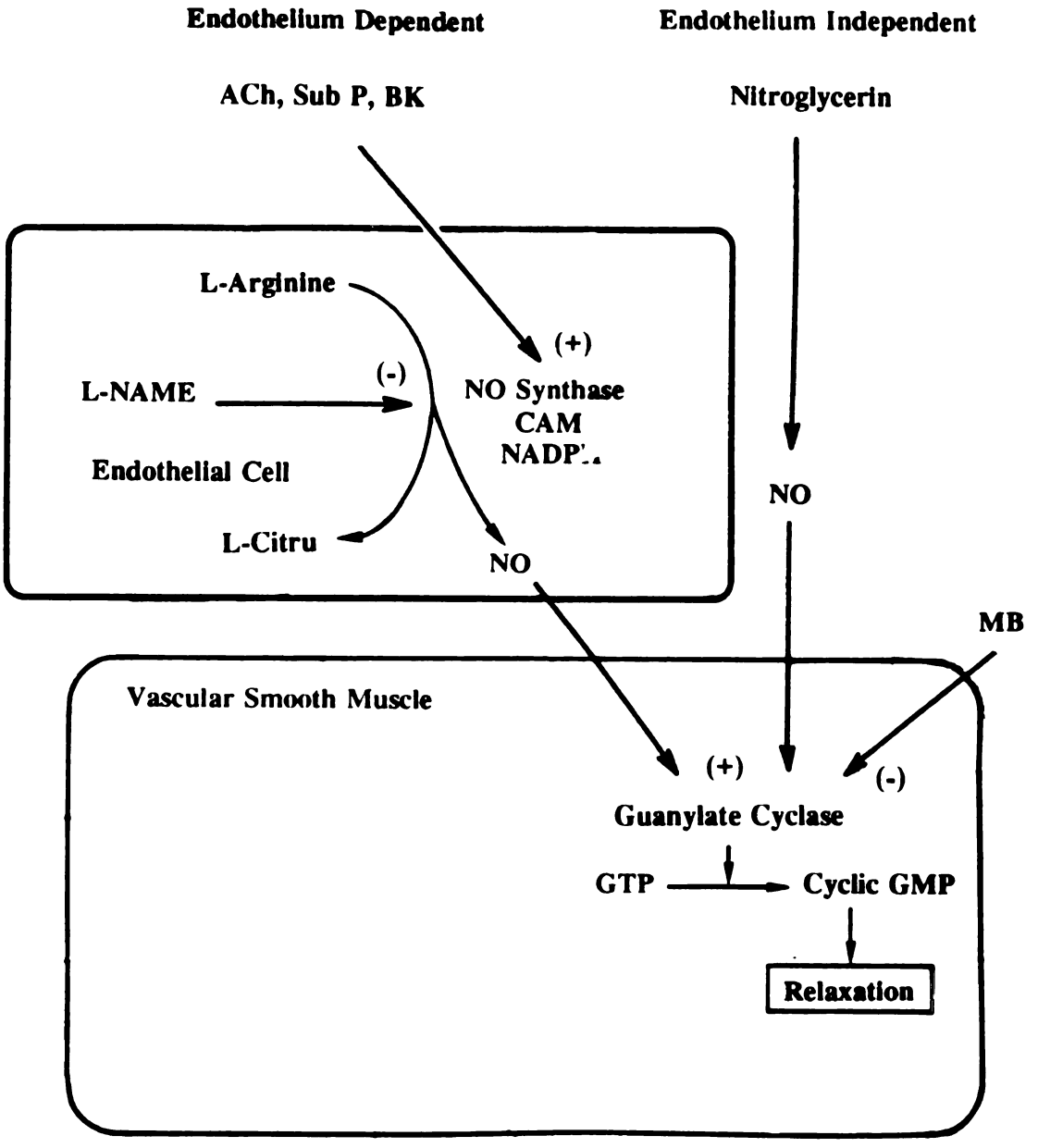



Figure 33. Hypothetical diagram of the mechanism of endothelium-dependent and endothelium-independent relaxation of vascular smooth muscle by nitric oxide. L-NAME, L-nitro-arginine methyl ester; L-Arg, L-arginine; L-Citru, L-Citrulline; NO, nitric oxide; CAM, calmodulin; NAPDH, nicotinamide adenine dinucleotide phosphate; GTP, guanosine triphosphate; MB, methylene blue; OxyHb, oxyhemoglobin; ACh, acetylcholine; Sub P, substance P; BK, bradykinin.

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