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Patricia K. Tithof D.V.M.

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ENDOTHELIAL CELLS AND THE CONTROL OF LYMPHATIC AND VASCULAR SMOOTH MUSCLE TONE: EFFECT OF THE FILARIAL PARASITE *Dirofilaria immitis*

By

Patricia K. Tithof D.V.M.

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ABSTRACT

ENDOTHELIAL CELLS AND THE CONTROL OF LYMPHATIC AND VASCULAR SMOOTH MUSCLE TONE: EFFECT OF THE FILARIAL PARASITE *Dirofilaria immitis*

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Patricia K. Tithof

Infection with Dirofilaria immitis depresses endothelium-dependent relaxation of the in vivo canine femoral artery. Experiments were designed to determine whether previous in vivo exposure to D. immitis depresses endothelium-dependent relaxation of isolated canine femoral artery, vein and thoracic duct. Methacholine dose-response relationships were examined in isolated preparations +/- mefenamic acid and/or indomethacin, methylene blue and N^G-nitro-L-arginine methyl ester (L-NAME).

Methacholine relaxation was not depressed in femoral artery or vein from heartworm infected dogs when compared to control. In preliminary experiments with thoracic duct, however, methacholine relaxation was significantly attenuated by in vivo exposure to heartworm. Methylene blue and L-NAME inhibited relaxation in femoral artery from control and heartworm infected dogs and in thoracic duct from control dogs, suggesting that NO is important in the response to methacholine. However, in preliminary studies, the depressant effect of methylene blue and L-NAME was less in femoral vein and thoracic duct from heartworm infected dogs when compared to control suggesting that NO may be less important in the relaxation of these vessels to methacholine. These data suggest that the depression of endothelium-dependent relaxation observed in the in vivo femoral artery from heartworm infected dogs is not demonstrable in systemic blood vessels in

vitro. However, previous exposure to heartworm may alter endothelium-dependent relaxation of the isolated thoracic duct.

Human filarial parasites reside in peripheral lymphatics, therefore an ultimate goal is to determine the effect of filarial parasites on peripheral lymphatic function. Rhythmic contractions are important in propulsion of lymph in peripheral vessels and the mechanism of these contractions is unknown. Experiments were designed determine whether an endothelium-derived factor mediates spontaneous contractions of bovine mesenteric lymphatics. Isolated rings were suspended in organ chambers for evaluation of contractile activity. Endothelial cells were removed either mechanically or chemically with collagenase. To determine whether an endothelium-derived factor is responsible for contractions, contractile activity was evaluated in endothelium-denuded rings "sandwiched" with endothelium-intact rings. Endothelial cell removal abolished spontaneous contractions. Contractions returned in denuded rings which were "sandwiched" with endothelium-intact rings, thus suggesting that an endothelium-derived diffusible factor mediates spontaneous contractions.

Since endothelial cells are important in the contractile response of peripheral lymphatics and D. immitis depresses endothelium-mediated responses it was hypothesized that heartworm-conditioned medium would depress contractions. In preliminary studies spontaneous contractile activity was abolished by exposure to heartworm-conditioned, but not control medium.

These results suggest that filarial parasites alter endothelium-mediated responses of isolated lymphatics. Filarial-induced alteration in lymphatic function may be important in the pathogenesis of filariasis.

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Introduction

Over 250 million people and countless animals are infected with filarial parasites. Despite extensive investigation, the pathogenesis of filarial diseases is not well understood. Clinical manifestations of human lymphatic filariasis range from inapparent infection to disabling lymphedema and elephantiasis (Weller et al., 1982; Piessens et al., 1980), and the pathogenesis has been attributed to outflow obstruction of lymphatics by the adult parasites or the immunological and inflammatory response of the host (Schacher & Sahyoun, 1967; Piessens et al., 1980; Nutman et al., 1987; Case et al., 1991). Neither of these mechanisms, however, adequately explain the complex clinical manifestations of these diseases (Kwan-Lim & Maizels, 1991; Case et al., 1991; Ottesen 1992).

Dirofilaria immitis, the canine heartworm, is a filarial parasite that is taxonomically related to the human filarial pathogens (Grieve et al., 1983). Whereas adult lymphatic filarial parasites reside in the lymphatics of humans (Hawking, 1977), adult D. immitis are found primarily in the right heart and pulmonary arteries of dogs (Jackson et al., 1966). Thus, the clinical manifestations of lymphatic filariasis are mostly due to abnormalities in lymphatic function (Hawking, 1977), whereas canine heartworm disease is characterized primarily by cardiovascular dysfunction (Calvert, 1987). Despite these differences, the pathogenesis of filariasis may be the same in humans and canines (Weil et al., 1982). Indeed, the spectrum of clinical symptoms seen in human filarial disease is also observed in dogs with D. immitis and ranges from inapparent infection to severe cardiopulmonary disease (Weil et al., 1982; Calvert, 1987). As in human filariasis, the pathogenesis of canine heartworm

disease has been attributed to pulmonary outflow obstruction by the adult parasites or the immunological and inflammatory response of the host (Atwell et al., 1985; Schaub et al., 1983; Rawlings, 1986; Knight, 1987). There is little direct experimental evidence, however, to support these mechanisms in the pathogenesis of canine heartworm disease.

Filarial parasites and parasite products are in direct contact with endothelial cells in both human lymphatic filariasis and canine heartworm disease, thus the interaction of filarial parasites with vascular and lymphatic endothelial cells may be important in the pathogenesis of filarial disease. Previous studies indicate that endothelial cell structure and function are altered by exposure to parasites and parasite products (Kwa et al., 1991; Schaub & Rawlings, 1980; Keith et al., 1983; Case et al., 1991). Endothelial cells play an important role in the control of underlying smooth muscle tone, and alteration of this control is characteristic of several cardiovascular diseases (Luscher et al., 1986; Kaiser et al., 1989a; Tesfamarian et al., 1989; Verbeuren et al., 1986; Knowles et al., 1990; Vallance et al., 1992). In view of the proximity of filarial parasites to vascular and lymphatic endothelial cells, and the emerging role for altered endothelial cell behavior in the pathogenesis of cardiovascular disease, Kaiser et al., (1987) postulated that altered endothelium-dependent vascular reactivity may be important in the pathogenesis of filariasis.

It was demonstrated that endothelium-dependent relaxation to acetylcholine is depressed in the in vivo femoral artery from dogs infected with D. immitis (Kaiser et al., 1987, 1989a). The depression is seasonal; it is seen in the spring, a time of maximal reproductive activity of the adult parasites, but not the fall, a more quiescent time (Stewart, 1975; Kaiser et al., 1987). Furthermore, the mechanism of relaxation is different in femoral artery from heartworm infected dogs when compared to control. Methylene blue, but not

indomethacin, significantly attenuates acetylcholine dilation in femoral artery from control dogs, suggesting that the NO/guanylate cyclase/cGMP system is primarily responsible for acetylcholine dilation in femoral artery from control dogs. In femoral artery from heartworm infected dogs, however, indomethacin depresses relaxation and methylene blue causes a biphasic response; enhanced relaxation at low concentrations and depressed relaxation at high concentrations of acetylcholine. These results suggest that the mechanism of acetylcholine relaxation is different in femoral artery from heartworm infected dogs when compared to control and involves two endothelium-derived relaxing factors; one is a cyclooxygenase product and the other is likely NO (Kaiser et al., 1987, 1989a).

Biologically active factors released by the adult parasites may be responsible for the filarial-induced alteration of endothelium-dependent relaxation (Kaiser et al., 1987; 1989a; 1990a, 1992). Since the same seasonal depression of endothelium-dependent relaxation seen in dogs with patent (microfilariae positive) infection is also seen in dogs with occult dirofilariasis (microfilariae negative), the adult parasites rather than the microfilariae are likely responsible for depression of endothelium-dependent relaxation (Kaiser et al., 1987). Adult parasites reside in the right heart and pulmonary arteries (Jackson et al., 1966), and depression of relaxation is seen in femoral artery, thus suggesting that filarial factors are responsible for the effect of D. immitis on femoral artery relaxation (Kaiser et al., 1987, 1990a). Therefore, experiments were designed using isolated rat aorta to characterize the parasite product(s) responsible for depression of relaxation. Short-term exposure of rat aorta to adult D. immitis, filarial-conditioned medium or serum from heartworm infected dogs depresses endothelium-dependent relaxation. The depression is

due, in part, to a filarial factor which may be filarial prostagladin D_2 (Kaiser et al 1990a, 1992; Lamb et al 1992).

Since previous experiments with femoral artery have been done in the presence of adult heartworms, the duration of filarial-induced depression of relaxation in systemic blood vessels is unknown. Therefore, a major goal of this study was to determine whether the depression and alteration of mechanism of endothelium-dependent relaxation observed in vivo is demonstrable in systemic vessels in vitro. Experiments were designed to test the hypothesis that previous in vivo exposure to D. immitis depresses and alters the mechanism of endothelium-dependent relaxation in the in vitro canine femoral artery and vein.

Because D. immitis is taxonomically related to the human filarial pathogens and because canine heartworm disease is prevalent in the United States, it is a useful model for studying human filarial disease (Grieve et al., 1983; Wong et al, 1983). Therefore, another purpose of this study was to determine whether endothelium-mediated responses of lymphatics are altered by acute and chronic exposure to D. immitis.

Only recently has the role of endothelial cells in the control of lymphatic smooth muscle tone been investigated. Ohhashi et al., (1991) demonstrated that endothelium-derived NO is responsible for the relaxation response to acetylcholine in the in vitro canine thoracic duct. Because endothelium-dependent responses have been demonstrated in canine thoracic duct, studies concerning the effects of D. immitis on lymphatics were initiated in canine thoracic duct. Experiments were designed to test the hypothesis that endothelium-dependent relaxation is depressed and the mechanism of relaxation is altered by previous in vivo exposure to D. immitis in the in vitro canine thoracic duct from heartworm infected dogs when compared to control.

Since factors released by the adult parasites depress endothelium-dependent relaxation in the vasculature (Kaiser et al., 1990a), filarial factors may also be responsible for depression of relaxation in lymphatics. Therefore, endothelium-dependent responses of the in vitro thoracic duct from control dogs was examined in the presence of heartworm-conditioned or control medium.

The thoracic duct is a conduit lymphatic and the flow of lymph in this vessel is driven primarily by passive forces (Ohhashi et al., 1991). In humans, filarial parasites inhabit peripheral lymphatics (Case et al., 1991). Although passive driving forces play a role in propulsion of lymph in peripheral lymphatics, a more important driving force is thought to be spontaneous contractile activity (Hall et al., 1965; McHale & Roddie, 1976; Roddie et al., 1980; Hargens & Zweifach, 1977; Benoit et al., 1989). If rhythmic contractions of lymphatics are important in the propulsion of lymph, then disruption of this activity by filarial parasites may be important in the pathogenesis of lymphedema and elephantiasis. Therefore, an ultimate goal of this research is to determine the effect of filarial parasites on phasic contractions of peripheral lymphatics. However, the mechanism of spontaneous contractions in peripheral lymphatics is not well understood (Elias et al., 1992; Bohlen & Lash, 1992).

There is considerable evidence to suggest that endothelial cells are important in the control of rhythmic lymphatic contractions. Previous reports indicate that some lymphatics spontaneously contract when studied in vitro while others do not. This variability has been attributed to damage during preparation of in vitro rings or strips (Mahwinney & Roddie, 1973; McHale & Roddie, 1976). Since damage to endothelial cells was responsible for the variable effect of acetylcholine on isolated blood vessels (Furchgott & Zawadzki,

1980), a similar explanation may account for the variability in contractile activity of isolated lymphatics. Johnston and coworkers (1981, 1983) and Allen et al., (1984) reported that a cyclooxygenase product derived from the lymphatic wall, which may be thromboxane A_2, is responsible for spontaneous contractions of isolated bovine mesenteric lymphatics. In the vasculature, endothelial cells are the primary source of thromboxane (Ingerman-Wojenski et al., 1981). If the same is true for lymphatics, then this is suggestive of a role for endothelial cells in spontaneous contractile activity. Finally, oxyhemoglobin when infused intraluminally, but not extraluminally, abolishes spontaneous contractions of bovine mesenteric lymphatics (Elias et al., 1992). Oxyhemoglobin, which acts primarily extracellularly because of its large molecular weight, is a well known inhibitor of endothelium-mediated responses (Gruetter et al., 1981; Ignarro et al., 1984). Thus, the inhibition of spontaneous contractions by intraluminal oxyhemoglobin suggests that this agent is acting primarily on endothelial cells. In light of these studies, experiments were designed to determine whether endothelial cells are important in spontaneous contractions of isolated lymphatics. Because bovine mesenteric lymphatics are structurally and functionally similar to human leg lymphatics and have been studied extensively in vitro, this preparation was chosen for the present study (Ohhashi et al., 1977). Experiments were designed to test the hypothesis that an endothelium-derived diffusible substance is responsible for spontaneous contractions of isolated rings of bovine mesenteric lymphatics. Additionally, preliminary studies were undertaken to determine the effect biologically active filarial factors on phasic contractions.

The following specific hypotheses were tested.

1. Infection with D. immitis depresses endothelium-dependent relaxation to methacholine in the in vitro femoral artery and vein from heartworm infected dogs when compared to control.

2. The mechanism of methacholine relaxation is different in femoral artery and vein from heartworm infected and control dogs.

a. In femoral artery and vein from control dogs, inhibitors of the NO/guanylate cyclase/cGMP system (methylene blue and L-NAME), but not inhibitors of cyclooxygenase (indomethacin and mefenamic acid), attenuate methacholine relaxation.

b. In femoral artery and vein from heartworm infected dogs, both inhibitors of the NO/guanylate cyclase/cGMP system (methylene blue and L-NAME) and inhibitors of cyclooxygenase (indomethacin or mefenamic acid) attenuate methacholine relaxation.

3. Exposure of the isolated thoracic duct from control dogs to heartworm-conditioned medium depresses endothelium-dependent relaxation to methacholine.

4. Infection with D. immitis depresses endothelium-dependent relaxation to methacholine in the in vitro thoracic duct from heartworm infected dogs when compared to control.

5. The mechanism of methacholine relaxation is different in thoracic duct from heartworm infected and control dogs.

a. In thoracic duct from control dogs, inhibitors of the NO/guanylate cyclase/cGMP system (methylene blue and L-NAME), but not inhibitors of cyclooxygenase (indomethacin and mefenamic acid), attenuate methacholine relaxation.

b. In thoracic duct from heartworm infected dogs, both inhibitors of the NO/guanylate cyclase/cGMP system (methylene blue and L-NAME) and inhibitors of cyclooxygenase (indomethacin or mefenamic acid) attenuate methacholine relaxation.

6. An endothelium-derived diffusible factor is responsible for spontaneous contractions in isolated rings of bovine mesenteric lymphatics.

7. Exposure of isolated rings of bovine mesenteric lymphatics to heartworm-conditioned medium depresses endothelium-dependent spontaneous contractile activity.

Review of the Literature

Endothelial cells and vascular smooth muscle relaxation

The role of endothelial cells in the control of vascular smooth muscle tone was first demonstrated by Furchgott and Zawadzki in 1980. Before this time, investigators reported different results with acetylcholine in vitro. Furchgott and Bhadrakom (1953) reported that acetylcholine constricted isolated strips of rabbit aorta at concentrations above10^{-7}M but caused no response at lower concentrations. Jeliffe (1962), however, using the same preparation, reported a relaxation response to acetylcholine at 10^{-9}M. Furchgott and Zawadzki (1980) postulated that accidental injury to endothelial cells during preparation of helical strips may account for these differences in response to acetycholine. They showed that acetylcholine relaxes isolated rabbit aorta when endothelial cells are present. Removal of endothelial cells or treatment with the muscarinic antagonist atropine, however, abolishes relaxation. Furchgott and Zawadzki postulated that a diffusible relaxing factor is released from endothelial cells in response to muscarinic receptor activation by acetylcholine. To test this hypothesis they measured vascular responses in preconstricted transverse strips of endothelium-denuded rabbit aorta mounted alone and together ("sandwiched") with longitudinal endothelium-intact strips. Endothelium-denuded strips do not relax to acetylcholine when mounted alone. When sandwiched with an endothelium-intact strip, however, relaxation is restored in the denuded strip.

These results suggest that endothelial cells produce a factor which is responsible for acetylcholine relaxation. They called this factor endothelium-derived relaxing factor or EDRF (Furchgott & Zawadzki, 1980).

Characterization of EDRF

The discovery of EDRF led to the identification of numerous pharmacological and physiological stimuli which cause endothelium-dependent relaxation. They include bradykinin, ATP, ADP, thrombin, substance P, the calcium ionophore A23187, histamine, serotonin, arachidonic acid, leukotrienes, platelets and increased blood flow (Chand & Altura, 1981; DeMey & Vanhoutte, 1981; DeMey et al., 1982; Van de Voorde & Leusen, 1983, Secrest & Chapnick, 1988; Cohen et al., 1983a & b; Hull et al, 1986; Kaiser et al, 1986). Endothelium-dependent relaxation has been demonstrated in vivo and in vitro in arteries, veins and resistance vessels of virtually every species examined, including man (Furchgott & Vanhoutte, 1989).

Endothelium-dependent responses however, are heterogeneous depending on which endothelium-dependent vasodilator, which blood vessel and which species is studied (DeMey et al 1982; Hoeffner & Vanhoutte, 1989; DeMey & Vanhoutte, 1982; Seidel & Rochelle, 1987; Vanhoutte & Miller,1985; Forstermann et al., 1984). These differences are attributed not only to variations in the concentration and chemical nature of EDRF, but also to differences in the response of smooth muscle to EDRF (DeMey et al 1982; Seidel & Rochelle 1987; DeMey & Vanhoutte, 1982; Miller & Vanhoutte, 1989). DeMey et al (1982) were the first to suggest that endothelium-dependent vasodilators cause release of different relaxing factors from endothelial cells. They showed that endothelial cells are required for relaxation to acetylcholine, arachidonic acid, thrombin and ATP in isolated canine femoral artery. The mechanism of relaxation, however, differs depending on the agent. Acetylcholine relaxation

is inhibited by ETYA (inhibitor of lipoxygenase and cyclooxygenase), but not by indomethacin (inhibitor of cyclooxygenase), whereas arachidonic acid relaxation is abolished by both ETYA and indomethacin. Relaxation to ATP and thrombin, however, are unaffected by either of these inhibitors. DeMey et al (1982) suggested that relaxation to acetylcholine, arachidonic acid, ATP and thrombin is mediated by different factors released by endothelial cells. Since this original observation, several endothelium-derived relaxing and contracting factors have been identified including NO (Palmer et al., 1987), prostacyclin (Moncada et al., 1976), adenosine (Pearson & Gordon, 1979), endothelium-derived hyperpolarizing factor (EDHF; Komori & Suzuki, 1987; Feletou & Vanhoutte 1988; Komori et al 1988), endothelin (Yanagisawa et al, 1988), and thromboxane (Ingerman-Wojenski et al., 1981).

The relaxing factor called EDRF by Furchgott and Zawadzki is now thought to be either nitric oxide (NO) or a closely related nitroso compound (Hutchinson et al., 1987; Ignarro et al., 1987a & b; Palmer et al., 1987). Ignarro and Furchgott were the first to postulate that EDRF is NO. Ignarro's suggestion was based on similarities of action between EDRF and a class of pharmacological agents referred to as nitrovasodilators. These drugs, which include nitroglycerin and sodium nitroprusside, act by releasing NO (Gruetter et al., 1981; Murad, 1986). EDRF, NO and nitrovasodilators cause relaxation by activating vascular smooth muscle soluble guanylate cyclase resulting in the conversion of GTP to cGMP (Gruetter et al., 1981; Rapoport & Murad, 1983; Ignarro et al., 1984, 1986). The mechanism of vascular smooth muscle relaxation by cGMP is unknown, but may result from activation of cGMP-dependent protein kinase, dephosphorylation of myosin light chain, inhibition of phosphatidylinositol breakdown and decreased cytosolic Ca^{++} concentration (Rapoport et al., 1983; Rapoport, 1986; Lincoln et al., 1990).

Methylene blue depresses relaxation in response to NO, nitrovasodilators, and endothelium-dependent agents by inhibiting guanylate cyclase and by binding and inactivating NO (Furchgott et al., 1984; Martin et al., 1985; Wolin et al., 1990). Oxyhemoglobin also inhibits relaxation to NO and endothelium-dependent agents by binding and inactivating extracellular NO (Gruetter et al, 1981; Rapoport & Murad, 1983; Ignarro et al 1984). Based on these observations, Ignarro suggested that EDRF is NO.

Furchgott suggested that EDRF is NO based on experiments with acidified nitrite. He showed that sodium nitrite, which liberates NO upon acidification, relaxes isolated rabbit aorta. This relaxation, as well as endothelium-dependent relaxation to acetylcholine, is inhibited by oxyhemoglobin and superoxide anion and potentiated by superoxide dismutase (SOD; Furchgott, 1988). Based on these studies, Furchgott suggested that EDRF and NO are the same.

Further evidence that EDRF is NO was provided by Palmer et al. (1987), Ignarro et al (1987) and Hutchinson et al (1987), using a cascade bioassay system, first described by Rubanyi et al. (1985) and modified by Gryglewski et al. (1986). Endothelium-intact blood vessels or cultured endothelial cells are perfused or superfused with Kreb's solution in the presence of agents which release EDRF (endothelium-dependent vasodilators). An endothelium-denuded ring or strip of blood vessel (detector) is attached to a force transducer and exposed to perfusate or superfusate from the endothelium-intact preparation (donor). The relaxation response of the detector ring provides a basis for the bioassay of EDRF released from the donor preparation. The half-life of EDRF is assessed by using multiple vascular strips at varying distances from the donor preparation

EDRF and Nitric Oxide

Palmer et al. (1987), Hutchinson et al. (1987) and Ignarro et al. (1987) demonstrated, with a cascade bioassay system, that EDRF released from porcine aortic endothelial cells, rabbit thoracic aorta or bovine pulmonary artery and vein is indistinguishable from NO in its half-life, biological activity, and effects of inhibitors and potentiators. Both substances have half-lives of 3-5 seconds (Ignarro et al., 1987; Palmer et al., 1987; Hutchinson et al., 1987) when perfused over tissues and 30 seconds (Palmer et al., 1987) when passed through polyethylene tubing. The half-lives of both substances are 30-40 seconds when perfused over tissues in the presence of SOD (Ignarro et al 1987). The relaxation response to NO and EDRF is inhibited by methylene blue (Ignarro et al., 1987a), oxyhemoglobin (Ignarro et al., 1987a & b; Palmer et al., 1987; Hutchinson et al., 1987;) superoxide anion (Ignarro et al, 1987b) and pyrogallol, a source of superoxide anion (Hutchinson et al, 1987; Ignarro et al 1987b). Finally, both NO and EDRF relax vascular smooth muscle and inhibit platelet aggregation by activating smooth muscle guanylate cyclase (Ignarro et al 1987a & b; Radomski et al 1987c).

The chemical identification of EDRF as NO or a closely related nitroso compound was provided by Palmer et al (1987) and Ignarro et al (1987a & b). Palmer and coworkers showed that the reaction of EDRF with ozone yields the same chemiluminescent product as the reaction between NO and ozone (Palmer et al 1987). Ignarro et al (1987b) demonstrated that NO and EDRF react in identical ways with hemoproteins. Spectrophotometric analysis revealed that the characteristic shift in the peak for deoxyhemoglobin caused by NO is duplicated by EDRF released from stimulated aortic endothelial cells (Ignarro et al 1987b). These studies provide evidence that the relaxing factor first described by Furchgott and Zawadzki is NO, or a closely related nitroso

compound from which NO is liberated (Ignarro et al, 1987a & b; Palmer et al 1987).

Biosynthesis of NO

Palmer and coworkers demonstrated in 1988 that NO is formed from L-arginine using cultured porcine aortic endothelial cells. Bradykinin-induced NO release, as measured by chemiluminescence and cascade bioassay, is enhanced by exposure of arginine-depleted endothelial cells to L-arginine. Palmer et. al. (1987) showed, using mass spectrometry, that release of ^{15}NO is similar in cells exposed to L-guanidino-^{15}N-arginine and L-^{15}N-arginine indicating that NO comes from the terminal guanido nitrogen atom (Palmer et al 1988a&b). D-arginine and other arginine analogues do not enhance bradykinin-induced NO production, suggesting that NO is formed specifically from L-arginine (Palmer et al 1987 a & b). Furthermore, an L-arginine analogue, N^G-monomethyl-L-arginine (L-nMMA), inhibits both endothelial cell NO generation and endothelium-dependent relaxation of rabbit aortic rings (Palmer et al., 1988b; Rees et al., 1989b). This inhibition is reversed by L-arginine, suggesting that L-nMMA competitively interacts with a NO-forming enzyme (Palmer et al 1988b; Rees et al., 1989b).

Mayer et al. (1989) and Mulsch et al. (1989) demonstrated in 1989 using cultured porcine aortic endothelial cells, that a cytosolic enzyme catalyzes the conversion of L-arginine to NO. They showed that L-arginine, in the presence of endothelial cell cytosol, activates soluble guanylate cyclase in a Ca^{++}/calmodulin and NADPH-dependent manner. L-arginine, however, has no effect on guanylate cyclase after heat denaturation of cytosolic proteins (Mayer et al., 1989). L-arginine-induced activation of guanylate cyclase is enhanced by SOD and inhibited by N^G-nitro-L-arginine and L-nMMA. These studies suggest that a cytosolic enzyme, now called nitric oxide synthase, is

responsible for conversion of L-arginine into a compound (presumably NO or a closely related nitroso species) which activates guanylate cyclase (Mayer et al 1989; Mulsch et al 1989).

Endothelial cell L-arginine, the substrate for NO synthase, may not be rate-limiting in the production of NO because it does not produce or enhance endothelium-dependent relaxation in most arteries (Amezcua et al., 1989; Gold et al 1989a&b; Aisaka et al 1989; Rees et al 1989b). In arginine-depleted arteries from many species, however, L-arginine causes a relaxation response which is inhibited by hemoglobin, methylene blue and the arginine analogues L-nMMA (Gold et al 1989a & b) or N^G-nitro-L-arginine (Schini & Vanhoutte, 1991) These results suggest that L-arginine is normally present in endothelial cells in amounts sufficient to saturate NO synthase (Schini & Vanhoutte, 1991; Gold et al 1989a &b).

Inhibition of endothelium-dependent relaxation by L-arginine analogues

Several structural analogues of L-arginine which inhibit endothelium-dependent relaxation have been identified. They include L-nMMA, N^G-nitro-L-arginine (NOARG), N^G-nitro-L-arginine methyl ester (L-NAME), N^G-amino-L-arginine, and N^G-amino-L-homoarginine. Inhibition of relaxation by arginine analogues can be reversed by L-arginine, suggesting that they act by competitively interacting with NO synthase (Palmer et al., 1988a&b; Moore et al., 1990; Kobayashi & Hattori, 1990; Fukoto et al., 1990; Rees et al., 1990a; Lambert et al., 1992). These agents have been utilized as "specific" inhibitors of NO synthesis, however, few studies have documented their effect on NO production (Palmer et al., 1988; Rees et al., 1989; Schmidt et al., 1990). Furthermore, recent reports suggest that arginine analogues have other actions besides competitive inhibition of NO synthesis.

Non-specific effects of arginine analogues were first suggested by the failure of L-arginine to reverse the effect of these agents (Jackson et al., 1991; Kirkeboen et al., 1992; Smith et al., 1992; Elsner et al., 1991). Jackson et al., (1991) reported that L-arginine reverses the inhibitory effect of L-nMMA, but not NOARG, on phenylephrine-induced phasic contractions in hamster aorta. NOARG at 0.07 uM/min and 0.56 uM/min inhibits basal blood flow in the in vivo canine femoral artery. L-arginine reverses the effect of low, but not high concentrations of NOARG (Kirkeboen et al., 1992). L-NAME and L-nMMA increase coronary perfusion pressure in the isolated rabbit heart and the effects of L-nMMA, but not L-NAME are reversed by L-arginine (Smith et al., 1992). L-arginine does not reverse the increase in systemic blood pressure elicited by intravenous NOARG in conscious dogs, thus suggesting a similar irreversibility with NOARG (Elsner et al., 1991).

. Further evidence that arginine analogues have effects other than competitive inhibition of NO synthase was provided by Peterson et al. (1992), Buxton et al. (1993) and Bogle et al. (1992). Peterson et al. (1992) reported that L-NAME and L-nMMA inhibit reduction of cytochrome C by ferrous iron and epinephrine. Arachidonic acid-acceleration of reduction of cytochrome C by ferrous iron is also prevented by L-NAME and L-nMMA. These results suggest that L-NAME and L-nMMA decrease EDRF/NO synthesis, not only by competitive inhibition of NO synthase, but also by an effect on electron transfer. The ability of arginine analogues to alter electron transfer suggests that they may inhibit many enzymes. An effect of arginine analogues on other enzymes besides NO synthase may contribute to the hemodynamic effects of these agents (Peterson et al., 1992).

Buxton et al. (1993) demonstrated with radioligand binding studies that L-NAME and other alkyl esters of L-arginine are muscarinic receptor

antagonists. L-NAME (100 uM), but not L-nMMA, inhibits contraction of endothelium-denuded strips of canine coronary artery by a mechanism which is not reversible with L-arginine. Binding of the muscarinic radioligand [^{3}H] quinuclidinyl benzilate to muscarinic receptors in whole canine aorta, atria, colon, submandibular gland, aortic endothelium and guinea pig brain is inhibited by L-NAME, but not L-arginine, NOARG or L-nMMA. These results suggest that the effects of L-NAME on muscarinic relaxation may be two-fold; inhibition of NO synthesis and antagonism of muscarinic receptors. L-arginine reverses the effect of L-NAME on NO synthase, but may not reverse the effect of L-NAME on muscarinic receptors.

L-NAME has been described as a potent inhibitor of endothelium-dependent relaxation in response to acetylcholine (Moore et al., 1990; Thiemermann et al., 1991). Thiemermann et al., (1990) showed that L-NAME (100 uM) inhibits acetylcholine relaxation of isolated rat aorta in a manner which is reversible with L-arginine (1mM). Moore et al., (1989) reported that L-nMMA, NOARG and L-NAME (30 uM) inhibit acetylcholine relaxation in isolated rabbit aorta. L-arginine (100 uM) reverses the effect of L-nMMA and NOARG, but not L-NAME. The failure of L-arginine to reverse L-NAME-induced inhibition of acetylcholine relaxation may be due to antagonism of muscarinic receptors by L-NAME.

Finally, L-nMMA and N^G-iminoethyl-L-ornithine, but not NOARG or L-NAME, inhibit endothelial cell transport of L-arginine by the saturable Na^+-independent Y^+ transport system (Bogle et al., 1992). Inhibition of arginine transport may be responsible, in part, for inhibition of endothelium-dependent relaxation by these agents.

These studies provide evidence that structural analogues have effects other than competitive inhibition of NO synthase. Therefore, it is important to

demonstrate that inhibition of endothelium-dependent relaxation by these agents is reversible with L-arginine.

Structural analogues of L-arginine have been used extensively both in vivo and in vitro to determine the role of NO in endothelium-dependent relaxation. Studies with L-nMMA and NOARG suggest that NO is important in mediating endothelium-dependent relaxation of canine femoral artery (Miller, 1991; Kirkeboen, 1992).

Role of NO in endothelium-dependent relaxation of canine femoral artery

Endothelium-dependent relaxation has been studied extensively in vivo and in vitro in canine femoral artery. Acetylcholine (DeMey & Vanhoutte, 1981, DeMey et al 1982; Angus & Cocks, 1983), ADP (DeMey & Vanhoutte, 1981), ATP (DeMey & Vanhoutte, 1981; DeMey et al, 1982), thrombin (DeMey & Vanhoutte, 1982 DeMey et al 1982), the calcium ionophore A23187 (Seidel & LaRochelle, 1987), and arachidonic acid (DeMey & Vanhoutte, 1982; Hoeffner et al, 1989) produce endothelium-dependent relaxation of in vitro femoral artery. Indomethacin does not alter endothelium-dependent responses to acetylcholine, ATP, ADP, or thrombin, suggesting that cyclooxygenase products are unimportant in the relaxation response to these stimuli (DeMey et al., 1982). Acetylcholine relaxation is inhibited by L-nMMA and L-nMMA-induced inhibition is reversed by L-arginine, thus providing evidence that NO or a closely related compound is responsible for acetycholine relaxation of canine femoral artery (Miller, 1991). Further evidence that NO is one EDRF in canine femoral artery is provided by bioassay experiments which suggest that the EDRF released under basal and stimulated conditions is similar to that described by Furchgott and Zawadzki in terms of half-life, response to

SOD, superoxide anion and oxyhemoglobin (Rubanyi et al, 1985; Rubanyi & Vanhoutte, 1986).

In the in vivo canine femoral artery, acetylcholine (Angus & Cocks, 1983; Hull et al., 1986; Kaiser et al., 1986), substance P (Angus & Cocks, 1983), and increased blood flow (Kaiser et al, 1986; Hull et al 1986) elicit endothelium-dependent vasodilation. Methylene blue inhibits the response to acetycholine and increased flow (Kaiser et al., 1986), and L-nMMA decreases basal blood flow and inhibits dilation to acetylcholine. The effect of L-nMMA on basal blood flow and acetylcholine dilation is reversed by L-arginine (Kirkeboen et al., 1992). These results suggest that NO or a closely related compound is released from femoral artery under basal and stimulated conditions (Kirkeboen et al., 1992).

Role of NO in endothelium-dependent relaxation of canine femoral vein

Acetylcholine (DeMey & Vanhoutte, 1982; Vanhoutte & Miller 1985; Miller & Vanhoutte 1989), ATP (Vanhoutte & Miller, 1985) and A23187 (Miller & Vanhoutte, 1989) produce endothelium-dependent relaxation of the in vitro canine femoral vein. Vanhoutte and Miller (1985) reported that indomethacin does not alter acetylcholine relaxation, suggesting that cyclooxygenase products are unimportant in the response to this agonist. This study, however, showed the effect of 10^{-5} M indomethacin at only one unreported concentration of acetylcholine. The effect of indomethacin on acetylcholine dose-response relationships has not been reported, therefore the role of cyclooxygenase products in acetylcholine relaxation in femoral vein is unknown.

Miller and Vanhoutte (1989), Miller (1991) and Vidal et al., (1991) examined the role of EDRF/NO in mediating relaxation to acetylcholine and the

calcium ionophore A23187 in the in vitro femoral vein. They suggested that the mechanism of relaxation varies with the preconstrictor. The relaxation response to acetylcholine and A23187 is not inhibited by methylene blue or LY83583 (lowers cGMP and generates superoxide anions) in rings preconstricted with norepinephrine (Miller & Vanhoutte, 1989; Miller, 1991). A23187 relaxation is associated with an increase in cGMP content of endothelium-intact, but not endothelium-denuded rings, suggesting that the accumulation of cGMP occurs in endothelial cells rather than in vascular smooth muscle. Methylene blue inhibits the increase in cGMP, but does not inhibit the relaxation response to A23187. These data suggest that the increase in cGMP elicited by A23187 is not required for relaxation (Vidal et al., 1991). Therefore, EDRF released by femoral vein preconstricted with norepinephrine may not act through vascular smooth muscle guanylate cyclase/cGMP. Since these experiments were conducted in the presence of indomethacin, cyclooxygenase products are also not involved (Miller & Vanhoutte, 1989; Miller, 1991; Vidal et al., 1991).

Different results are obtained when prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is used as the preconstricting agent. With $PGF_{2\alpha}$ preconstriction, methylene blue and LY83583 in the presence of indomethacin, attenuate acetylcholine and A23187 relaxation (Miller & Vanhoutte, 1989). These results suggest that an EDRF which acts through vascular smooth muscle guanylate cyclase/cGMP is important in the relaxation response to acetylcholine and A23187 when $PGF_{2\alpha}$ is the preconstricting agent. Whether this EDRF is NO is uncertain. NOARG, but not L-nMMA, inhibits acetylcholine relaxation, however, the authors failed to demonstrate reversibility of NOARG-inhibition with L-arginine. The lack of effect of L-nMMA, in addition to the failure to demonstrate reversibility with

L-arginine suggests that the inhibitory effect of NOARG on acetylcholine relaxation may be non-specific.

These studies provide evidence that femoral vein produces at least two different EDRF's in response to acetycholine and A23187. One factor acts through cGMP/guanylate cyclase, and the mechanism of action of the other factor is unknown (Miller & Vanhoutte, 1989; Miller, 1991; Vidal et al., 1991).

EDRF/NO and resistance vessels

Investigation of endothelium-dependent relaxation in resistance vessels has been hindered by difficulties in removing endothelial cells from isolated perfused organs (Bhardwaj & Moore, 1988). Several methods have been described for removing or inactivating endothelial cells in resistance beds including light dye (Koller et al., 1989a&b), gossypol (Pohl et al., 1987), collagenase (Furchgott et al., 1987), saponin (Samata et al., 1986), sodium deoxycholate (Warner et al., 1989), CHAPS (Bhardwaj & Moore, 1988), and air treatment (Eskinder et al., 1990). There are problems, however, with these techniques. Chemical removal of endothelial cells by in vivo infusion of collagenase, air, or detergents results in tissue edema, thrombosis, damage to vascular smooth muscle, and obstruction of capillary beds by cellular debris (Furchgott et al., 1987; Bhardwaj & Moore, 1988). These problems are not encountered with endothelial cell inactivation by gossypol or light-dye treatment. The mechanism of action of these agents, however, is uncertain. Gossypol has multiple effects which include inhibition of lipoxygenase and cellular oxidorectases and interference with membrane phospholipids (Pohl et al., 1987). Which effect is responsible for inhibition of endothelium-dependent responses is unknown. The mechanism of endothelial cell impairment by light dye treatment is also unknown (Koller et al., 1989a) thus making difficult the interpretation of results obtained using this technique. Arginine analogues

have been used to study the role of NO in regional circulations, however, the effect of arginine analogues may not be on endothelial cells since many other cell types are present which are capable of synthesizing NO (Bredt & Snyder 1989; Forstermann et al 1990; Bredt et al 1991; Ignarro et al 1990; Torphy et al 1986; Li & Rand, 1989; Boeckxstaens et al 1990; Hata et al 1990; Toda et al 1990; Shuttleworth et al 1991; Rimele et al 1988; McCall et al 1989; Masini et al 1991).

Despite these problems several studies suggest that endothelial cells play an important role in regulating arteriolar smooth muscle tone. Endothelium-dependent vasodilation has been documented in the autoperfused rabbit hindlimb (Forstermann et al., 1987; Pohl et al., 1987), the PSS-perfused rat and rabbit mesenteric and rat renal vasculature (Furchgott et al, 1987; Bhardwaj & Moore, 1988, 1989), and the rat cremaster muscle preparation (Koller et al., 1989a&b; Koller & Kaley, 1990 a; Koller et al., 1991). Agents which produce endothelium-dependent dilation of resistance vessels include acetylcholine (Furchgott et al., 1987), ATP (Pohl et al., 1987), substance P (Pohl et al., 1987) and increased blood flow (Koller & Kaley, 1990). Acetylcholine produces vasodilation in the mesenteric, renal, cremaster muscle, and hindlimb vascular preparations. Removal or injury of endothelial cells by collagenase, light-dye, gossypol, CHAPS, sodium deoxycholate or air treatment abolishes relaxation to acetylcholine, but not to endothelium-independent agents (e.g. NO, nitroglycerin, adenosine; Bhardwaj & Moore, 1989; Furchgott et al., 1987; Koller et al., 1989a&b; Pohl et al., 1987; Warner et al., 1989).

Methylene blue (Bhardwaj & Moore, 1989), L-nMMA and NOARG (Moore et al., 1990) inhibit acetylcholine dilation in rat renal and mesenteric vascular beds. L-nMMA and NOARG-induced inhibition is partially reversed by L-arginine suggesting that NO/guanylate cyclase/cGMP mediates the relaxation response to acetycholine in these tissues. L-nMMA increases

coronary perfusion pressure in the isolated perfused rabbit heart (Smith et al., 1992) and constricts rat and rabbit cerebral (Faraci, 1991; Humphries et al., 1991), and rat mesenteric vascular beds (Moore et al., 1990). The effect of L-nMMA is reversed by L-arginine suggesting basal release of NO in these vascular beds (Smith et al., 1992, Faraci, 1991, Moore et al., 1990). These data provide evidence that NO or a closely related compound is released basally and upon stimulation in several vascular beds. These studies failed to demonstrate, however, that arteriolar endothelial cells are the source of NO. NO production has been demonstrated recently in many other cells types including neurons, platelets, vascular smooth muscle and inflammatory cells; arginine analogues inhibit NO production in any of these cells (Bredt & Snyder 1989; Forstermann et al 1990; Bredt et al 1991; Ignarro et al 1990; Torphy et al 1986; Li & Rand, 1989; Boeckxstaens et al 1990; Hata et al 1990; Toda et al 1990; Shuttleworth et al 1991; Rimele et al 1988; McCall et al 1989; Masini et al 1991).

Other cells that produce NO

Neurons in the central (Bredt & Snyder 1989; Forstermann et al 1990; Bredt et al 1991) and peripheral nervous system (Ignarro et al 1990; Torphy et al 1986; Li & Rand, 1989; Boeckxstaens et al 1990; Hata et al 1990; Toda et al 1990; Shuttleworth et al 1991) produce NO as do neutrophils (Rimele et al 1988; McCall et al 1989), vascular smooth muscle (Moritoki et al., 1991), macrophages (Tayeh & Marletta, 1989), mast cells (Masini et al 1991) and platelets (Radomski et al 1990a,b&c). NO has many functions. It is likely an intercellular messenger of the central nervous system where it may be important in long term potentiation (Bohme et al 1991; Schuman & Madison, 1991). NO may act, in the autonomic nervous system, as a non-cholinergic/non-adrenergic neurotransmitter (Boeckstaens et al 1990; Ignarro et al 1989; Torphy et al 1986; Li & Rand, 1989; Hata et al 1990; Toda et al 1990; Shuttleworth et al 1991). It is

also a cytotoxic agent produced by inflammatory cells (Effron et al., 1991; Liew et al., 1991, 1992), and may be important in immunity against intracellular microoganisms, as well as extracellular pathogens, such as fungi and helminths which are too large to be phagocytosed (Granger et al., 1986; Denis 1991; Liew et al., 1991, 1992; Adams et al., 1990). EDRF/NO may also play an important role in the interaction between endothelial cells and platelets (Radomski et al., 1987a,b,c; Nguyen et al., 1991; Cohen et al., 1983a&b).

EDRF/NO and platelets

Endothelial cells are important regulators of platelet-vessel wall interactions. Prostacyclin and NO released from cultured porcine aortic endothelial cells synergistically inhibit bradykinin-induced aggregation and adhesion of human platelets (Radomski et al., 1987b; Radomski et al., 1987a,b,c). NO inhibits thrombin-induced platelet aggregation by increasing cGMP, phosphorylation of protein kinase C and preventing the rise in intracellular Ca^{++} concentration. Aggregating platelets and platelet-derived factors, including serotonin and adenine nucleotides, relax endothelium-intact canine coronary arteries, but constrict endothelium-denuded preparations, suggesting that endothelial cells modulate the effect of platelets on vascular tone (Cohen et al., 1983a & b; Houston et al., 1986).

The production of NO by many cell types as well as the diverse actions of the NO/guanylate cyclase/cGMP system suggest that NO plays a physiologically significant role in cell-to-cell communication (McCall & Vallance, 1992).

Physiological significance of endothelium-dependent relaxation

Wide spread endothelium-dependent relaxation in many species (e.g. reptiles, amphibia, bony fishes and man) suggests that endothelial cell control of vascular smooth muscle tone is ancestral (Collier & Vallance 1989; Miller et al.,1984). Also, endothelial cells are not the only lining cells that produce

substances affecting smooth muscle tone. Airway epithelium and gut mucosa produce similar substances (Flavahan et al 1985; Gallavan & Jacobson, 1982). The prevalence of endothelium- and epithelium-mediated smooth muscle responses, as well as their phylogenetic occurrence suggest that they are important physiologically.

Vasoactive factors released by endothelial cells may be important in regulation of blood pressure and regional blood flow. Intravenous infusion of L-nMMA increases mean arterial pressure in the guinea pig, rat and rabbit (Aisaka et al 1989; Rees et al., 1989a; Whittle et al., 1989), inhibits the fall in blood pressure caused by infusion of acetylcholine in rats and rabbits (Rees et al., 1989a; Whittle et al., 1989), and decreases resting and acetylcholine-induced blood flow in hindquarter, mesenteric, and renal vascular beds of chronically instrumented rats (Gardiner et al 1990). It also causes a sustained increase in coronary perfusion pressure and inhibits acetylcholine vasodilation in Langendorff-perfused rabbit hearts (Amezcua et al., 1989). Infusion of L-nMMA and NOARG causes a decrease in resting and acetylcholine-induced human forearm blood flow (Vallance et al 1989) and intravenous NOARG vasoconstricts coronary, cerebral, renal and duodenal vascular beds of the conscious rabbit (Humphries et al., 1991). These studies indicate that NO is released basally and upon stimulation in several vascular beds. Basally released NO may be important in regulation of blood pressure and regional blood flow (Vallance et al 1989, Gardiner et al 1990; Aisaka et al 1989; Rees et al 1989a).

Endothelium-dependent vasodilation in response to increased blood flow may promote vasodilation during active hyperemia. Flow-mediated vasodilation, which occurs in conduit and resistance-sized blood vessels, may contribute to increased tissue perfusion during periods of high demand

(Kaiser et al., 1986; Hull et al., 1986; Koller & Kaley, 1990). In exercising skeletal muscle, for example, increasing metabolism causes microvascular vasodilation. The resulting increase in blood flow causes further vasodilation by stimulating flow-dependent mechanisms, which contribute to further increases in flow (Hull et al., 1986; Koller & Kaley, 1990). Alteration of flow-mediated vasodilation may be important in the pathogenesis of exercise intolerance associated with some cardiovascular diseases.

A methylated arginine analogue, N^G, N^G-dimethylarginine (ADMA) has been isolated recently from human plasma. It inhibits NO production in isolated macrophages, elicits a dose-dependent rise in systemic blood pressure in guinea pigs, and causes a dose-dependent decrease in forearm blood flow in humans. L-arginine reverses these effects, suggesting that ADMA is an endogenous agent which competitively inhibits NO synthesis. The presence of circulating arginine analogues suggest that a mechanism exists for regulating plasma NO levels. Changes in circulating levels of these analogues may be important in the pathogenesis of disease (Vallance et al., 1992).

Endothelium-dependent relaxation and cardiovascular disease

A role for altered endothelium-dependent responses in the pathogenesis of cardiovascular disease has emerged. Many diseases that alter vascular reactivity are associated with changes in endothelium-dependent relaxation. These include diabetes mellitus (Mayhan, 1989; Tesfamarian et al 1989, 1990, 1992; Hattori et al 1991), renal failure (Vallance et al., 1992), heart failure (Kaiser et al 1989b), hypertension (Luscher et al., 1986; Mayhan, 1990), atherosclerosis (Verbeuren et al 1986, 1990; Guerra et al 1989; Girerd et al, 1990; Cooke et al 1991), coronary vasospasm (Vanhoutte & Shimokawa, 1989b), vascular occlusion-reperfusion injury (Headrick et al 1990; Mehta et al 1989a & b), endotoxic shock (Knowles et al 1990; Rees et al 1990b; Kilbourn et al 1990;

Thiemermann & Vane, 1990; Busse & Mulsch, 1990; Salvenimi et al., 1990), chronic obstructive pulmonary disease (Dinh-Xuan et al., 1991), lymphatic filariasis (Kaiser et al., 1991) and canine heartworm disease (Kaiser et al, 1987, 1989a, 1990, 1992). Pathological alterations in endothelium-dependent relaxation can occur anywhere in the transduction mechanism from endothelial cell receptor activation to vascular smooth muscle relaxation. Disease may affect endothelium-dependent relaxation by altering endothelial cell receptors, post-receptor signalling mechanisms or biosynthesis, degradation or smooth muscle responsiveness to endothelium-derived vasoactive factors. Chronic renal failure, lymphatic filariasis, endotoxemia, and atherosclerosis are described below to illustrate some of the mechanisms by which disease alters endothelium-dependent relaxation.

Chronic Renal Failure

Vallance et al., (1992) demonstrated that plasma concentrations of the endogenous arginine analogue, ADMA, are elevated in patients with endstage renal failure, while plasma arginine levels are depressed. ADMA concentration increases in proportion to serum creatinine suggesting that as renal function deteriorates, ADMA elimination is progressively impaired. As ADMA concentrations rise, NO synthesis may be inhibited. Inhibition of NO synthesis by ADMA may contribute to hypertension, as well as to impaired immune function associated with this disease (Vallance et al, 1992).

Lymphatic filariasis

Endothelium-dependent relaxation is depressed in the in vitro abdominal aorta of rats infected with the filarial parasite, Brugia pahangi. Responses in thoracic aorta, however, are not different from control. Furthermore, the mechanism of acetylcholine relaxation is altered in abdominal, but not thoracic aorta of Brugia infected rats when compared to

control. Indomethacin, which does not alter acetylcholine relaxation in abdominal aorta from control rats or thoracic aorta from Brugia infected rats, significantly depresses relaxation in Brugia abdominal aorta. NOARG abolishes relaxation to acetylcholine in control abdominal aorta, however, in abdominal aorta from Brugia infected rats, acetylcholine still causes significant relaxation in the presence of NOARG. Methylene blue inhibits relaxation in abdominal aorta from both control and Brugia infected rats, however, the relaxation in aorta from Brugia rats is significantly greater than control. These data suggest that chronic infection with Brugia pahangi depresses and alters the mechanism of endothelium-dependent relaxation in the abdominal aorta. In control abdominal aorta, the NO/guanylate cyclase/cGMP system is primarily responsible for acetylcholine relaxation. However, in abdominal aorta from Brugia infected rats, two factors are important; one is a cyclooxygenase product and the other is likely NO (Kaiser et al., 1991).

The reason that endothelium-dependent relaxation is altered in the abdominal, but not the thoracic aorta of rats with Brugia infection is unknown, however, it could be related to the inflammatory response invoked by the adult parasites. The adults reside in the abdominal cavity in the periaortic lymphatics where they cause a localized inflammatory reaction characterized by a 3-6 fold increase in mast cells, and accumulation of eosinophils and other mononuclear cells (Kaiser et al., 1990b). A similar reaction is not observed in the region of the abdominal aorta of control rats or the thoracic aorta of rats with Brugia infection. Inflammatory cells present within the vascular wall of Brugia abdominal aorta could alter endothelial cell-dependent relaxation by release of vasoactive substances when acutely stimulated in the muscle bath. Alternatively, chronic release of vasoactive agents by inflammatory cells may result in chronic alteration in the behavior

of endothelial cells. Further investigation is necessary to determine the mechanism of altered endothelium-dependent relaxation in rats with chronic Brugia infection (Kaiser et al., 1990b, 1991).

Endotoxemia

Endotoxemia is characterized by hypotension, decreased vascular responsiveness to constrictors and enhanced production of vascular smooth muscle cGMP (Suffredini et al, 1989; McKenna, 1989: Wakabayashi et al 1987). These alterations have been attributed to increased production of NO in endothelial cells and vascular smooth muscle (Busse & Mulsch, 1990; Rees et al., 1990b; Knowles et al., 1990; Salvemini et al., 1990).

Salvemini et al., (1990) reported that incubation of bovine aortic endothelial cells with endotoxin enhances their ability to inhibit platelet aggregation. SOD enhances and oxyhemoglobin attenuates the effect of endotoxin-treated endothelial cells on platelet aggregation indicating that EDRF is responsible for enhanced ability of endothelial cells to inhibit platelet aggregation. Treatment of endothelial cells with L-nMMA reduces the antiaggregatory effect of endotoxin. The reduction of activity by L-nMMA is reversed by L-arginine, but not D-arginine. These results suggest that endothelial cell production of NO is enhanced by endotoxin (Salvemini et al., 1990).

Knowles et al., (1990) and Rees et al., (1990b) reported that cGMP formation is enhanced and the contractile response to phenylephrine is decreased in rings of rat aorta after in vivo (Knowles et al., 1990) or in vitro (Rees et al., 1990b) exposure to endotoxin. Enhanced production of NO is time-dependent and can be prevented by the inhibitors of protein synthesis cycloheximide and dexamethasone. Because these effects are also prevented by

arginine analogues, it has been suggested that endotoxin stimulates de novo synthesis of an inducible NO synthase (Rees et al., 1990b; Knowles et al., 1990).

Similar results were reported by Busse and Mulsch (1990) with endothelium-denuded rabbit aorta and cultured rabbit vascular smooth muscle. Incubation with tumor necrosis factor and interleukin-1 attenuates the contractile response to norepinephrine and causes an increase in cGMP production in de-endothelialized rabbit aortic rings. Cultured rabbit vascular smooth muscle incubated with cytokines exhibit an 11-fold elevation in cGMP content and a significant increase in production of the stable metabolite of the L-arginine pathway, NO_2^-, when compared to untreated cells. Cytokine-induced increases in cGMP is time-dependent; 12 hours of incubation with cytokines is required before significant differences in cGMP content are observed between control and cytokine-treated preparations. Cyclic GMP accumulation and depression of norepinephrine contraction can be abolished by NOARG and cycloheximide suggesting that an inducible NO synthase is responsible for these effects (Busse & Mulsch, 1990).

To assay for NO synthase activity, activation of purified guanylate cyclase was measured in the presence of cytosolic preparations of de-endothelialized rabbit aorta and cultured vascular smooth muscle. Cytosol obtained from cytokine-treated segments, in the presence of L-arginine and NADPH, increases guanylate cyclase activity. Guanylate cyclase activity is not observed with cytosol from control or cycloheximide/cytokine-treated segments. Cytokine-treated cultured vascular smooth muscle cells exhibit a 7-10 fold increase in guanylate cyclase activity when compared to control. Accumulation of cGMP in cytosolic preparations is inhibited by N^G-nitro-L-arginine and cycloheximide and is absolutely dependent on the presence of L-arginine and NADPH. These data suggest that an inducible NO

synthase is present in vascular smooth muscle from cytokine-treated rabbit aorta (Busse & Mulsch, 1990).

Excessive production of NO by vascular smooth muscle and endothelial cells may contribute to hypotension associated with gram negative sepsis. If this is true, then NO synthase inhibitors may be therapeutically useful in treatment of gram negative sepsis. Arginine analogues, when given in vivo, reverse hypotension in rats treated with endotoxin (Thiemermann & Vane, 1990) and dogs treated with tumor necrosis factor (Kilbourn et al., 1990). Low doses of L-nMMA caused a significant and sustained increase in blood pressure and generalized hemodynamic stability in two patients with life-threatening hypotension associated with sepsis. (McCall & Vallance, 1992). These studies suggest that arginine analogues may be therapeutically useful in the treatment of gram negative sepsis.

Hypercholesterolemia

Atherosclerosis affects vascular reactivity by changing vascular morphology, EDRF production and smooth muscle function. Verbeuren et al (1986) demonstrated that endothelium-dependent relaxation to acetylcholine and ATP is depressed in thoracic aorta, but not in pulmonary artery, in rabbits on a diet high in cholesterol. There were histological changes in aorta, but not pulmonary artery, suggesting that depression of relaxation is related to morphological changes in the vessel wall (Verbeuren et al 1986). These changes, which consist of fatty streak formation, may depress endothelium-dependent relaxation by creating a diffusion barrier which limits transport of EDRF from endothelium to vascular smooth muscle (Shimokawa & Vanhoutte, 1989). Morphological alterations, however, are not the only cause of vascular dysfunction in hypercholesterolemia since

endothelium-dependent relaxation is also depressed in morphologically normal resistance vessels from hypercholesterolemic rabbits.

Many studies show that hypercholesterolemia depresses EDRF production (Guerra et al 1989; Verbeuren et al, 1990; Shimokawa & Vanhoutte, 1989). Guerra et al (1989) and Verbeuren et al (1990) demonstrated, using bioassay experiments, that hypercholesterolemic donor rabbit aorta produces significantly less relaxation in control endothelium-denuded detector rings. This suggests that hypercholesterolemia depresses endothelium-dependent relaxation by altering production, release or degradation of EDRF.

Girerd et al (1990) and Cooke et al (1990) suggested that the effect of hypercholesterolemia on endothelial cell function is due to alterations in NO production from L-arginine. Cooke et al (1990) demonstrated that in vivo administration of L-arginine, but not D-arginine reverses the depression of acetylcholine relaxation in in vitro thoracic aorta from hypercholesterolemic rabbits. Similarly, Girerd (1990) demonstrated that vasodilation of hindlimb resistance vessels is enhanced in cholesterol-fed, but not control rabbits, by intravenous L-arginine. They proposed that hypercholesterolemia depresses endothelium-dependent relaxation by either causing intracellular depletion of L-arginine or by altering the metabolism of L-arginine to NO (Girerd et al, 1990; Cooke et al 1991).

Others have suggested that hypercholesterolemia depresses endothelium-dependent relaxation by altering endothelial cell receptor or post-receptor signalling mechanisms (Kugiyama et al 1990). Kugiyama et al (1990) demonstrated that in vitro exposure of rabbit aorta to oxidized low-density lipoprotein (ox-LDL), a factor elevated in the serum of hypercholesterolemic rabbits and humans, almost abolishes relaxation to the receptor-mediated endothelium-dependent vasodilators, acetylcholine,

substance P and ATP. Relaxation to the calcium ionophore A23187, which acts independent of receptor function, however, is only partially inhibited by ox-LDL. Defatted albumin, a carrier with high avidity for lysophospholipids, was used to decrease the content of lysolecithin in ox-LDL to determine whether the effects of ox-LDL are due to lysolecithin. Albumin treatment reduces the elevated lysolecithin content of ox-LDL to that of native LDL and also attenuates the inhibitory effect of ox-LDL on endothelium-dependent relaxation. Furthermore, incubation of arteries with lysolecithin mimicks the depression of relaxation observed with ox-LDL. These data suggest that lysolecithin is responsible for ox-LDL-induced depression of relaxation. Since lysolecithin, but not lecithin, activates GTPase activity of the G protein-like Ras protein, and acetylcholine, substance P and ATP receptors are linked to G-proteins, the authors suggested that lysolecithin may depress endothelium-dependent relaxation to these agonists by altering G-protein function (Kugiyama et al., 1990). However, since relaxation to the calcium ionophore is also depressed by ox-LDL other mechanisms besides alteration of G-protein function are probably involved.

It is uncertain whether hypercholesterolemia affects vascular responsiveness to EDRF. Verbeuren et al (1990) reported that EDRF released by donor thoracic aorta from control rabbits produces less relaxation in detector abdominal aorta from hypercholesterolemic rabbits than in control detector tissue. In contrast, Guerra et al (1989) reported that detector thoracic aorta from hypercholesteremic rabbits displays enhanced relaxation to EDRF released from donor thoracic aorta. Relaxation to the endothelium-independent agents, sodium nitroprusside and nitroglycerin, have also been variable (Shimokawa & Vanhoutte, 1989; Verbeuren et al, 1986).

The reasons for these discrepancies are unknown, but could be due to differences in experimental models in terms of severity of atherosclerosis.

It appears that hypercholesterolemia/atherosclerosis depresses endothelium-dependent relaxation in rabbit aorta by a variety of mechanisms which may include attenuation of EDRF production by alteration of endothelial cell receptor and post-receptor signalling mechanisms, and by alteration in biosynthetic pathways for production of EDRF. Also, morphological changes may create a diffusion barrier which limits transport of EDRF from endothelial cell to vascular smooth muscle. Hypercholesterolemia may also impair vascular smooth muscle responsiveness to vasodilators.

Disease alters endothelium-dependent relaxation by a variety of mechanisms which include changes in production, release, degradation or effect of EDRF, as well as other vasoactive factors produced by the vessel wall. Abnormal endothelium-dependent relaxation may be important in the pathogenesis of many cardiovascular diseases. Kaiser et al (1987, 1989a,1990a, 1992) showed that Dirofilaria immitis, the canine heartworm, depresses endothelium-dependent relaxation by release of biologically active factor(s). Depression of endothelium-dependent relaxation by parasite products may be important in the pathogenesis of human and animal filarial disease.

Canine heartworm disease

Kaiser et al (1987, 1989a) demonstrated that D. immitis depresses acetylcholine vasodilation in the in vivo canine femoral artery. The depression is seasonal, occurring in the spring, a time of maximal reproductive activity of the adult worms (Stewart, 1975). There is no difference in acetylcholine dilation between infected and noninfected dogs in the fall, a more quiescent time for the parasites. Dilation response to sodium nitroprusside is not different between heartworm infected and control dogs,

suggesting that vascular smooth muscle/guanylate cyclase/cGMP mechanisms are unaffected by D. immitis. The cyclooxygenase inhibitor, indomethacin, depresses acetylcholine dilation in femoral artery from dogs infected with heartworm, but does not depress acetylcholine dilation in control. Topical application of piriprost potassium, the inhibitor of 5-lipoxygenase, has no effect on acetylcholine dilation in control, but enhances acetylcholine dilation in dogs with D. immitis. This suggests that a cyclooxygenase product is important in acetylcholine dilation in femoral artery from heartworm infected dogs. The enhancement of dilation by piriprost potassium may be secondary to the shunting of arachidonic acid from the lipoxygenase to the cyclooxygenase pathway. Another possibility is that lipoxygenase products are involved in filarial-induced depression of relaxation. Methylene blue, which almost abolishes dilation in control femoral artery, causes a biphasic response in femoral artery from D. immitis infected dogs. At low concentrations of acetylcholine, dilation is enhanced and at high concentrations of acetylcholine, dilation is depressed by methylene blue. These results suggest that the mechanism of endothelium-dependent relaxation is different in the in vivo femoral artery of heartworm infected dogs when compared to control and involves a cyclooxygenase product.

Dogs with occult (microfilariae negative) heartworm infection have the same seasonal responses as those with patent (microfilariae positive) infections suggesting that adult parasites, rather than microfilariae, are involved in depression of endothelium-dependent vasodilation. Since the adult parasites are in the right heart and pulmonary arteries and depression of relaxation occurs in the femoral artery, Kaiser et al. (1987, 1989a) postulated that circulating filarial factors may be responsible for alteration of endothelium-dependent relaxation.

The effect of filarial factors on endothelium-dependent relaxation of the in vitro rat aorta

In vitro experiments were designed to test the hypothesis that filarial factors depress endothelium-dependent relaxation (Kaiser et al 1990a, 1992). Exposure of isolated rat aorta to adult heartworms depresses relaxation to acetylcholine. Since filarial parasites are capable of releasing acetylcholinesterases (Rathaur et al., 1987), dose-response relationships were examined to carbachol, a muscarinic agonist resistant to hydrolysis by cholinesterases. Because carbachol relaxation is depressed in the presence of D. immitis, it is unlikely that filarial cholinesterases are responsible for depression of endothelium-dependent relaxation. Dose-response relationships were examined to the calcium ionophore A23187, an endothelium-dependent agent which acts independent of receptors, to rule out an effect of parasites on receptor cell function. Since endothelium-dependent relaxation is depressed in response to A23187, it is unlikely that filarial parasites modify endothelial cell receptor function. Dose-response relationships to norepinephrine and nitroglycerin were examined to determine the effect of heartworm on vascular smooth muscle function. Since norepinephrine constriction and nitroglycerin relaxation are not depressed by heartworm, it is unlikely that these parasites alter guanylate cyclase/cGMP or vascular smooth muscle function.

Acetylcholine relaxation was examined in the presence of heartworm-conditioned and control medium to assess the stability of factor(s) responsible for depression of endothelium-dependent responses. Exposure of rat aorta to D. immitis-conditioned medium depresses acetylcholine relaxation, suggesting that the effect of D. immitis is due to a relatively stable filarial factor. To determine the size of the factor, experiments were performed with D.

immitis placed in dialysis tubing of different molecular weight (MW) cut-off. Acetylcholine relaxation is depressed with D. immitis in dialysis tubing of 1000 MW cut-off, but not 100 MW cut-off, suggesting that the factor is between 100 and 1000 MW.

Other studies have focused on the chemical nature of filarial factors that depress endothelium-dependent relaxation. Filarial parasites absorb and metabolize arachidonic acid into several cyclooxygenase products (Liu & Weller, 1988, 1989, 1991; Liu et al 1990; Longworth et al, 1985, 1987, 1988). Liu and Weller demonstrated that parasite-derived prostanoids inhibit mammalian platelet aggregation indicating that filarial arachidonic acid metabolites alter mammalian cell function (Liu & Weller, 1991). Therefore, Kaiser et al. (1990a, 1992) postulated that one filarial factor responsible for depression of endothelium-dependent relaxation is a cyclooxygenase product (Kaiser et al., 1990a).

In vitro exposure of both rat aorta and D. immitis to indomethacin (50 uM) reverses the filarial-induced depression of relaxation (Kaiser et al., 1990a) suggesting the importance of mammalian and/or filarial cyclooxygenase products. Pretreatment of the parasites with indomethacin and aspirin reverses filarial-induced depression of acetylcholine relaxation, whereas treatment of the vascular ring does not alter filarial-induced depression. These data suggest that filarial rather than mammalian metabolites of arachidonic acid are involved in depression of relaxation.

Chloroform extraction of heartworm-conditioned medium was done in order to acquire samples for biochemical analysis and to manipulate the concentration of biologically active factor in heartworm-conditioned medium. Dose-response relationships to the factor were assessed to determine if it is a direct vasoconstrictor. Dose-response relationships to the filarial factor at

concentrations five orders of magnitude less than to two times greater than the concentration known to depress relaxation did not cause vasoconstriction, suggesting that the factor does not depress relaxation by producing an opposing constriction response. Examination of chloroform extracted heartworm-conditioned medium using gas chromatography-mass spectrometry (GC-MS) shows two peaks not present in chloroform-extracted control PSS or heartworm-conditioned PSS prepared with aspirin-treated parasites. One peak has a retention time and a mass chromatographic profile of prostaglandin D_2 (PGD_2) standard. The other is unidentified.

If filarial PgD_2 is responsible for depression of relaxation then exogenous PgD_2 should mimic the effect of heartworm-conditioned medium. Incubation of control rat aorta with PGD_2 at concentrations which do not cause a direct vasoconstriction (10^{-12}, 10^{-11}, 10^{-10}M) mimics the filarial-induced depression of relaxation at low concentrations, but not at high concentrations of acetylcholine. This suggests that a filarial cyclooxygenase product, possibly filarial PGD_2, is partly responsible for depression of relaxation. The unidentified peak, in addition to the failure of PGD_2 to mimic the effects of D. immitis at all concentrations of acetylcholine implicates more than one filarial factor in depression of endothelium-dependent relaxation.

Exposure of isolated rat aorta to serum from heartworm infected, but not control dogs depresses endothelium-dependent relaxation of in vitro rat aorta, thus suggesting that the filarial factor circulates in the blood (Lamb et al. 1992). If biologically active filarial factors circulate, then they are potentially capable of affecting all endothelial cells. Adverse effects of filarial factors on endothelium-dependent relaxation may be important in the pathogenesis of canine heartworm disease.

Description of D. immitis

D. immitis belongs to the superfamily Filarioidea. Adult male parasites are 12-20 cm long and 0.7-0.9 mm wide with a spirally coiled tail. Females are 25-31 cm long and 1.0-1.3 mm wide and produce motile larvae called microfilariae. Microfilariae numbers are higher in the summer, the mosquito season, than in the winter suggesting seasonal production of microfilariae by adult parasites (Sawyer, 1974). Adult parasites are in the pulmonary arteries and right heart and microfilariae circulate in the blood (Jackson et al., 1966).

Lifecycle of D. immitis

Dogs are infected with heartworm by an intermediate host, the mosquito. Infective larvae (L_3) of D. immitis enter subcutaneous tissues when the mosquito feeds and undergo two molts over a period of 3 months. This phase is terminated when young adults (L_5) migrate to the pulmonary arteries where they grow to adult size (Kume & Itagaki, 1955). Female heartworms are gravid 5 to 6 months after infection and circulating microfilariae can be detected in 6 to 7 months (Orihel, 1961). The infection is considered "patent" when both adults and microfilariae are present; it is "occult" when microfilariae cannot be detected (Otto, 1977). A mosquito must feed on a dog with a patent infection (microfilariae positive) and ingest immature larvae (microfilariae) in order to transmit the disease to another animal. The larvae undergo essential phases of development in the mosquito. When this development is complete, larvae can infect another dog thereby completing the life cycle (Taylor, 1960; Orihel, 1961).

Clinical signs of heartworm disease

Ogburn et al., (1977) reported no clinical signs in 28 of 58 (48%) dogs with microfilariae of D. immitis in the peripheral blood. Likewise, Calvert

(1987) reported that 55-60% of heartworm-positive dogs seen at the University of Georgia Veterinary Teaching Hospital exhibited no symptoms or only mild clinical signs of heartworm infection. These studies indicate that the majority of dogs infected with heartworm are asymptomatic (Ogburn et al., 1977; Calvert, 1987). Moderate clinical cardiopulmonary signs of heartworm disease characterized by frequent coughing and lethargy were reported in 24-33% of infected dogs. Only 10% of D. immitis infected dogs were reported to have severe cardiopulmonary symptoms of heartworm disease which include exercise intolerance, exertional dyspnea, tachypnea, chronic coughing, dyspnea, syncope, ascites and limb edema (Rawlings et al., 1977; Ogburn et al., 1977; Calvert, 1987). These symptoms have been attributed to filarial-induced pulmonary vascular damage, pulmonary hypertension and subsequent heart failure (Rawlings, 1983; Keith et al., 1983; Calvert, 1987).

Pathogenesis of pulmonary hypertension

Pulmonary hypertension is attributed to the obstruction of blood flow by adult parasites and parasite-induced endarteritis, fibrosis and thromboembolism (Keith et al, 1983; Rawlings, 1983). Early lesions, before myointimal proliferation is evident, include disruption and loss of endothelial cells and adherence of leukocytes and platelets to damaged endothelium and exposed subendothelial surfaces (Keith et al, 1983). These changes occur as early as 4 days after transplantation of adult worms into the vascular system of dogs (Keith et al, 1983).

Disruption of the endothelial cell layer is followed by an inflammatory response characterized by accumulation of eosinophils, histiocytes and activated macrophages (Hirth et al., 1966; Keith et al., 1983; Schaub & Rawlings, 1980). The intima becomes thickened due to fibrosis and myointimal hyperplasia and villous lesions develop which proliferate into the lumen and

grow around the adult heartworms. Villous formation, fibrosis and myointimal proliferation occur as early as 30 days following transplantation of adult worms into the vascular system of uninfected dogs (Schaub & Rawlings, 1980). Transfusion of microfilariae, however, does not cause myointimal proliferation (Schaub & Rawlings, 1980). Also, histological lesions rarely develop in systemic arteries which are exposed to high concentrations of microfilariae. When histological lesions do occur in systemic vessels, they are associated with ectopic adult parasites. This suggests that adult parasites rather than microfilariae are responsible for myointimal proliferation (Atwell, 1980; Liu et al., 1966; Schaub & Rawlings, 1980).

Pathogenesis of myointimal proliferation

The pathogenesis of myointimal proliferation in canine heartworm disease is unclear (Knight, 1987). Atwell et al (1985) attributed histopathological changes to physical trauma to the vessel wall by adult parasites because polyvinyl chloride threads placed inside canine pulmonary arteries cause pathological changes similar to those caused by D. immitis. Others report lesions in pulmonary veins distal to the residence of the adult worms, suggesting that vascular lesions are caused by factors other than physical trauma (Schaub & Rawlings, 1980).

Adult parasites may release substances which cause morphological alterations in the host's pulmonary vasculature. Histopathological alterations may be due to a direct effect of parasite products on mammalian endothelial cells or secondary to activation of the host's immune response (Schaub et al., 1980). Studies so far have failed to demonstrate that alterations in host immunity contribute to myointimal proliferation. A search for immune complexes and IgE within actively proliferating lesions has failed to identify these components (Knight, 1983). Cell-mediated immunity to D. immitis antigen

has been described in lymphocytes (Welch et al., 1979) and splenocytes (Grieve et al., 1979), however, their importance in the pathogenesisis of heartworm disease is unknown.

It is likely that parasite products have a direct effect on host cell function. Schaub et al (1983) reported that aspirin (325 mg/day, orally) decreases myointimal proliferation in dogs infected with heartworm suggesting that cyclooxygenase products may be responsible for histological changes associated with heartworm infection. Mammalian, as well as parasite cyclooxygenase activity is inhibited by aspirin (Liu & Weller, 1990; Liu et al., 1990). The authors attributed the effect of aspirin to inhibition of the host's inflammatory response (Schaub et al, 1983). An alternate explanation is that filarial cyclooxygenase products are responsible for pulmonary pathology. The pathogenesis of pulmonary hypertension in heartworm disease is unknown, but has been attributed to morphological alterations in the pulmonary vasculature.

Relationship between myointimal proliferation and pulmonary hypertension

Rawlings (1986) attributed pulmonary hypertension to outflow obstruction of pulmonary arteries as a result of myointimal proliferation, thrombosis and fibrosis. He correlated the number of adult worms with the severity of pulmonary endarteritis and fibrosis. The greater the number of worms, the more widely distributed and the greater the severity of pulmonary arterial lesions (Rawlings, 1986). There is no correlation, however, between the number of worms, the severity of pulmonary lesions and the severity of pulmonary hypertension and/or exercise intolerance (Rawlings et al, 1977, Rawlings, 1981).

In one study, dogs were inoculated with 100 infective larvae and all had "severe alterations typical of heartworm disease" on thoracic radiographs and pulmonary arteriograms, but none developed exercise intolerance (Rawlings, 1981). Courtney et al. (1985) reported exercise intolerance in dogs infected with as few as one heartworm. One heartworm is insufficient to obstruct pulmonary outflow or to cause significant obstructive arteritis in pulmonary arteries suggesting that other factors besides pulmonary outflow obstruction are important in the pathophysiology of heartworm disease.

Rawlings et al (1977) reported an increase in mean pulmonary artery pressure and total pulmonary vascular resistance in response to hypoxia that was increased in dogs with heartworm. However, radiographic and arteriographic evaluation showed little difference in the severity of pulmonary disease between control and heartworm infected dogs. These results indicate that dogs infected with D. immitis, without significant radiographic and arteriographic signs of pulmonary outflow obstruction, have an increased pulmonary hypertensive response to hypoxia. Rawlings et al (1977) suggested that hypoxic vasoconstriction is increased in dogs infected with heartworm due to an increase in vascular smooth muscle responsiveness or vasoactive substances released by inflammatory cells, adult parasites or microfilariae. Increased smooth muscle responsiveness is unlikely since Mupanomunda et al. (1993) reported that contractile responses to norepinephrine, histamine and serotonin are not different in the in vitro pulmonary artery from heartworm infected dogs when compared to control. In fact, in pulmonary artery from dogs with D. immitis, constrictions are depressed rather than enhanced in response to $PGF_{2\alpha}$ and the thromboxane analogue, U44069 (Mupanomunda et al., 1993).

An alternate hypothesis is that the potentiation of hypoxic pulmonary vasoconstriction is secondary to filarial-induced depression of endothelium-dependent relaxation. Dinh-Xuan et al., (1991) demonstrated that endothelium-dependent relaxation is depressed in humans with pulmonary hypertension secondary to chronic obstructive pulmonary disease. Furthermore, antagonists of endothelium-dependent relaxation enhance the constriction response to hypoxia in the isolated perfused rat lung, suggesting that EDRF attenuates hypoxic vasoconstriction (Brashers et al., 1988; Mazmanian et al., 1989; Adnot et al 1991). Mupanomunda et al., (1992) reported that endothelium-dependent relaxation is depressed in the in vitro pulmonary artery of heartworm infected dogs. If depressed endothelium-dependent relaxation is due to decreased EDRF, then accentuated hypoxic vasoconstriction in D. immitis infected dogs may be secondary to the loss of EDRF.

The pathogenesis of pulmonary hypertension associated with canine heartworm disease is not well understood. A novel hypothesis is that biologically active filarial factors depress endothelium-dependent relaxation. Understanding the pathogenesis of pulmonary hypertension associated with canine heartworm disease may provide insight into the mechanism of filarial diseases, including human filariasis.

Canine heartworm disease as a model of human filariasis

Important human filarial pathogens include Wuchereria bancrofti, Brugia timori, Brugia malayi, Loa Loa and Onchocerca volvulus. Filarial nematodes that infect humans have narrow specificity for vertebrate hosts. Onchocerca volvulus and Wuchereria bancrofti, the two major human filarial pathogens, infect only humans (Taylor et al., 1990; Ellenberger et al., 1992). Because insect vectors are required in transmission of filarial disease, it is necessary not only to have a colony of infected mammals, but also one of

infected insects for obtaining infective larvae. It is difficult to develop adequate experimental models of human filariasis because of narrow host specificity and the requirement of an insect vector in propogation of filarial disease. D. immitis is taxonomically and behaviorally related to the major human filarial pathogens (Grieve et al, 1983) and the incidence of heartworm infection is high in the U.S. In a study by Wong et al., (1983) 83% of dogs euthanized at animal shelters in the United States were infected with D. immitis. Thus heartworm infection in dogs provides a useful, naturally occurring animal model of human filarial disease (Weil et al., 1982).

Human filarial disease

More than 200 million people worldwide are infected with filarial parasites (Nutman et al., 1984). Onchocerca volvulus infection is an important cause of blindness, Loa loa is responsible for calabar swellings (localized angioedema of the extremities) and Wuchereria bancrofti and Brugia species are primarily responsible for lymphatic filariasis (Nutman et al., 1986; Piessens et al., 1980; WHO, 1984). Adult parasites of Onchocerca volvulus and Loa loa live in the dermis and subcutaneous tissues of the host, whereas adults of Wuchereria bancrofti and Brugia species are in the lymphatic system. The microfilariae of W. bancrofti, L. loa, and Brugia circulate in the blood and O. volvulus microfilariae inhabit the skin and subcutaneous tissues (Ukoli, 1984).

Treatment, eradication or prevention of filarial diseases has generally been unsuccessful. Diethylcarbamazine (DEC) is used to treat or prevent filariasis, however, its side effects are often more severe than the disease (Wilson, 1950; Otto et al., 1953). Severe side effects which include headache, fever, nausea, vomiting and dizziness cause refusal of patients to comply with therapeutic regimens, and subsequent failure of preventative, as well as curative therapy with DEC (Wilson, 1950; Partono et al., 1981). Mechanisms

concerning immunity against filarial infection are not well understood, and attempts to produce vaccines against filarial pathogens have so far been unsuccessful (Abraham et al., 1988; Oikawa et al., 1992). Eliminating filarial disease by eradicating the insect vector has also been unsuccessful (Philipp et al., 1988). Therefore, despite extensive effort, the incidence of filarial infection remains high. New therapies in treatment, eradication and/or prevention of filarial diseases are important. Better understanding of the pathogenesis of human filariasis will contribute to the development of new therapeutic regimens.

Pathogenesis of lymphatic filariasis

The pathogenesis of lymphatic filariasis is poorly understood despite extensive investigation (Piessens et al., 1980; Case et al 1991; Kwa et al, 1991). Symptoms of filarial disease are variable, ranging from inapparent infection to disabling elephantiasis (Piessens et al., 1980; Nutman et al., 1987; Oikawa et al, 1992). Many people infected with filarial parasites have no clinical signs (Ottesen et al., 1977, 1982; Piessens et al., 1980; Weller et al., 1982), whereas others suffer from acute episodic adeno-lymphangitis or "filarial fever" characterized by fever, enlargement of lymph nodes (usually inguinal), and lymphedema of the affected extremity. The fever lasts for 3-5 days and acute adenolymphangitis resolves in 6-7 days (Ottesen et al., 1977, 1982; Piessens et al., 1980; Weller et al., 1982). The duration and severity of lymphedema may get progressively worse with recurrent episodes of filarial fever. The most severe manifestation of lymphatic filariasis is elephantiasis which is characterized by chronic disfiguring and debilitating edema and inflammation of the extremities (Ottesen et al., 1977, 1982; Weller et al., 1982). Other important symptoms of lymphatic dysfunction are chyluria (lymph in the urine) and hydrocoele (lymph in the scrotum) which are thought to develop from the

formation of fistulae between lymphatics and genitourinary organs (Johnston, 1955). Chronic lymphedema and hydrocoele are relatively uncommon manifestations of filarial disease. In an epidemiologic study in the Cook Islands, where bancroftian filariasis is endemic, chronic lymphobstructive lesions were observed in only 36 out of 459 people. Elephantiasis was seen in 12 patients and 24 had hydrocoeles (Weller et al., 1982).

Lymphedema and elephantiasis have been attributed to physical obstruction of afferent lymphatic outflow by the adult parasites or the immunological response of the host (Schacher & Sahyoun, 1967; Piessens et al., 1980; Nutman et al., 1987). Neither mechanism adequately explains the complex clinical manifestations of human filarial disease (Kwan-Lim & Maizels, 1991; Ottesen, 1992). Physical obstruction of lymphatic outflow by adult parasites is unlikely to be the cause of lymphedema because lymphedema has been reported in patients infected with Onchocerca volvulus (Wolfe et al 1974) and Loa Loa (Grove & Schneider 1981; Paleologo et al., 1984), filarial parasites which live in skin and subcutaneous tissues rather than in the lymphatic system. An immunodeficient infant infected with Brugia species presented initially with swelling of the left leg which progressed to involve the right leg, both arms, the perineum and the face. The infant was microfilaremic, however, biopsy of the left inguinal lymph node failed to reveal adult parasites. The lymphedema persisted after the patient became amicrofilaremic (Simmons et al.,1984). Ngan et al. (1977) reported that lymphatic obstruction, as assessed by lymphography, was not responsible for lymphangiectasis in 90 patients with chyluria. They suggested that toxic parasite products or the host's immune response may be responsible for disturbances in flow dynamics of lymphatic vessels. Tani et al (1987) showed

that placement of adult Dirofilaria immitis into the peritoneal cavity of dogs causes dilation of popliteal, inguinal, para-aortic, and aortic lymphatics, as well as cisterna chyli and thoracic duct. These studies suggest that mechanisms other than obstruction of lymphatic outflow by adult parasites is responsible for lymphedema and elephantiasis. Tani et al., (1987) attributed lymphatic dysfunction to immunological reactions of the host in response to substances released by the worms.

Variations in the host's immunological response to filarial parasites may be responsible for the diversity of clinical symptoms in filarial disease (Ottesen et al., 1977, 1982; Piessens et al., 1980; Nutman et al., 1987a; Hussain et al., 1987). In many studies an attempt has been made to correlate the degree of immunological reactivity of the host with the severity of clinical symptoms. Asymptomatic patients are generally microfilaremic, whereas amicrofilaremia is frequently seen in patients with severe manifestations of filarial infection. Therefore, it has been suggested that severe symptoms are seen in patients that mount a vigorous immune response against the parasites (Ottesen et al., 1977). Several studies suggest that asymptomatic microfilaremic patients are frequently immunologically hyporeactive to parasites and parasite antigens (Ottesen et al., 1977, 1982; Piessens et al., 1980; Nutman et al., 1987). They produce low levels of antifilarial IgG1, IgG2 and IgG3, but make large quantities of IgG4, the IgG subclass that inhibits allergic responsiveness (Hussain et al., 1987; Ottesen, 1992). Furthermore, lymphocytes from patients with asymptomatic microfilaremia fail to respond to filarial antigens in cellular proliferation assays, but retain their reactivity to nonparasitic antigens. Lymphokine production by parasite-stimulated peripheral blood mononuclear cells are also deficient in these individuals (Nutman et al., 1987b). Patients with elephantiasis, however, are often amicrofilaremic and

mount vigorous parasite-specific immune responses characterized by high quantities of parasite-induced lymphokine, and parasite-specific IgM and IgG production. They produce minimal IgG4 responses, however, when compared to asymptomatic microfilaremic individuals (Nutman et al., 1987a; Piessens et al., 1980; Ottesen et al., 1977,1982; Hussain et al., 1987). These results suggest that quantitative and qualitative differences in the immune response of the host may explain the diversity of clinical symptoms associated with filarial disease (Ottesen, 1989; Grieve et al. 1983; Kwan-lim & Maizels, 1990). Differences in immune responsiveness, however, do not explain the pathogenesis of lymphedema in microfilaremic patients that are hyporesponsive to parasite antigens (Ottesen 1992).

Studies with the immunodeficient nude mouse suggest that there may be two pathogenic mechanisms that cause lymphedema and elephantiasis (Vincent et al., 1984; Vickery et al., 1991). Infection with Brugia sp. induces proliferation of lymphatic endothelium, lymphatic dilatation, lymphedema and elephantiasis in immunodeficient nude mice. This response is not associated with obliterative cellular reactions and can be reversed by removal of the worms (Vincent et al., 1984). If immunodeficient mice are made immunocompetent by reconstitution with spleen cells from mice previously sensitized to filarial antigens, then inflammatory reactions to the parasites result in obliterative lymph thrombus formation, perilymphangitis, lymphedema and elephantiasis (Vickery et al., 1991). These results suggest that lymphedema and elephantiasis can be caused by two discrete pathogenic mechanisms: one depends on a competent immune system and the other does not. Lymphedema in the absence of a competent immune system may be due to a direct effect of parasite products on lymphatic function (Ottesen, 1992). Filarial parasites release a variety of potentially immunosuppressive

substances which aid in the parasites evasion of the host's immune system (Lal et al., 1990; Wadee et al., 1987; Liu et al., 1992). These substances which include arachidonic acid metabolites, high molecular weight proteins and phosphocholine may have a direct effect on lymphatic function and this may be important in the pathogenesis of lymphedema and elephantiasis (Ottesen, 1992). In order to define mechanisms by which lymphedema may result from alterations in lymphatic function, it is necessary to understand the anatomy and physiology of the lymphatic system.

Lymphatic system

Anatomy

The lymphatic system is a closed circulation that returns fluid, macromolecules and cells to the circulation from the interstitial space (Leak, 1984; Ryan, 1989). It begins in the periphery as a network of blind-ended capillaries. Lymphatic capillaries empty into collecting vessels that converge into collecting ducts to eventually drain into the thoracic duct, the largest vessel of the lymphatic system. The thoracic duct communicates with the vascular system by the subclavian vein (Leak, 1984; Ryan, 1989).

Lymphatic capillaries are thin walled tubes with a single layer of endothelial cells. Collecting lymphatics have an intimal layer of endothelial cells, a middle layer of smooth muscle and an outer adventitial layer composed of collagen, elastic fibers, blood vessels and nerves. Blood supply is more extensive in lymphatics, (Ohhashi et al., 1977), but its nerve supply is less than in arteries and veins (Roddie et al., 1980; McHale et al., 1988).

Certain anatomical features of the lymphatic system aid in the transport of lymph. An elaborate system of anchoring filaments attach lymphatic capillaries and collecting vessels to the interstitial matrix. These stretch lymphatic capillaries and ducts when the interstitium is swollen by excess

fluid. Stretching increases the width of intercellular junctions and allows large molecules and cells to enter thereby promoting transport of excess interstitial fluid and protein (Leak, 1984; Ryan, 1989). All collecting lymphatics have valves that prevent backflow of lymph. The functional unit of the lymphatic, the lymphangion, is composed of an inlet and outlet valve and the lymphatic segment in between.

Physiology

Extrinsic forces that govern lymph flow

The lymphatic system was once considered a network of passive conduits, and lymph flow was thought to be driven primarily by lymph formation and external forces. Muscle activity, active and passive body movements, respiration, blood vessel pulsations, and massage aid in lymph flow by externally compressing lymphatic vessels. Also, a pressure gradient along the lymphatic vessel is created by respiratory movements. During inspiration, intrathoracic pressure decreases and intrabdominal pressure increases to create a pressure gradient between the abdominal and thoracic portions of the thoracic duct. This drives lymph flow centrally. Passive forces may be the primary factor in driving lymph flow in conduit lymphatics which contain little smooth muscle (i.e. thoracic duct), however in peripheral lymphatics with an extensive tunica media, active propulsion of lymph may be the primary driving force (Hall et al., 1965; Hargens & Zweifach, 1977; McHale & Roddie, 1976; Ohhashi et al., 1978, 1980; Roddie et al., 1980; Benoit et al., 1989).

Intrinsic forces that govern lymph flow

Spontaneous rhythmic contractions of lymphatic vessels provide a major force driving lymph flow (Hall et al., 1965; Ohhashi et al., 1978; Leak, 1984). The importance of lymphatic contractions in mammals was first suggested by Ranvier in 1889 (Hall et al., 1965). Since that original

observation, lymphatic contractions have been described both in vivo and in vitro in both central and peripheral lymphatics of the sheep, cow, rat, guinea-pig, bat, mouse and man (Kinmouth & Taylor, 1956; Hall et al., 1965; McHale & Roddie, 1976; Hargens & Zwefach, 1977; Reddy & Staub, 1981).

The importance of lymphatic contractility in propulsion of lymph

Hall et al. (1965) reported that peristaltic contractions of various lymphatic vessels in the unanesthetized sheep have a frequency of 1-30 per minute and a pulse pressure of 1-25 mm Hg. Both pulse pressure and contraction frequency increase in response to increasing lymph flow produced by intravenous infusion of Ringer's solution. Distal occlusion of a lymphatic vessel causes mean lymph pressure to rise and contraction frequency to increase. Although contraction amplitude increases with increasing pressure, it decreases at very high pressures. Muscular, circulatory and respiratory movements increase intralymphatic pressure however, their effects are small compared to the intraluminal pressure generated by spontaneous contractions. The authors concluded that lymphatic contractile activity is primarily responsible for the propulsion of lymph from the periphery to the thoracic duct, and suggested that both frequency and amplitude of contractions increase with lymph production, so there is a balance between the rate of lymph production and the rate of lymph removal from the periphery (Hall et al., 1965).

McHale and Roddie (1976) and Roddie et al. (1980) showed that spontaneous contractile activity propels lymph. They studied spontaneously contractile segments of bovine mesenteric lymphatics in vitro and reported that inlet and outlet pressures are different during contraction. They suggested that because initial hydrostatic pressure was zero, this pressure gradient results from lymphatic vessel contraction.

Hargens and Zweifach (1977) studying mesenteric lymphatics of the rat and guinea pig in vivo suggested that lymphangions undergo coordinated contractions which favor unidirectional flow of lymph. A lymphangion contracts in response to increased hydrostatic pressure. A critical pressure is reached during contraction which causes the inlet valve to close, the outlet valve to open, and lymph to flow into a downstream lymphangion. As the lymphangion fills, contraction begins and the cycle repeats. They proposed that lymphangions behave as simultaneously contracting pumps arranged in series which move lymph centrally.

Ohhashi et al. (1980) reported similar results for isolated one-lymphangion segments of bovine mesenteric lymphatics. Coordinated contractions propogate along the lymphangion from the inlet to outlet valve. Calculated ejection fractions vary from 45 to 65%. Increasing intraluminal pressure results in an increase in the rate of spontaneous contractions, whereas the amplitude of contractions decrease beyond an optimal intraluminal pressure.

McHale and Roddie (1976) reported that alterations in transmural pressure affect the frequency and amplitude of spontaneous contractions of isolated bovine mesenteric lymphatics. They found that transmural pressure and contraction frequency vary linearly, but an optimum transmural pressure exists for contraction amplitude. Pressures above optimum decrease contraction. They suggested that decreasing contraction amplitude at high transmural pressures may be due to overstretching of smooth muscle.

Spontaneous lymphatic contractions play an important role in the propulsion of lymph. With high rates of lymph production, increasing contraction frequency and amplitude increase lymph flow. The mechanisms relating lymph flow and pressure to contraction frequency and amplitude are

not known. How lymphatic smooth muscle contracts spontaneously is also unknown. Lymphatic smooth muscle may be similar to cardiac muscle and certain smooth muscle fibers may have inherent rhythmicity. This is unlikely, however, since lymphatic vessels are frequently inactive, becoming contractile in response to fluid distention (Hargens & Zwefach, 1977). Increases in pressure may stimulate neural reflexes or humoral pathways which mediate lymphatic contraction. Spontaneous contractions may also be myogenic and increases in pressure may invoke intrinsic stretch-dependent contractions of lymphatic smooth muscle (Benoit et al., 1989). Endothelial cells or smooth muscle may also produce vasoactive agents which mediate spontaneous contractile activity. Multiple mechanisms are undoubtedly important in the control of lymphatic smooth muscle contraction.

Nervous control

Because spontaneous contractions persist in vivo after death, and occur in vitro in the presence of tetrodotoxin, a nerve blocker, they are probably not neurogenic (Hall et al., 1965; Hargens & Zweifach, 1977; McHale et al., 1980). The nervous system may be important, however, in modulating lymphatic smooth muscle activity. There is a sparse noradrenergic innervation in lymphatics (McHale et al., 1980). Stimulation of intramural noradrenergic nerves or application of norepinephrine in vitro increases both frequency and force of contraction in bovine mesenteric lymphatics (McHale et al., 1980; McHale et al., 1988). Stimulation of the lumbar sympathetic chain increases hindlimb lymph flow in the sheep, suggesting noradrenergic innervation in the living animal (McGeown et al., 1987).

Humoral control

There is no evidence that humoral agents mediate spontaneous lymphatic contractions, but they may be important modulators. Serotonin,

bradykinin, prostaglandin $F_{2\alpha}$, histamine, dopamine, and endothelin contract lymphatic smooth muscle whereas ATP, ADP, adenosine, atrial natriuretic peptide, prostaglandin E_2 , and interleukin-1 suppress lymphatic contractility (Ohhashi et al., 1978, 1990; Hanley et al., 1989; Dobbins & Dabney, 1991; Watanabe et al., 1988). These agents may be produced in both physiological and pathophysiological conditions to play an important role in modulating lymph flow. Because lymphatics contract spontaneously in vitro, it is unlikely that contractile activity is mediated by nerves or circulating substances. Mechanisms operating within the vessel wall are more likely responsible.

Myogenic mechanisms

Benoit et al. (1989) studied the mechanism of augmented stroke volume and contraction frequency associated with increased lymph formation in rat mesenteric lymphatics in vivo. They suggested that increased contraction amplitude and frequency with increased lymph production is due primarily to intrinsic stretch-dependent mechanisms caused by increased preload. This was shown by the effects of fluid volume expansion on end-diastolic diameter, contraction frequency, ejection fraction, stroke volume, rate of pressure development (dP/dt) and contractility. Intravenous administration of fluids increases contraction frequency, end-diastolic diameter, stroke volume and ejection fraction, but rate of pressure development and contractility are not altered in response to increased lymph formation. Acute increases in lymph flow by plasma dilution are not associated with changes in the inotropic properties of lymphatic smooth muscle. They suggested that augmented stroke volume and contraction frequency result from intrinsic stretch-dependent smooth muscle mechanisms invoked by increased preload but did not discuss the nature of this stretch-dependent mechanism.

Stretch-dependent constriction has been studied extensively in blood vessels including rabbit ear artery (Hwa & Bevan, 1986), rat portal vein (Johansson, 1989), dog basilar (Katusic et al., 1987) and carotid artery (Rubanyi, 1988), and cat cerebral artery (Harder, 1987). Bevan and Laher proposed that membrane-bound Ca^{++}-entry pathways are activated by pressure or stretch in vascular smooth muscle (Bevan & Laher, 1991). Others suggest that endothelial cells and endothelium-derived products mediate stretch-dependent constriction (Rubanyi, 1988; Katusic et al., 1987; Harder, 1987; Sumpio & Widman, 1990). Endothelial cells mediate stretch or pressure-induced vasoconstriction in cerebral arteries of the cat (Harder, 1987) and carotid (Rubanyi, 1988) and basilar arteries of the dog (Katusic et al., 1987). Cultured bovine aortic endothelial cells produce increased quantities of the potent vasoconstrictor, endothelin in response to pulsatile stretch (Sumpio & Widman, 1990).

Endothelial cells also mediate rhythmic contractions in blood vessels. Jackson et al. (1988, 1991) showed that L-nMMA, NOARG or endothelial cell removal abolishes phenylephrine-induced spontaneous contractions in isolated hamster aorta. Rhythmic contractions are restored by L-arginine in L-nMMA-treated preparations. Sodium nitroprusside, an exogenous source of NO, restores oscillations in NOARG treated vessels. These results indicate that endothelium-derived NO is responsible for rhythmic contractions of hamster aorta (Jackson et al., 1991).

Endothelium-dependent, as well as endothelium-independent mechanisms mediate stretch-induced and agonist-induced contraction of vascular smooth muscle (Rubanyi, 1988; Katusic et al., 1987; Harder, 1987; Sumpio & Widman, 1990; Bevan & Laher, 1991; Furchgott & Vanhoutte, 1989). Endothelial cells mediate contractions by releasing vasoactive agents such as

cyclooxygenase products, endothelin and NO (Jackson et al., 1991; Katusic et al., 1987; Sumpio & Widman, 1990). Thus vasoactive agents released by lymphatic endothelial cells may be important in phasic contractile activity of lymphatics.

Cyclooxygenase products

Arachidonic acid metabolites from the lymphatic vessel wall may be responsible for spontaneous contractions of lymphatics (Johnston & Gordon, 1981; Johnston & Feuer, 1983; Allen et al., 1984). Inhibition of cyclooxygenase with aspirin or indomethacin abolishes spontaneous contractions in bovine and ovine mesenteric lymphatics both in vivo and in vitro (Johnston & Gordon, 1981; Johnston & Feuer, 1983; Allen et al., 1984). In addition, the thromboxane synthase inhibitor, UK37248, abolishes, whereas the PGH_2 analogue, U46619, restores spontaneous contractions of bovine mesenteric lymphatics (Johnston & Gordon, 1981; Johnston & Feuer, 1983; Allen et al., 1984). These studies suggest that cyclooxygenase products derived from the vessel wall are important in generation of spontaneous lymphatic contractions. The site of eicosanoid production, however, is unknown. In the vascular system, endothelial cells rather than smooth muscle, are the primary source of thromboxane (Ingerman-Wojenski et al., 1981). If the same is true for lymphatics, then spontaneous contractions may be dependent on endothelium-derived thromboxane.

Endothelial cells

Recent studies have focused on the role of endothelial cells in control of lymphatic smooth muscle tone. Ohhashi et al. (1991) demonstrated that lymphatic endothelial cells mediate smooth muscle relaxation. Acetylcholine elicits relaxation in the in vitro canine thoracic duct, a lymphatic which does not contract spontaneously. Relaxation is abolished by endothelial cell

removal however, sandwiching an endothelium-denuded transverse strip of thoracic duct with an endothelium-intact longitudinal strip restores relaxation in the denuded strip. Oxyhemoglobin, methylene blue and L-nMMA abolish acetylcholine relaxation suggesting that NO released from thoracic duct endothelial cells mediates the relaxation response to acetylcholine.

NO release has also been demonstrated in endothelial cells from lymphatic capillaries. Bohlen and Lash (1992) reported that lymphatic capillaries in the submucosa of rat intestine release vasoactive agents, including NO, in response to endothelium-dependent vasodilators. They demonstrated using videomicroscopy, that microiontophoresis of acetylcholine or bradykinin onto the wall of lymphatic capillaries causes relaxation in nearby arterioles. The arteriolar relaxation response was 80% of that invoked by direct application of acetylcholine or bradykinin to the arteriolar wall. Because application of endothelium-dependent vasodilators to nearby connective tissue produced little or no relaxation, relaxation was not due simply to diffusion of endothelium-dependent vasodilators to the arteriole. Microiontophoresis of L-nMMA onto the lymphatic capillary wall before treatment with acetylcholine abolished arteriolar relaxation. This suggests that acetylcholine stimulates lymphatic endothelial cells to release NO which relaxes neighboring arterioles. Treatment of lymphatic capillaries with bradykinin also produced relaxation of nearby arterioles. Because this response was not inhibited by L-nMMA another endothelium-derived factor is involved. Vasoactive agents, including NO, released from lymphatic capillary endothelial cells may be important in modulating the tone of nearby vascular smooth muscle. Because lymphatic endothelial cells are a substantial fraction of the total population of endothelial cells in the submucosal layer of the

intestine, relaxing factors released by lymphatic endothelial cells may provide an important source of NO (Bohlen & Lash, 1992).

Hanley et al. (1992), using isolated perfused five lymphangion segments (2 cm in length) of bovine mesenteric lymphatics, investigated the role of endothelial cells in mediating lymphatic pumping in response to changes in transmural pressure. They reported that lymphatic pumping increases with increasing transmural pressure up to a peak pressure (between 8 and 12 cm H_2O), and then declines at higher pressures. Removal of endothelial cells by denudation with silk suture does not alter lymphatic pumping in response to changes in transmural pressure, suggesting that endothelial cells are not required for transmural pressure-induced lymphatic pumping. They examined endothelium-denuded and endothelium-intact sections of vessel using silver nitrate staining and electron micropscopy and reported removal of greater than 90% of endothelial cells. The sections examined, however, may not represent the entire lymphatic vessel. It is difficult to remove endothelial cells from valves which contain more than one surface covered by endothelium. Since they did not examine valve sections, it is unknown whether these regions were endothelium-denuded. Furthermore, most investigators use chemicals to remove endothelial cells from small vessels like lymphatics because chemical denudation is more effective at removing the entire endothelial cell surface without destroying smooth muscle (Furchgott et al., 1987; Ralevic et al., 1989; Eskinder et al., 1990; Koller et al., 1989; Pohl et al., 1987). The effect of chemical denudation on pumping activity was not reported in this study.

Elias et al. (1992), demonstrated that endothelial cells play an obligatory role in pumping activity of bovine mesenteric lymphatics after exposure to oxyhemoglobin. They demonstrated that infusion of oxyhemoglobin into the

lumen of isolated perfused 5-lymphangion segments of bovine mesenteric lymphatics abolishes their pumping activity. Pumping is restored by increases in transmural pressure in endothelium-intact preparations, but not in endothelium-denuded preparations, suggesting an obligatory role for endothelial cells in lymphatic pumping after exposure to oxyhemoglobin (Elias et al., 1992). The mechanism of action of oxyhemoglobin on lymphatic pumping is unknown. Oxyhemoglobin inhibits endothelium-dependent relaxation by binding and inactivating EDRF/NO and this effect has been reported in isolated canine thoracic duct (Ohhashi et al. 1991). Oxyhemoglobin also inhibits phasic contractions of hamster aorta by binding and inactivating NO (Jackson et al., 1988, 1991). Thus inhibition of endothelium-derived NO may account for the effects of oxyhemoglobin on lymphatic pumping.

The role of endothelial cells in controlling lymphatic contractile activity is not well understood. Recent studies suggest that lymphatic endothelial cells release vasoactive factors (Ohhashi et al., 1991; Bohlen & Lash, 1992). The role of these factors in regulating lymphatic smooth muscle function requires further investigation.

MATERIALS AND METHODS

I. EXPERIMENTAL MODEL: Mongrel dogs of either sex were obtained from the University Laboratory Animal Resources at Michigan State University. Heartworm infected dogs were identified by having microfilariae of D. immitis in the peripheral blood. At necropsy, all dogs designated as infected with D. immitis had adult parasites in the right heart and pulmonary arteries, but no evidence of other cardiovascular or systemic disease. No control dogs had adult parasites or microfilariae.

Dogs were euthanized with an overdose of intravenous pentobarbital. The heart and lungs were removed and placed in cold physiological saline solution (PSS) containing (in mM): NaCl-127; KCl-4.7; $CaCl_2$-2.5; $MgSO_4$-1.2; KH_2PO_4-1.2; $NaHCO_3$-15; glucose-5.5; EDTA-0.03). The right ventricle and pulmonary arteries were opened and adult worms removed, counted, sexed and placed in RPMI culture media. D. immitis was used in preparation of heartworm-conditioned medium.

II. EXPERIMENTAL PREPARATIONS

A. Isolated vessels:

(1) Canine femoral artery: After dogs were euthanized with pentobarbital, both femoral arteries were removed, and placed in cold PSS. Vessels were dissected free of surrounding connective tissue, 5 mm rings prepared and suspended in 10 ml organ chambers filled with warm (37°C) PSS oxygenated with 95% O_2/5% CO_2. Endothelial cells were removed from some rings by rubbing the intimal surface with a sanded pipet tip. Rings were suspended by two silk sutures connected to both a stationary rod in the organ chamber and a Grass force transducer (Model FT03C). Changes in isometric tension were continuously recorded on a Grass polygraph (Model 7B).

Experiments were performed in fall and spring to determine if heartworm (HW) infection seasonally influences endothelium-dependent relaxation in vitro. Spring refers to the time in Michigan when heartworm-conditioned medium depresses endothelium-dependent relaxation of in vitro rat aorta; fall is the period when it does not (Kaiser et al 1990).

In 22 out of 32 experiments (11 HW and 11 controls), rings were weighed (wet weight), placed in a 70° C oven for 24 hours and reweighed (dry weight). There were no significant differences in wet (HW=.021 ± .002 gm vs. Control=.018 ± .00002 gm; $p>0.05$) or dry weights (HW=.007 ± .001 gm vs. Control=.006 ± .0003 gm; $p>0.05$) in rings from control and heartworm infected dogs. Femoral artery from heartworm infected dogs is designated as "heartworm femoral artery" and femoral artery from control dogs as "control femoral artery".

(2) Canine femoral vein: After dogs were euthanized with pentobarbital, both femoral veins were removed and placed in cold PSS. Vessels were dissected free of surrounding connective tissue, transverse strips (4-5 mm) were prepared and suspended in 10 ml organ chambers for measurement of isometric tension as described in methods section II.A.(1.). Femoral vein from heartworm infected dogs is designated as "heartworm femoral vein" and femoral vein from control dogs as "control femoral vein".

(3) Bovine mesenteric lymphatics: Bovine mesenteric lymphatics were obtained from the Michigan State University meat laboratory. Cattle were killed by a "captive bolt" technique and exsanguinated. After evisceration, mesenteric lymph nodes and lymphatic vessels were removed, transported to the laboratory and placed in warm (39°C) oxygenated (95% O_2/5% CO_2) PSS. Mesenteric vessels were dissected in warm oxygenated PSS to prevent solidification of mesenteric fat. Lymphatic vessels were ligated distally with

silk suture and lymph nodes injected with warm PSS. This procedure distends the lymphatic for easier visualization and dissection. Rings (5-7 mm) were cut and cannulated with a small glass cannula which was used to thread two silk sutures through the lumen of the vessel. Rings were suspended in organ chambers filled with warm (39°C) oxygenated (95% O_2/5% CO_2) PSS for measurement of isometric tension as described in methods section II.A.(1.). In some experiments, endothelial cells were removed by everting the ring and rubbing the intimal surface with moistened filter paper (Ohhashi et al., 1991) or by addition of Type I collagenase to the bath (500-600 U/ml) for 30 minutes.

(4) **Canine thoracic duct:** Dogs were euthanized with an overdose of intravenous pentobarbital. To aid in visualization, the thoracic duct was distended by injection of cold PSS into the mesenteric lymph nodes. A segment (10-15 cm) of thoracic duct was ligated, transected and placed in cold PSS. Rings (5-7 mm) were cut and cannulated with a small glass cannula which was used to thread two silk sutures through the lumen of each ring. Rings were suspended in organ chambers for measurement of isometric tension as described in methods section II.A.(1.). In some experiments, endothelial cells were removed by everting the rings and gently rubbing the luminal surface with wetted filter paper (Ohhashi et al., 1991). Thoracic duct from control dogs is designated as "control thoracic duct" and thoracic duct from heartworm infected dogs is designated as "heartworm thoracic duct".

B. MATERIALS

(1) **Femoral artery:** A cumulative dose-response relationship was obtained for the endothelium-dependent vasodilator methacholine (10^{-10} to 3 x 10^{-5}M), endothelium-independent vasodilator sodium nitroprusside (10^{-9} to 3 x 10^{-4}M), and the vasoconstrictor norepinephrine (10^{-10} to 3 x 10^{-5}M). After preconstriction with norepinephrine (ED_{60-75}), dose-response relationships

to methacholine were measured in the presence and absence of inhibitors of cyclooxygenase [mefenamic acid (10^{-5}M) and indomethacin (10^{-5}M)]; the inhibitor of guanylate cyclase [methylene blue (10^{-5}M; Kaiser et al 1991)]; and the inhibitor of nitric oxide synthesis [N-nitro-L-arginine methyl ester (L-NAME; 1.5 x 10^{-5}M; Moore et al 1990)]. To determine the specificity of L-NAME, L- and D- arginine at 20 fold molar excess (3 x 10^{-4}M; Mulsch & Busse, 1990) were added to L-NAME-treated and untreated vessels at the end of methacholine dose-response relationships.

(2) **Femoral vein:** In preliminary experiments, dose response-relationships were obtained for the endothelium-dependent vasodilator, methacholine (10^{-10} to 3 x 10^{-6}M) and the vasoconstrictors, norepinephrine (10^{-10} to 3 x 10^{-5}M) and $PGF_{2\alpha}$ (2.1 x 10^{-10} to 6.3 x 10^{-5} M). Dose-response relationships to methacholine were examined in strips preconstricted with either norepinephrine (ED_{30}; Miller & Vanhoutte, 1989) or prostaglandin $F_{2\alpha}$ (ED_{30}; Miller & Vanhoutte, 1989). In strips preconstricted with $PGF_{2\alpha}$, experiments were done in the presence and absence of the inhibitor of cyclooxygenase [mefenamic acid (10^{-5}M)]; the inhibitor of guanylate cyclase [methylene blue (3 x 10^{-5}M; Martin et al., 1985)]; and the inhibitor of NO synthesis [L-NAME; 3 x 10^{-5}M; Moore et al., 1990]. D-arginine, followed by L-arginine at 20 fold (6 x 10^{-4}M; Busse & Mulsch, 1990; Busse & Mulsch, 1991) and 60 fold molar excess (1.8 x 10^{-3}M; Busse & Mulsch, 1990) were added after completion of methacholine dose-response relationships to determine the specificity of L-NAME inhibition of relaxation.

(3) **Bovine mesenteric lymphatics:** Spontaneous contractions and norepinephrine constriction (3 x 10^{-5}M) were examined in lymphatic rings before and after endothelial cell removal. In preliminary experiments, phasic contractions were studied in the presence and absence of indomethacin (10^{-5}

M), aspirin (2.78 x 10^{-4}M; Kaiser et al., 1992), methylene blue (10^{-5} M; Kaiser et al., 1991), and L-NAME (3 x 10^{-5} M; Moore et al., 1990) +/-D- and L-arginine (9 x 10^{-4} M; Mulsch & Busse, 1990).

(4) Thoracic duct:

(a). To determine the effect of filarial factors on endothelium-dependent relaxation of canine thoracic duct, dose-response relationships to methacholine (10^{-10} to 3 x 10^{-6}M) and sodium nitroprusside (10^{-9} to 3 x 10^{-4}M) were obtained in preliminary experiments. Experiments were done on paired rings preconstricted with a submaximal concentration of norepinephrine (10^{-5} M; Ohhashi & Takahashi, 1991) in the presence of control medium or heartworm-conditioned medium.

(b). Dose-response relationships to methacholine were obtained in rings of thoracic duct from heartworm infected and control dogs to determine the effect of in vivo exposure to heartworm on endothelium-dependent relaxation in vitro. Experiments were done on rings preconstricted with norepinephrine (10^{-5} M; Ohhashi & Takahashi, 1991) in the presence and absence of mefenamic acid (10^{-5} M), methylene blue (10^{-5} M; Kaiser et al., 1991), and L-NAME (1.5 x 10^{-5} M; Moore et al., 1990). L- and D-arginine (6 x 10^{-4} M; Mulsch & Busse, 1990) were added at the end of methacholine dose-response relationships.

C. Heartworm-conditioned medium

Heartworm-conditioned medium depresses endothelium-dependent relaxation of the in vitro rat aorta (Kaiser et al., 1990, 1992). It was prepared by incubation of 1 male and 1 female heartworm per 10 ml of room temperature, unoxygenated PSS for 30 minutes. Room temperature, unoxygenated PSS (control medium) served as control. Endothelium-dependent relaxation of canine thoracic duct and spontaneous contractile activity of bovine

mesenteric lymphatics were examined in the presence of control and heartworm-conditioned medium. Control and heartworm-conditioned media were prepared during the spring and stored frozen until use. Frozen medium was thawed to room temperature in a water bath. After equilibration of the lymphatic, regular PSS was removed and control or heartworm-conditioned medium was added to the organ chamber.

IV. PROTOCOLS

A. Femoral artery: To insure that experiments were performed with rings at optimum passive tension, length-tension experiments were performed after 30 minute equilibration. Each ring was stretched to the optimum point on its length-tension curve by measuring the contractile response to norepinephrine (3×10^{-6} M) at different levels of stretch (7.5 gm-15 gm). Rings were equilibrated for 15 minutes between each contractile response. To insure that endothelial cells had been removed from denuded preparations, the relaxation response to acetylcholine (2×10^{-6} M) was determined after norepinephrine preconstriction at optimal length. Exclusion criteria were: (1) Endothelium-denuded rings that did not constrict to norepinephrine or relaxed more than 10% to acetylcholine. (one control and 2 heartworm rings relaxed more than 10% to acetylcholine and were omitted). 2) Endothelium-intact rings that failed to constrict to norepinephrine (two control and 3 heartworm rings failed to constrict to norepinephrine and were omitted).

(1) Norepinephrine dose-response relationships:

Dose-response relationships to norepinephrine were obtained for endothelium-intact (+EC) and denuded (-EC) control and heartworm femoral artery to determine the effect of heartworm infection on vascular smooth

muscle function. Between-group comparisons (+EC from HW vs. Control) and within-group comparisons (+EC vs. -EC) were made.

(2) Methacholine dose-response relationships: To determine the effect of heartworm infection on endothelium-dependent relaxation, dose-response relationships to methacholine were obtained for endothelium-intact and denuded heartworm and control femoral artery. Between-group comparisons (+EC from HW vs. Control) and within-group comparisons (+EC vs. -EC) were made. Between group comparisons (HW: Spring vs. Fall and Control: Spring vs. Fall) were also made.

(3) Sodium nitroprusside dose-response relationships: To determine the effect of heartworm infection on the vascular smooth muscle guanylate cyclase/cGMP system, dose-response relationships to sodium nitroprusside were examined in the spring for endothelium-intact and denuded rings Between-group comparisons (HW vs. Control) were made. Within-group comparisons (+EC vs. -EC) were also made.

(4) Methacholine dose-response relationships +/- cyclooxygenase inhibitors: Methacholine dose-response relationships were examined with endothelium-intact rings in the presence and absence of mefenamic acid or indomethacin to determine the role of cyclooxygenase products in methacholine relaxation. Experiments were done in fall and spring, in control and heartworm femoral artery. Cyclooxygenase inhibitors were added 30 minutes before norepinephrine preconstriction and were present throughout the experiment. Within-group comparisons were made between control or heartworm femoral artery ± cyclooxygenase inhibitors.

(5) Methacholine dose-response relationships +/-methylene blue: Methacholine dose-response relationships were examined in the presence and absence of methylene blue to determine the importance of the

vascular smooth muscle guanylate cyclase/cGMP system in methacholine relaxation. Methylene blue was added 30 minutes before norepinephrine preconstriction and was present throughout the experiment. Within-group comparisons were made between control or heartworm femoral artery ± methylene blue.

(6) Methacholine dose-response relationships +/-L-NAME: Dose-response relationships were obtained for endothelium-intact rings in the presence and absence of L-NAME to determine the importance of nitric oxide in methacholine relaxation. L-NAME was added to the bath 30 minutes before norepinephrine preconstriction and was present throughout the experiment. Within-group comparisons were made between control or heartworm femoral artery ± L-NAME.

(7) Methacholine dose-response relationships +/-D- and L-arginine: To determine the specificity of L-NAME, the response to D- and L-arginine, was determined at the end of methacholine dose-response experiments in L-NAME-treated and untreated rings. Two minutes after addition of the highest concentration of methacholine, D-arginine was added to the bath. Fifteen minutes after the addition of D-arginine, L-arginine was added. Responses to D- and L-arginine were evaluated at 15 minutes (Kaiser et al., 1991). Between-group comparisons (L-NAME treated rings from HW vs. Control) were made.

B. Femoral vein:

Because the mechanism of relaxation varies with the preconstrictor in femoral vein (see literature review, pg. 17), methacholine relaxation was examined in rings preconstricted with either norepinephrine or $PGF_{2\alpha}$.

PROTOCOL 1-Methacholine dose-response relationships in femoral vein preconstricted with $PGF_{2\alpha}$

Modified length-tension experiments were performed after 30 minutes of equilibration. Strips were stretched to the optimum point on their length-tension curve as determined by measuring the contractile response to $PGF_{2\alpha}$ (0.3 uM) at each level of stretch. Strips were equilibrated for 15 minutes between each level of stretch and contractile response. In the first 6 experiments (3 HW, 3 control), optimum passive tension was 750-1000 mg in heartworm and control rings. This level of passive tension is consistent with that reported in the literature (Miller, 1991; Vidal et al., 1991). Therefore, strips were equilibrated between 750-1000 mg optimum tension in the last 5 experiments (3 control and 2 HW).

(1) $PGF_{2\alpha}$ dose-response relationships: Dose-response relationships to $PGF_{2\alpha}$ were obtained for endothelium-intact strips of control and heartworm femoral vein to determine the effect of heartworm infection on vascular smooth muscle function. Between group comparisons (HW vs. control) were made.

(2) Methacholine dose-response relationships: Dose-response relationships to methacholine were examined in endothelium-intact strips of control and heartworm femoral vein preconstricted with $PGF_{2\alpha}$. Between-group comparisons (HW vs. control) were made.

(3) Methacholine dose-response relationships +/-mefenamic acid: Methacholine dose-response experiments were done on endothelium-intact strips with and without mefenamic acid to determine the role of cyclooxygenase products in methacholine relaxation. Experiments were done in control and heartworm femoral vein. Mefenamic acid was added 30 minutes before $PGF_{2\alpha}$ preconstriction and was present throughout the experiment. Within-group comparisons were made between control or heartworm femoral vein ± mefenamic acid.

(4) Methacholine dose-response relationships +/-methylene blue: Methacholine dose-response experiments were done in the presence and absence of methylene blue to determine the importance of the vascular smooth muscle guanylate cyclase/cGMP system in methacholine relaxation. Methylene blue was added 30 minutes before $PGF_{2\alpha}$ preconstriction and was present throughout the experiment. Within-group comparisons were made between control or heartworm femoral vein ± methylene blue.

(5) Methacholine dose-response relationships +/-L-NAME: Dose-response relationships were obtained for endothelium-intact strips in the presence and absence of L-NAME to determine the importance of nitric oxide in mediating methacholine relaxation. L-NAME was added to the bath 30 minutes before $PGF_{2\alpha}$ preconstriction and was present throughout the experiment. Within-group comparisons were made between control or heartworm femoral vein ± L-NAME.

(6) Methacholine dose-response relationships +/- D- and L-arginine: The response to D- and L-arginine was determined at the end of methacholine dose-response experiments in L-NAME-treated and untreated strips to determine the specificity of L-NAME. Two minutes after addition of the highest concentration of methacholine, D-arginine was added to the bath. Fifteen minutes after addition of D-arginine, L-arginine was added to the bath. Responses to D- and L-arginine were evaluated at 15 minutes (Kaiser et al., 1991). Between-group comparisons (L-NAME treated rings from HW vs. Control) were made.

PROTOCOL 2-Methacholine dose-response relationships in femoral vein preconstricted with norepinephrine

(1) Norepinephrine dose-response relationships: Dose-response relationships to norepinephrine were obtained for

endothelium-intact strips of control and heartworm femoral vein to determine the effect of heartworm infection on vascular smooth muscle function. Between-group comparisons (HW vs. control) were made.

(2) Methacholine dose-response relationships: Dose-response relationships to methacholine were examined in endothelium-intact strips of control and heartworm femoral vein to determine the effect of heartworm infection on endothelium-dependent relaxation. Between-group comparisons (HW vs. control) were made.

(3) Methacholine dose-response relationships +/- L-NAME: Dose-response relationships to methacholine were examined in endothelium-intact strips of control and heartworm femoral vein in the presence and absence of L-NAME to determine the importance of NO in methacholine relaxation. L-NAME was added to the bath 30 minutes before norepinephrine preconstriction and was present throughout the experiment. Within-group comparisons were made in control and heartworm femoral vein +/-L-NAME.

C. Bovine mesenteric lymphatics

Rings were equilibrated until spontaneous contractions began (15-60 minutes). Rings which failed to contract spontaneously within 60 minutes were not used. Optimum passive tension was determined by measuring the amplitude of spontaneous contractions at different levels of passive stretch (250-750 mg). Rings were equilibrated at optimum passive tension until contractions became regular in frequency and amplitude (30-60 minutes).

PROTOCOL 1: The effect of endothelial cell removal on spontaneous lymphatic contractions.

(1) Endothelial cell removal: Frequency and amplitude of spontaneous contractions were measured before and after removal of

endothelial cells to determine if the endothelium is responsible for phasic contractile activity of bovine mesenteric lymphatics. Endothelial cells were removed mechanically by everting the ring and rubbing the intimal surface with moistened filter paper (Ohhashi & Takahashi, 1991) or chemically with collagenase (Furchgott & Zawadzki, 1980). When endothelial cells were removed mechanically, three rings were used, a ring from which endothelial cells were removed (treated) and two endothelium-intact rings, a time control and a space control. The time control remained in the bath for the duration of the experiment to determine the effect of time on phasic contractions. The space control was removed from the bath for the same time period as the treated ring to rule out an effect on phasic contractions of removing and resuspending the rings. When endothelial cells were removed chemically, collagenase was added to the bath for 30 minutes and then removed by washing the ring with warm PSS. An untreated ring served as time control. Amplitude and frequency of contraction were compared between endothelium-intact and endothelium-denuded rings before and after removal of endothelial cells. Contractile responses to norepinephrine were measured before and after endothelial cell removal to determine whether collagenase or mechanical removal of endothelial cells damaged smooth muscle.

(2) Endothelium-intact/endothelium-denuded sandwich: A "sandwich" preparation similar to that described by Furchgott and Zawadzki was used to determine if an endothelium-derived diffusible substance is responsible for spontaneous contractile activity (for review of "sandwich" see lit. review pg. 1). Instead of closely apposing an endothelium-denuded transverse strip with an endothelium-intact longitudinal strip, however, two rings (an endothelium-denuded detector and an endothelium-intact donor ring) were suspended in the same organ chamber in close contact without

touching. This modification was necessary because bovine mesenteric lymphatics contain both longitudinal and circular smooth muscle (Ohhashi et al., 1977). Since both layers of smooth muscle exhibit spontaneous contractile activity, it is impossible to obtain an endothelium-intact preparation which is not spontaneously active. Therefore, by placing the rings close to each other without touching, spontaneous activity in donor rings would not affect activity in detector rings. After "sandwiching" spontaneous contractions were monitored in both rings for 30 minutes. One ring (either donor or detector) was then removed and resuspended in a separate organ chamber, the baths washed and spontaneous contractions again monitored in both rings. Contraction amplitude and frequency were measured in donor and detector rings before, during, and after sandwiching. Contraction frequency was compared within groups (before vs. during "sandwiching" and before vs. after "sandwiching") and between groups (detector vs. donor). Similar comparisons were made for contraction amplitude. The time for contractions to begin during "sandwiching" and the time for contractions to end after "sandwiching" were measured in detector rings. At the end of some experiments, donor and detector rings were placed in 10% formalin for histopathological evaluation. In other experiments, after spontaneous contractions reoccurred in the donor ring, its endothelial cells were removed and it was used in an endothelium-denuded/endothelium-denuded sandwich.

(3) Endothelium-denuded/endothelium-denuded sandwich: Spontaneous contractions were evaluated in endothelium-denuded detector rings that were sandwiched with endothelium-denuded donor rings. At the end of some experiments, rings were placed in 10% formalin for histopathological evaluation.

PROTOCOL 2: The effect of cyclooxygenase inhibitors on spontaneous contractile activity.

To determine whether cyclooxygenase products mediate phasic contractile activity of bovine mesenteric lymphatics, spontaneous lymphatic contractions were evaluated in preliminary experiments, in the presence and absence of indomethacin, aspirin and mefenamic acid. Contractile activity was evaluated immediately after addition of the inhibitor.

PROTOCOL 3: The effect of methylene blue on spontaneous contractile activity.

Spontaneous contractions were evaluated in preliminary experiments in the presence and absence of methylene blue to determine whether smooth muscle guanylate cyclase/cGMP plays an important role in phasic contractile activity of bovine mesenteric lymphatics. Contractile activity was evaluated immediately after addition of the inhibitor.

PROTOCOL 4: The effect of L-NAME on spontaneous contractile activity.

Spontaneous contractions were evaluated in preliminary experiments in the presence and absence of L-NAME to determine whether nitric oxide mediates phasic contractile activity of bovine mesenteric lymphatics. Contractile activity was evaluated immediately after addition of the inhibitor.

PROTOCOL 5: The effect of D- and L-arginine on spontaneous contractile activity

The response to D- or L-arginine was assessed to determine whether the effect of L-NAME on phasic lymphatic contractions was due to a specific effect on NO synthesis. D- or L-arginine was added 30 minutes after treatment with L-NAME and the effects evaluated after 15 minutes. The effect of L-arginine was also determined in control rings in the absence of L-NAME.

PROTOCOL 6: The effect of heartworm-conditioned medium on spontaneous contractile activity.

Spontaneous contractile activity was evaluated in preliminary experiments in the presence of heartworm-conditioned or control medium to determine the effect of filarial factors on phasic lymphatic contractions.

PROTOCOL 7: The effect of prostaglandin D_2 on spontaneous contractile activity.

Previous experiments suggest that filarial prostaglandin D_2 may be partly responsible for depression of endothelium-dependent relaxation in the in vitro rat aorta (Kaiser et al., 1992). If filarial PGD_2 mediates the effect of heartworm-conditioned medium on spontaneous contractile activity, then this effect should be mimicked by exogenous PGD_2. Therefore, in preliminary experiments, dose-response relationships to PGD_2 were obtained. Since PGD_2 at a concentration of 10^{-12} depresses acetylcholine relaxation of in vitro rat aorta, concentrations chosen for this study ranged from 3×10^{-12} to 3×10^{-5}M.

D. Thoracic duct

(1) Effect of heartworm-conditioned medium on relaxation of isolated thoracic duct from control dogs

Experiments were performed with the in vitro thoracic duct from control dogs in the presence of control or heartworm-conditioned medium to determine whether filarial factors depress endothelium-dependent relaxation. Rings were equilibrated for 90 minutes at a passive tension of 500 mg which is optimum for canine thoracic duct (Ohhashi & Takahashi, 1991). The rings were preconstricted with norepinephrine (10 uM) and the relaxation response to a single dose of acetylcholine (2 uM) determined. Rings which failed to constrict to norepinephrine (1 HW and 1 Control) or to relax to acetylcholine (0 rings) were not used. Acetylcholine relaxation was compared between rings (HW vs.

Control) before exposure to heartworm-conditioned or control medium to insure that endothelium-dependent relaxation was similar in the two groups. Rings were washed, re-equilibrated for 45 minutes and PSS removed from the organ chamber and replaced with control or heartworm-conditioned media. Rings were preconstricted with norepinephrine (10 uM) and dose-response relationships to methacholine or sodium nitroprusside obtained.

(1) Norepinephrine constriction responses: The constriction response to norepinephrine was compared in each ring before and after addition of control or heartworm-conditioned medium to determine if heartworm-conditioned medium affects lymphatic smooth muscle function. Within-group comparisons (before vs. after addition of conditioned media) and between-group comparisons (HW-conditioned vs. control medium) were made.

(2) Methacholine dose-response relationships: Dose-response relationships to methacholine (10^{-10} to 3×10^{-6}M) were obtained for rings of thoracic duct from control dogs in the presence of heartworm-conditioned or control medium. Between-group comparisons (HW-conditioned vs. control media) were made.

(3) Sodium nitroprusside dose-response relationships: Dose-response relationships to the endothelium-independent agent, sodium nitroprusside (10^{-9} to 3×10^{-6} M) were examined to determine the effect of heartworm-conditioned medium on lymphatic smooth muscle guanylate cyclase/cGMP function. Between-group comparisons (HW-conditioned vs. control media) were made.

(2) Effect of previous in vivo exposure to <u>D. immitis</u> on endothelium-dependent relaxation of the in vitro canine thoracic duct.

Experiments were done in thoracic duct from control and heartworm infected dogs to determine if heartworm infection causes depression of endothelium-dependent relaxation in vitro. Rings were equilibrated at optimum tension (500 mg) for 30 minutes, preconstricted with norepinephrine (10 uM) and relaxed with acetylcholine (2 uM). Rings which failed to constrict to norepinephrine were omitted (3 HW and 1 control).

(1) Methacholine dose-response relationships:

Dose-response relationships to methacholine were obtained for endothelium-intact rings of control and heartworm thoracic duct. Between group comparisons (HW vs. control) were made.

(2) Methacholine dose-response relationships +/- mefenamic acid: Methacholine dose-response relationships were obtained for endothelium-intact rings in the presence and absence of mefenamic acid to determine the role of cyclooxygenase products in mediating methacholine relaxation. Experiments were done in control and heartworm thoracic duct. Mefenamic acid was added 30 minutes before norepinephrine preconstriction and was present throughout the experiment. Within-group comparisons were made between control or heartworm thoracic duct ± cyclooxygenase inhibitors.

(3) Methacholine dose-response relationships +/- methylene blue: Methacholine dose-response relationships were constructed for endothelium-intact rings in the presence and absence of methylene blue to determine the importance of the vascular smooth muscle guanylate cyclase/cGMP system in methacholine relaxation. Methylene blue was added 30 minutes before norepinephrine preconstriction and was present throughout the experiment. Within-group comparisons were made between control or heartworm thoracic duct ± methylene blue.

(4) Methacholine dose-response relationships +/- L-NAME: Dose-response relationships were established for endothelium-intact rings in the presence and absence of L-NAME to determine the importance of NO in mediating methacholine relaxation. L-NAME was added to the bath 30 minutes before norepinephrine preconstriction and was present throughout the experiment. Within-group comparisons were made between heartworm or control thoracic duct ± L-NAME.

(5) Methacholine dose-response relationships +/-D- and L-arginine: To determine the specificity of L-NAME, the response to D- and L-arginine was determined at the end of methacholine dose-response experiments in L-NAME-treated and untreated rings. Two minutes after addition of the highest concentration of methacholine, D-arginine was added to the bath. Fifteen minutes after addition of D-arginine, L-arginine was added. Responses to D- and L-arginine were evaluated at 15 minutes (Kaiser et al., 1991). Between-group comparisons (L-NAME treated rings from HW vs. control) were made.

V. ANALYSIS

A. Statistical Analysis:

Data are expressed as mean ± standard error of the mean (SEM), with $p<0.05$ as the criterion of statistical significance. Ring weights are expressed in grams. Constriction responses are expressed as grams of active tension. Relaxation responses are expressed as percent, with norepinephrine preconstriction taken as 0% relaxation. Ring weights and all dose-response relationships were analyzed by one way analysis of variance (ANOVA), and least significant difference (LSD). The response to D- or L-arginine in the presence of methacholine was expressed in percent, with 0% indicating no response and

100% indicating complete relaxation. Within group (treated vs untreated) and between group (HW vs. control) comparisons of the response to single concentrations of a drug were made using ANOVA with LSD unless otherwise noted.

(1) Femoral artery: Norepinephrine, methacholine and sodium nitroprusside dose-responses were compared between groups (HW vs. Control) and within groups (+EC vs. -EC). Methacholine dose-responses, in the presence and absence of inhibitors, were compared within groups (treated vs. untreated).

(2) Femoral vein: Norepinephrine, Prostaglandin $F_{2\alpha}$ and methacholine dose-responses were compared between groups (HW vs. Control). Methacholine dose-responses, in the presence and absence of inhibitors, were compared within groups (treated vs. untreated).

(3) Bovine mesenteric lymphatics: The contractile response to norepinephrine was compared within groups (same ring before and after endothelial cell removal) using a paired t-test and between groups (+EC vs. -EC) using ANOVA with LSD. Contraction frequency was measured by determining the number of contractions/minute over a 10 minute period and contraction amplitude was the peak contraction that occurred during the same period. Contraction frequency and amplitude were compared between groups (+EC vs. -EC) and within groups (before vs. during and before vs. after sandwiching) using ANOVA with LSD.

(4) Thoracic duct

(a) Effects of heartworm-conditioned medium on relaxation of isolated thoracic duct: Sodium nitroprusside and methacholine dose-responses were compared between groups (HW vs. control medium). Norepinephrine constriction was compared within groups (before

vs. after treatment with conditioned medium) and between groups (HW vs. control medium). The relaxation response to acetylcholine was assessed before addition of control or heartworm-conditioned media and was compared between groups (HW vs. control medium). Within group comparisons were analyzed using a paired t-test; between group comparisons were analyzed using ANOVA with LSD.

(b) Effect of previous in vivo exposure to D. immitis on endothelium-dependent relaxation of the in vitro canine thoracic duct: Norepinephrine and methacholine dose-response relationships were compared between groups (HW vs. control). Methacholine dose-responses, in the presence and absence of inhibitors, were compared within groups (treated vs. untreated).

B. Histopathology: An accurate method to identify endothelial cells is to detect the presence of factor VIII related antigen using immunohistochemical techniques (Yong & Jones, 1989). This procedure was used to determine the presence or absence of endothelial cells in rings of bovine mesenteric lymphatics. Endothelium-denuded and endothelium-intact rings were fixed with 10% buffered formalin and embedded in paraffin. After enzyme digestion the sections were rinsed in buffer and placed in 3% hydrogen peroxide for 5 minutes. Rabbit anti-human Factor VIII antibody was diluted 1:200 in buffered saline and incubated on sections for 30 minutes at room temperature. Samples were washed several times then a biotinylated goat-anti-rabbit antibody was added for a further 10 minutes incubation and rinsed with buffered saline. Avidin-biotinylated horseradish peroxidase complex was applied and incubated for 30 minutes. The sections were washed several times, stained with AEC

chromagen and examined under light microscopy in a blind fashion for the presence of endothelial cells.

Sections were ranked from 1 through 5 with rankings of 4 or 5 representing an intact endothelial cell layer and rankings of 1,2 or 3 representing the absence of endothelial cells.

VI. Drugs and Chemicals: Acetylcholine chloride, acetyl-β-methylcholine chloride (methacholine), arterenol (bitartrate salt), ascorbate, aspirin, Type I collagenase, N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), L-arginine hydrochloride, D-arginine hydrochloride, indomethacin, mefenamic acid and sodium nitroprusside were obtained from Sigma Chemical Co. (St. Louis, MO). Methylene blue was obtained from Merck Chemical Co. (Rahway, NJ). $PGF_{2\alpha}$ and PGD_2 were obtained from Caymen Chemical (Ann Arbor, MI). Cyclooxygenase inhibitors were mixed 1:4 by weight with sodium carbonate in distilled water. Methylene blue and sodium nitroprusside were mixed in distilled water. $PGF_{2\alpha}$ and PGD_2 were mixed in ethanol and diluted in ascorbate. All other drugs were mixed and diluted in ascorbate. Drugs were mixed fresh daily and stored in the refrigerator until use.

RESULTS

A. FEMORAL ARTERY

(1) Norepinephrine dose-response relationships: Norepinephrine caused a dose-dependent constriction in all groups studied. Norepinephrine constriction was not significantly different in endothelium-intact rings from heartworm infected dogs when compared to control in fall (Figure 1) or spring (HW: n=7; Control: n=9). Endothelial cell removal did not significantly alter dose-responses in either group in fall (Figure 1) or spring (HW: n=3; Control: n=4).

(2) Methacholine dose-response relationships: Methacholine caused a dose-dependent relaxation in all endothelium-intact rings. Methacholine relaxation was not different in heartworm femoral artery when compared to control in either fall or spring (Figure 2A and 2B). However, methacholine relaxation was significantly depressed in femoral artery from heartworm infected dogs studied in spring when compared to responses obtained in fall (Figure 2C). A similar seasonal depression of relaxation was seen in femoral artery from control dogs studied in spring when compared to fall (Figure 2C). Removal of endothelial cells abolished methacholine relaxation in all groups (fall: HW n=8; control n=7; spring: HW n= 4; control n=3).

(3) Sodium nitroprusside dose-response relationships: Relaxation to sodium nitroprusside was significantly enhanced by removal of endothelial cells in femoral artery from heartworm infected and control dogs (Figure 3). Sodium nitroprusside relaxation was not depressed in endothelium-intact rings from heartworm infected dogs when compared to control, nor were there differences in relaxation of endothelium-denuded rings from heartworm infected and control dogs (Figure 3).

(4) Methacholine dose-response relationships +/- cyclooxygenase inhibitors: In femoral artery from control dogs, methacholine relaxation was not altered by mefenamic acid (Figure 4A) or indomethacin in either fall or spring. Neither mefenamic acid (Figure 4B) nor indomethacin (Figure 4C) depressed methacholine relaxation in femoral artery from heartworm infected dogs in either fall or spring.

(5) Methacholine dose-response relationships +/- methylene blue: In fall and spring, methylene blue significantly depressed methacholine relaxation in femoral artery from control (Figure 5A) and heartworm infected dogs (Figure 5B).

6) Methacholine dose-response relationships +/- L-NAME: In fall and spring, L-NAME significantly depressed methacholine relaxation in femoral artery from control (Figure 6A) and heartworm infected dogs (Figure 6B).

(7) The effect of D- and L-arginine on methacholine response in L-NAME-treated rings: In the presence of methacholine, L-arginine, but not D-arginine, caused significant relaxation in L-NAME treated rings from all groups. L-arginine relaxation was not significantly different in L-NAME treated rings from heartworm infected dogs when compared to control in fall (HW=21.7 ± 6.5%; n=8 vs. control=29.4 ± 8.7%; n=7; $p>0.05$) or spring (HW=13.8 ± 4.1%; n=6 vs control=17.8 ± 6.9%; n=6; $p>0.05$).

FIGURE 1

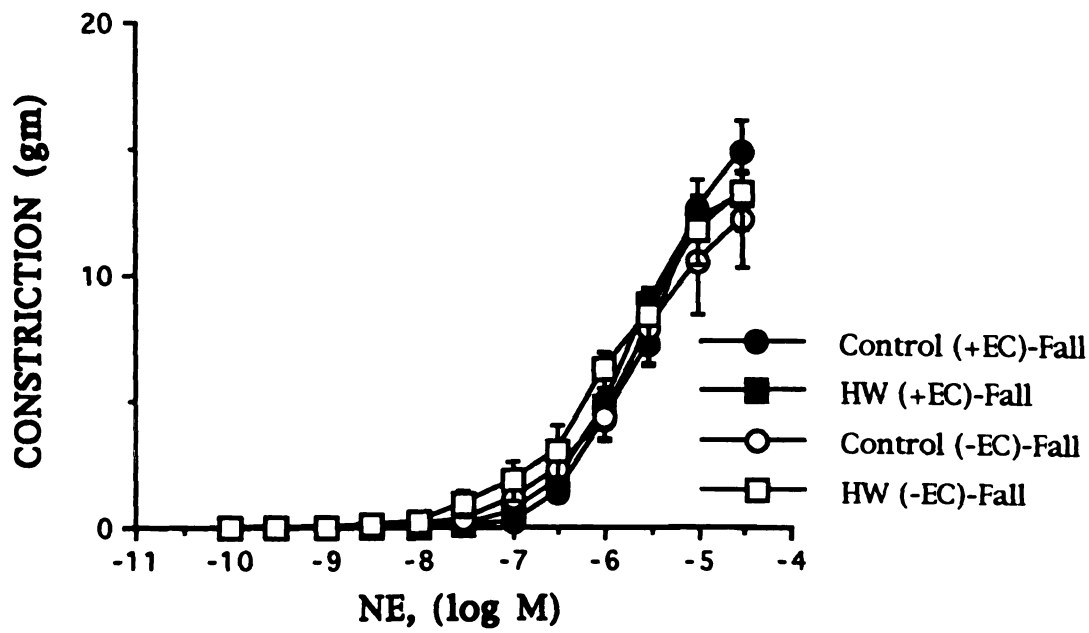

FIGURE 1: Norepinephrine constriction in rings of femoral artery from heartworm infected (■; n=8) and control dogs (●; n=7) studied in fall. Norepinephrine constriction was not significantly different in rings from heartworm infected dogs (HW) when compared to control. Removal of endothelial cells did not alter norepinephrine constriction in femoral artery from heartworm infected (□; n=8) or control dogs (○; n=6)

FIGURE 2A: FALL

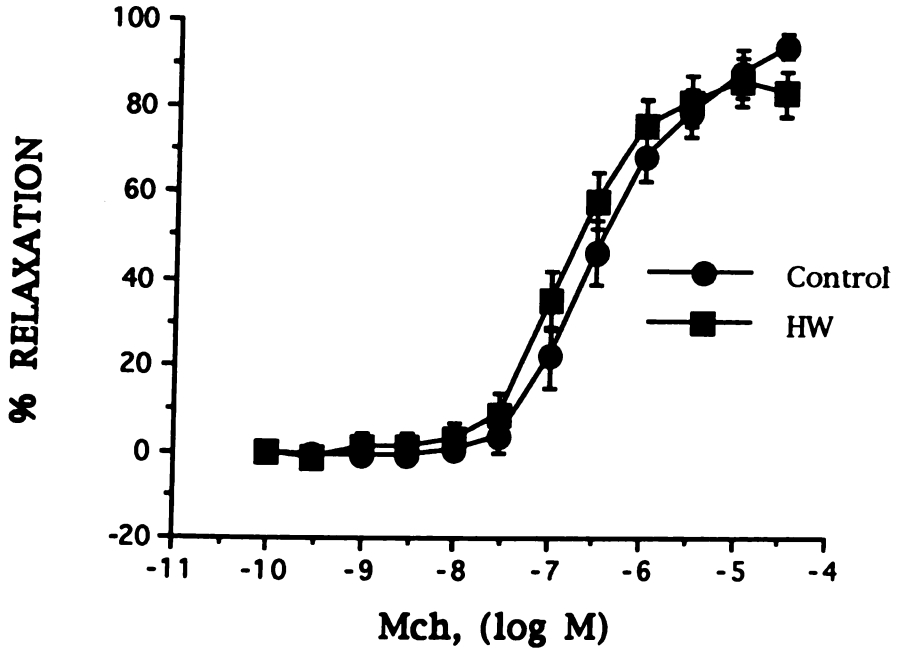

FIGURE 2A: Methacholine (Mch) relaxation in rings of femoral artery from control and heartworm infected dogs (HW) studied in fall. Methacholine relaxation was not significantly different in femoral artery from heartworm infected dogs (■; n=9) when compared to control (●; n=7).

FIGURE 2B: SPRING

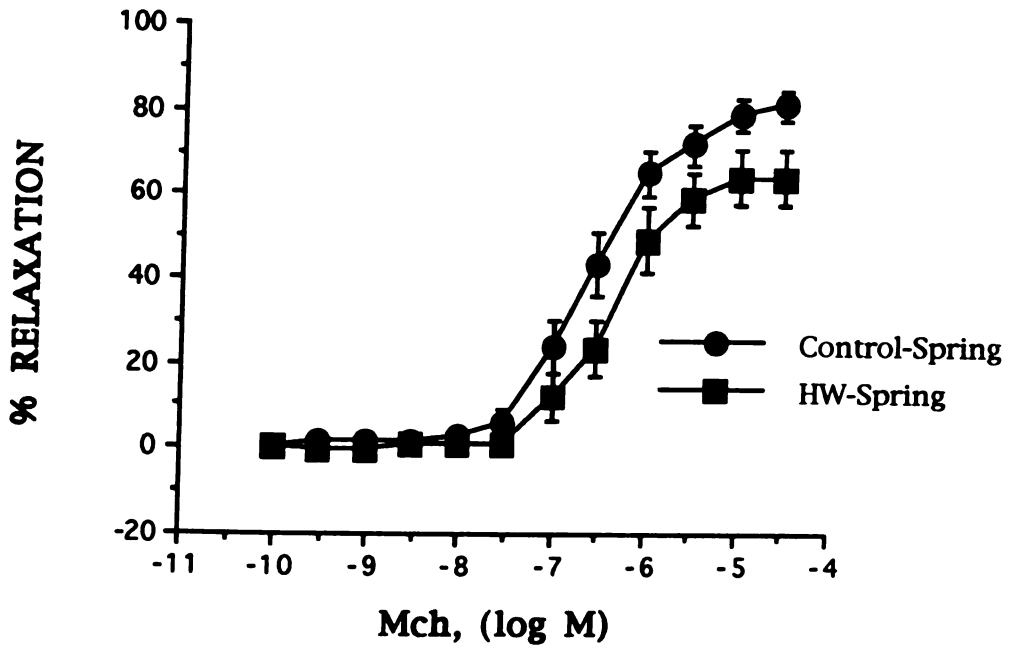

FIGURE 2B: Methacholine (Mch) relaxation in rings of femoral artery from control and heartworm infected dogs (HW) studied in spring. Methacholine relaxation was not significantly different in femoral artery from heartworm infected dogs (■; n=7) when compared to control (●; n=9).

FIGURE 2C: SPRING VS. FALL

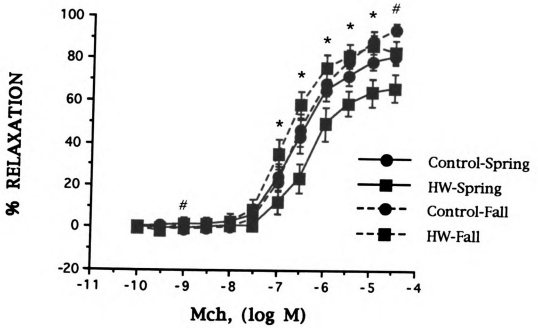

FIGURE 2C: Methacholine (Mch) relaxation in femoral artery from control (●) and heartworm infected dogs (HW; ■) studied in fall (broken lines) and spring (solid lines). Methacholine relaxation is depressed at two concentrations of methacholine in femoral artery from control dogs studied in the spring (#) when compared to responses obtained in fall. Methacholine relaxation is also depressed in femoral artery from heartworm infected dogs studied in spring (*) when compared to fall; $p<0.05$.

FIGURE 3

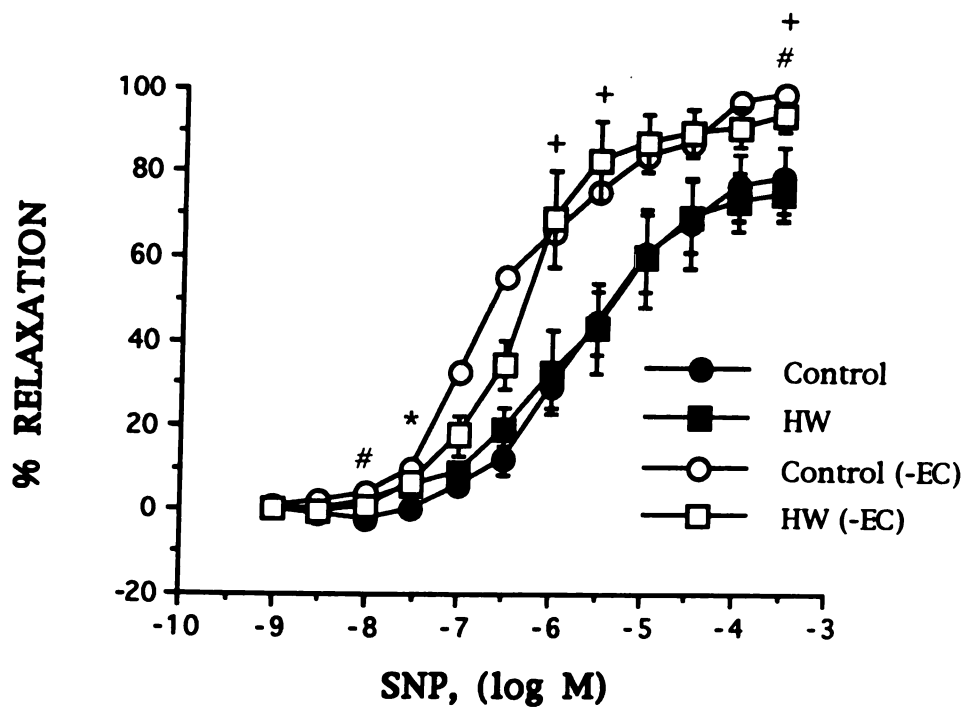

FIGURE 3: Dose-response relationships to sodium nitroprusside (SNP) in endothelium-intact (closed symbols) and endothelium-denuded (open symbols) rings of femoral artery from control (●; n=4) and heartworm infected dogs (HW; ■; n=5). Endothelium-denuded rings from control (#) and heartworm infected dogs (+) relaxed significantly more than endothelium-intact rings; $p<0.05$. Relaxation was not depressed, and in fact was enhanced at one concentration of SNP in endothelium-intact rings of heartworm femoral artery (*) when compared to endothelium-intact control rings; $p<0.05$. Relaxation was not different in endothelium-denuded rings from heartworm (n=4) and control dogs (n=4). Error bars are omitted from control(-EC) for clarity.

FIGURE 4A

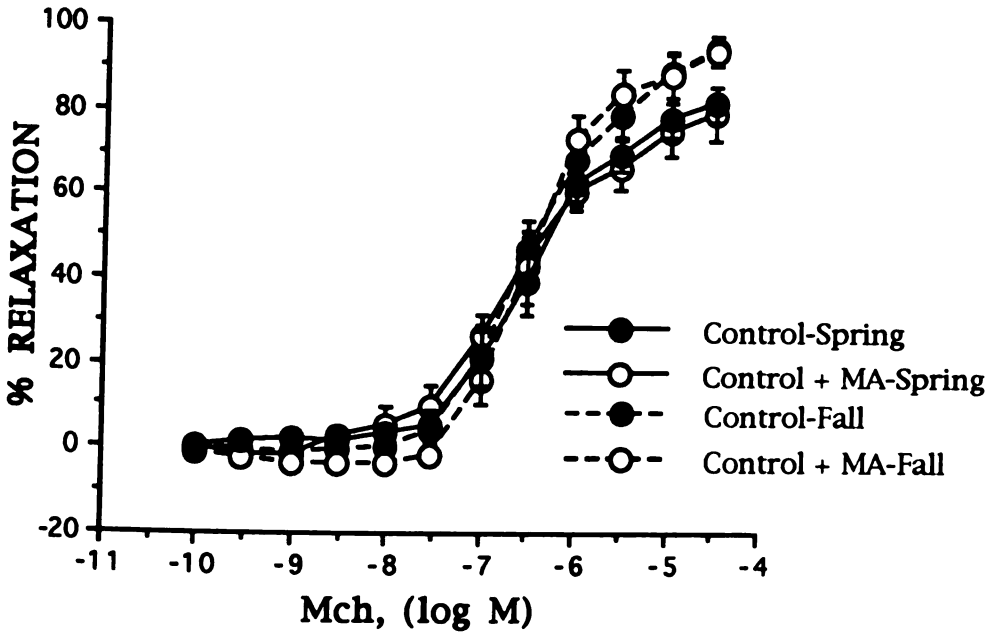

FIGURE 4A: Methacholine (Mch) relaxation in paired rings (n=7) of femoral artery from control dogs studied in fall (broken line) and spring (solid line), in the presence (O) and absence (●) of mefenamic acid (MA; 10^{-5} M). Mefenamic acid did not significantly alter methacholine relaxation.

FIGURE 4B

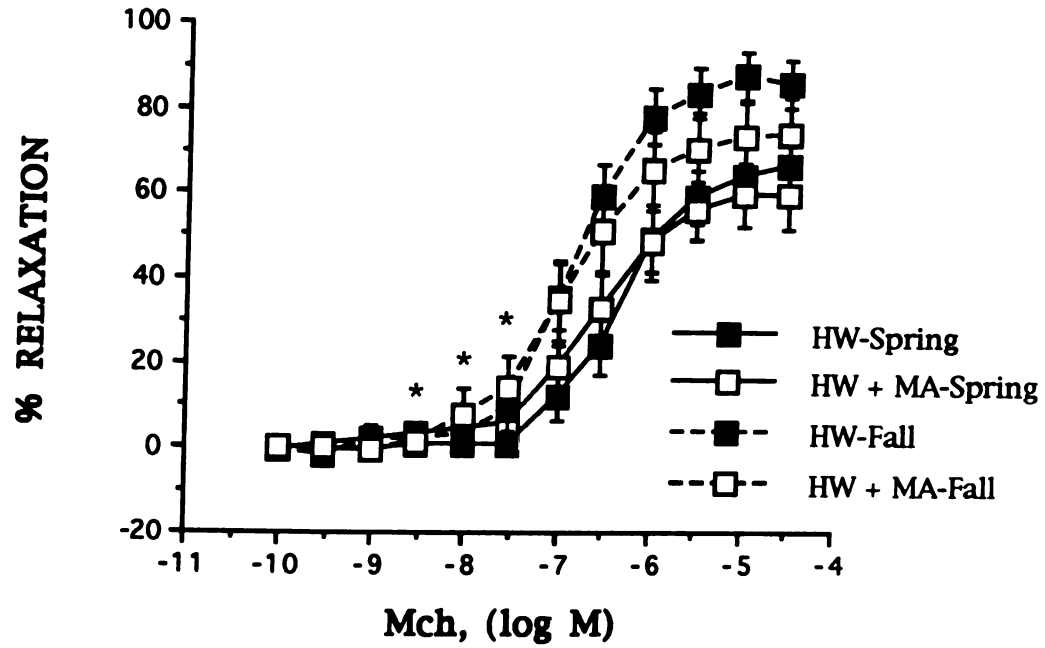

FIGURE 4B: Methacholine relaxation in paired rings of femoral artery from heartworm infected dogs (HW) studied in fall (broken line; n=8) and spring (solid line; n=7), in the presence (❑) and absence (■) of mefenamic acid (10^{-5} M). Mefenamic acid did not depress, and in fact, enhanced relaxation at three concentrations of methacholine in spring (*), but not fall; $p<0.05$.

FIGURE 4C

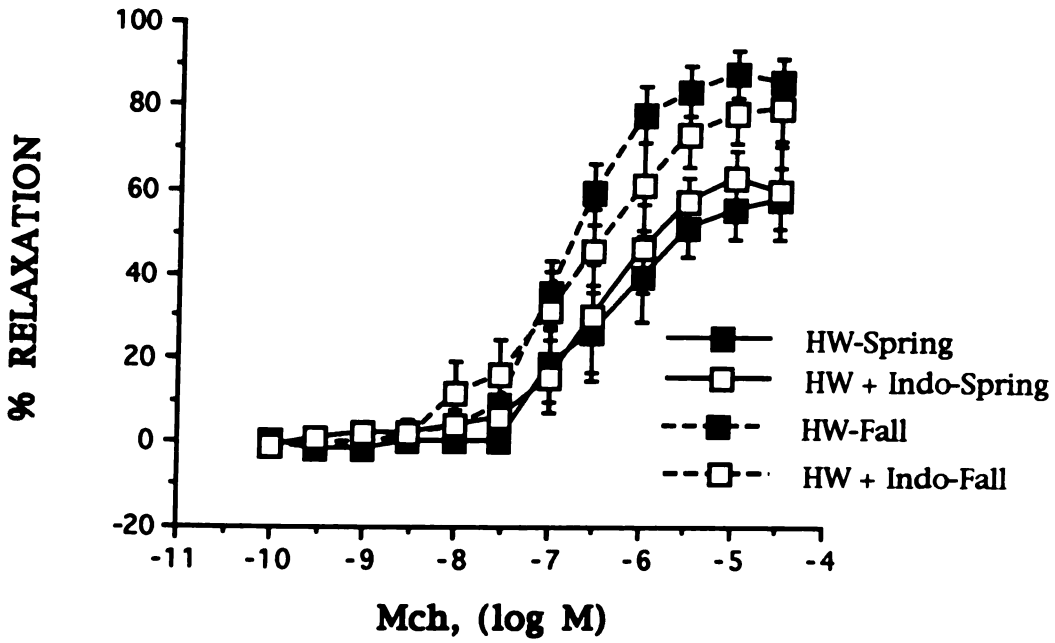

Figure 4C: Methacholine relaxation in paired rings of femoral artery from heartworm (HW) infected dogs studied in fall (broken lines; n=7) and spring (solid lines; n=4) in the presence (□) and absence (■) of indomethacin (Indo; 10^{-5} M). Indomethacin did not alter methacholine relaxation in fall or spring.

FIGURE 5A

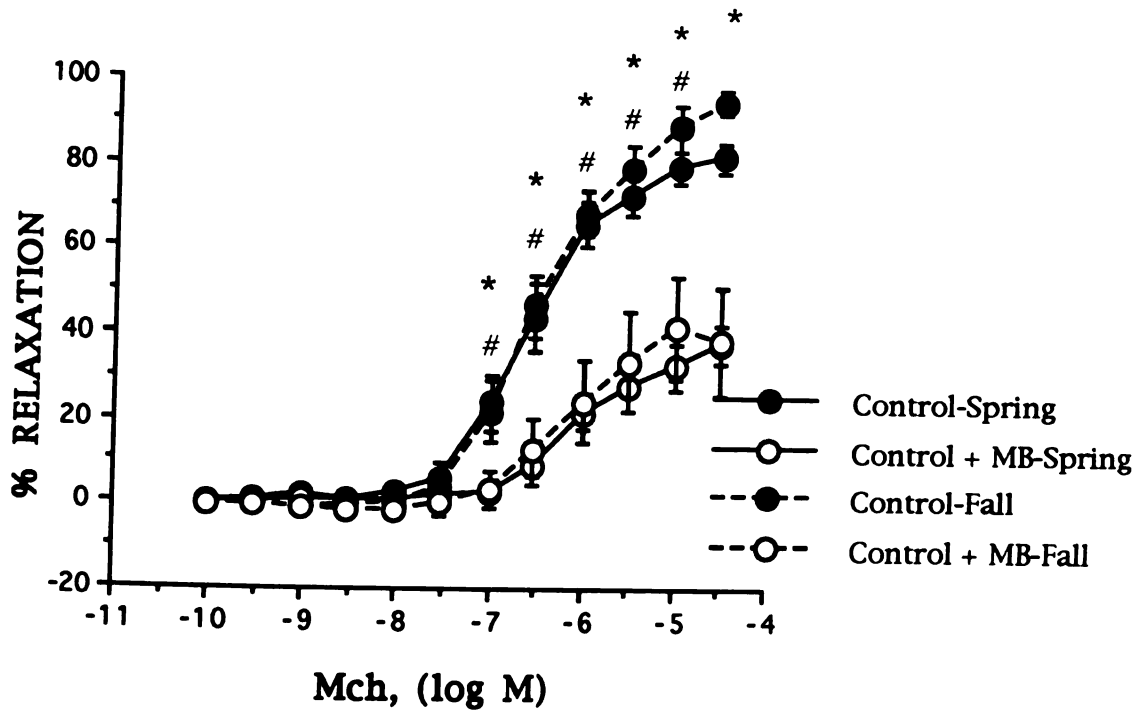

FIGURE 5A: Methacholine relaxation in paired rings of femoral artery from control dogs studied in fall (broken lines; n=4) and spring (solid lines; n=9) in the presence (❑) and absence (■) of methylene blue (MB; 10^{-5} M). Methylene blue significantly depressed methacholine relaxation in fall (#) and spring (*); $p<0.05$.

FIGURE 5B

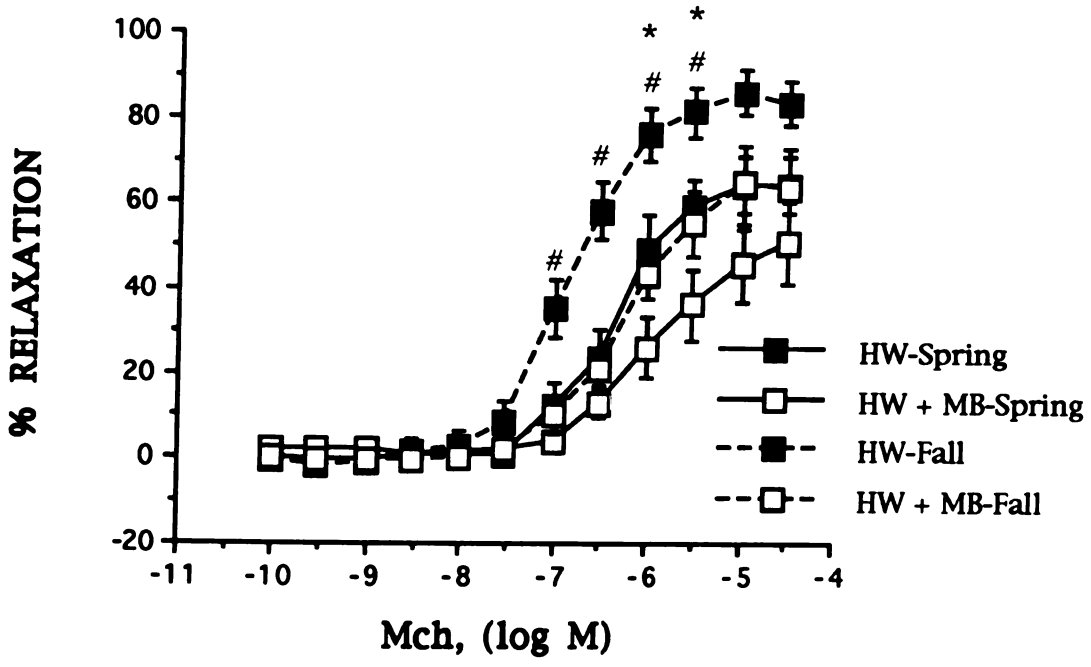

FIGURE 5B: Methacholine relaxation in paired rings of femoral artery from heartworm infected dogs (HW) studied in fall (broken lines; n=9) and spring (solid lines; n=7) in the presence (□) and absence (■) of methylene blue (MB; 10^{-5} M). Methylene blue significantly depressed methacholine relaxation in fall (#) and spring (*); $p<0.05$.

FIGURE 6A

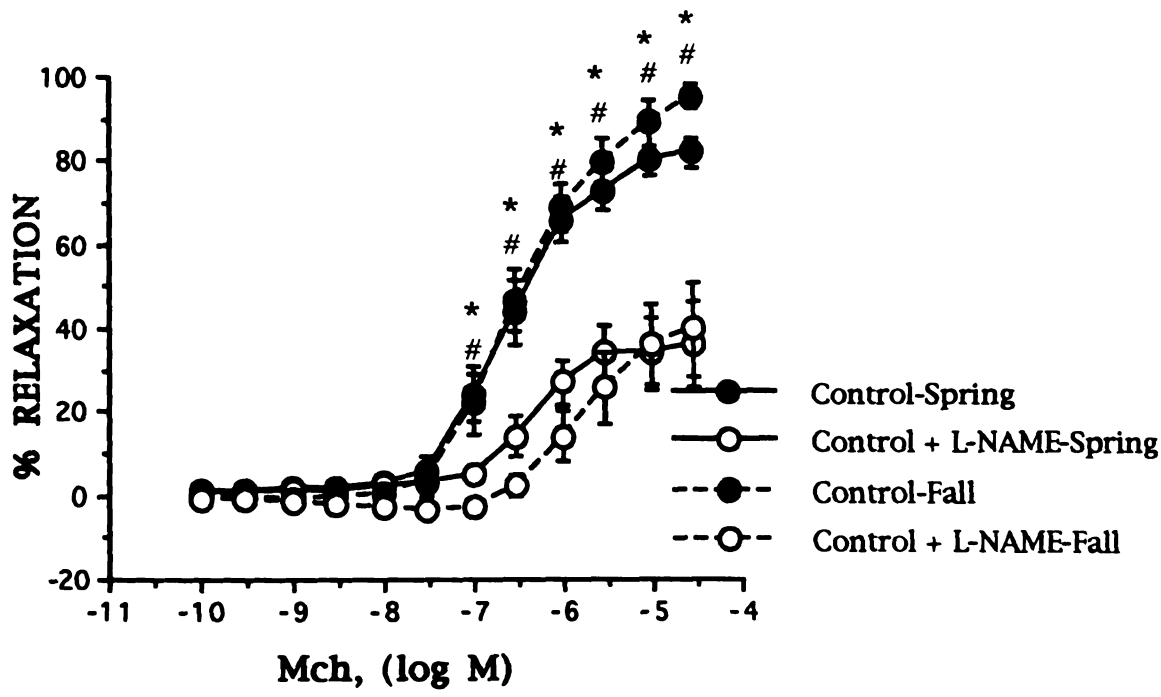

FIGURE 6A: Methacholine (Mch) relaxation in paired rings of femoral artery from control dogs studied in fall (broken lines; n=7) and spring (solid lines; n=8) in the presence (○) and absence (●) of N-nitro-L-arginine methyl ester (L-NAME; 1.5×10^{-5} M). L-NAME significantly depressed methacholine relaxation both in fall (#) and spring (*); $p<0.05$.

FIGURE 6B

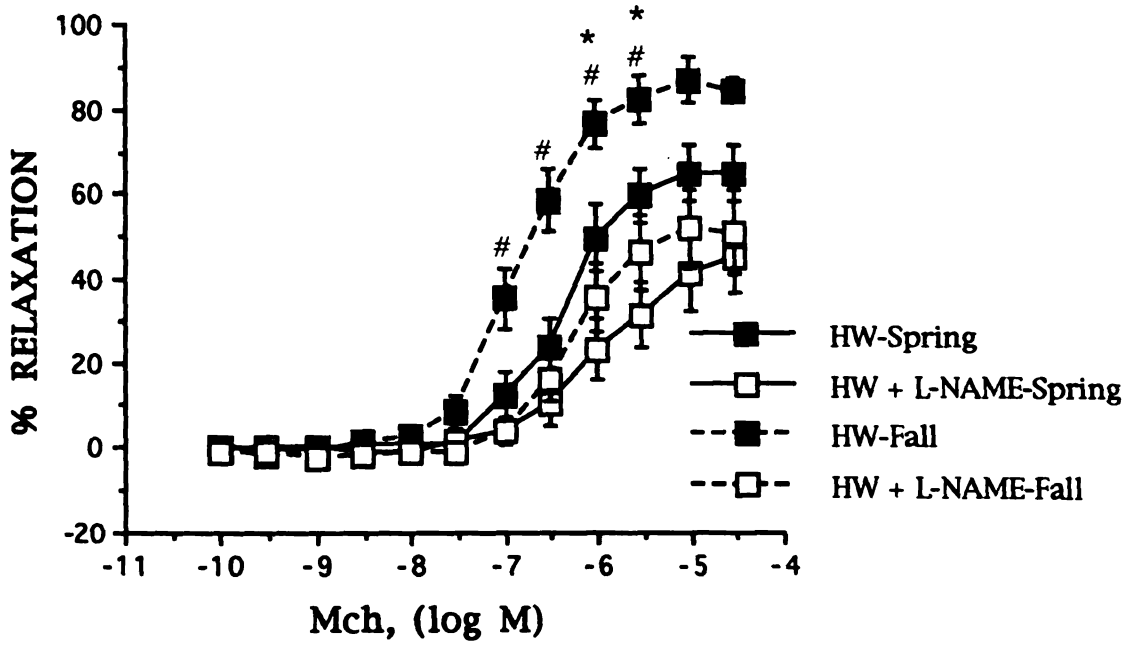

FIGURE 6B: Methacholine relaxation in paired rings of femoral artery from heartworm infected dogs (HW) studied in fall (broken lines; n=9) and spring (solid lines; n=7) in the presence (□) and absence (■) of N-nitro-L-arginine methyl ester (L-NAME; 1.5 x 10^{-5} M). L-NAME significantly depressed methacholine relaxation in fall (#) and spring (*); $p<0.05$.

B. FEMORAL VEIN-PRELIMINARY DATA

(1) Norepinephrine and $PGF_{2\alpha}$ dose-response relationships: Norepinephrine and $PGF_{2\alpha}$ caused a dose-dependent constriction response in heartworm and control femoral vein. Norepinephrine constriction in heartworm femoral vein was not significantly different from control (Figure 7). Maximum $PGF_{2\alpha}$ constriction was 400 mg for heartworm (n=1) and 600 mg for control femoral vein (n=1).

(2) Methacholine dose-response relationships: Methacholine caused a dose-dependent relaxation response in all endothelium-intact strips of femoral vein. Methacholine relaxation was not different in heartworm femoral vein when compared to control in strips preconstricted with norepinephrine (Figure 8) or $PGF_{2\alpha}$ (Figure 9).

(3) Methacholine dose-response relationships +/- mefenamic acid: In preliminary studies, when either $PGF_{2\alpha}$ (Figure 10) or norepinephrine (HW: n=2; Control: n=2) was used as the preconstricting agent, mefenamic acid did not significantly alter methacholine relaxation in femoral vein from either control or heartworm infected dogs.

(4) Methacholine dose-response relationships +/- methylene blue: Methylene blue depressed methacholine relaxation in femoral vein from control and heartworm infected dogs preconstricted with $PGF_{2\alpha}$. (Figure 11).

(5) Methacholine dose-response relationships +/- L-NAME: In preliminary studies, L-NAME almost abolished the relaxation response to methacholine in femoral vein from control dogs when $PGF_{2\alpha}$ was the preconstricting agent (Figure 12). However, in control strips preconstricted with norepinephrine, methacholine relaxation was not depressed by L-NAME (maximum relaxation: Control=65% n=2 and L-NAME=82% n=2). In heartworm femoral vein, methacholine relaxation with and without L-NAME was not significantly different in strips preconstricted with $PGF_{2\alpha}$ (Figure 12) or norepinephrine(n=1).

(6) Methacholine dose-response relationships in L-NAME-treated strips +/- D- and L-arginine: L-arginine (0.6 mM and 1.8 mM) partially reversed L-NAME-induced depression of relaxation in control (15% at 0.6mM; n=1 and 17% at 1.8 mM; n=1) and heartworm infected dogs (5% at 0.6 mM; n=1 and 50% at 1.8mM; n=1). D-arginine did not cause relaxation in either group at any dose.

FIGURE 7

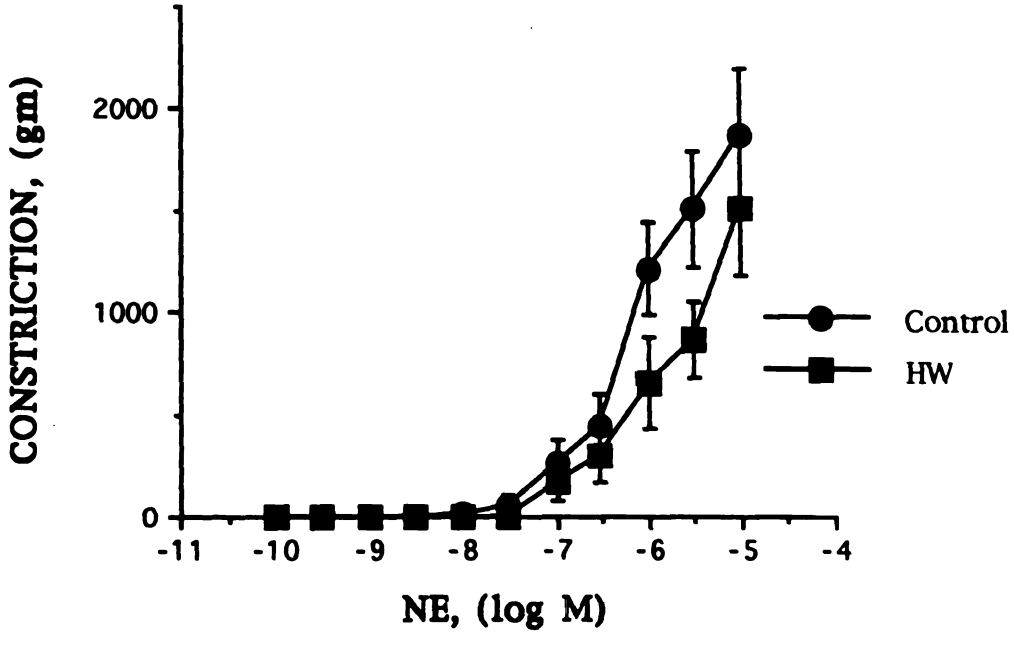

FIGURE 7: Norepinephrine constriction in strips of femoral vein from heartworm infected (HW; ■; n=3) and control dogs (●; n=3) studied in spring. Norepinephrine constriction was not significantly different in femoral vein from heartworm infected dogs when compared to control.

FIGURE 8

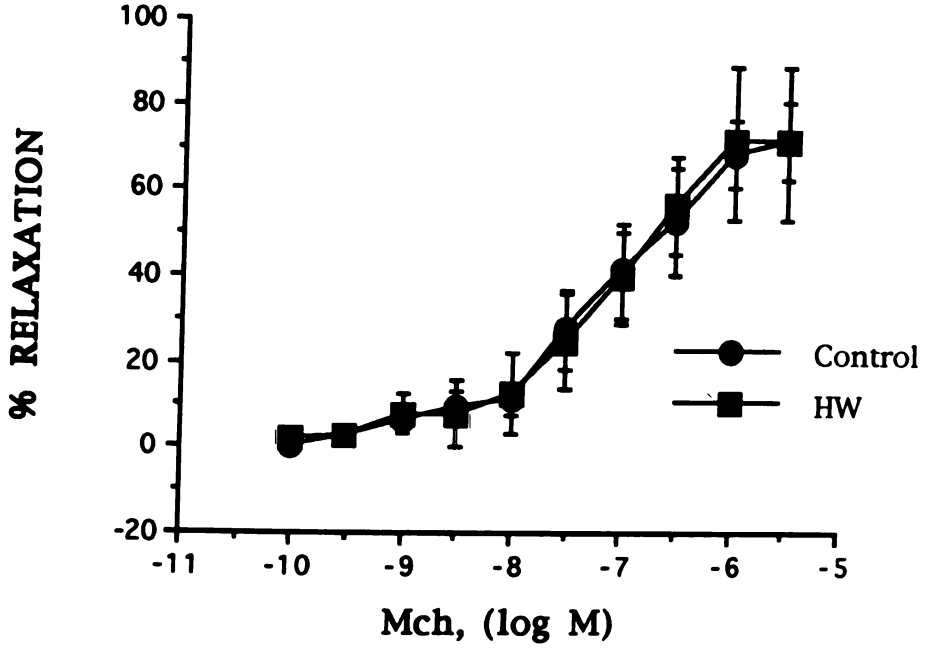

FIGURE 8: Methacholine (Mch) relaxation in norepinephrine preconstricted strips of femoral vein from control and heartworm infected dogs (HW) studied in spring. Relaxation in control (●; n=3) and heartworm infected dogs (■; n=3) was not significantly different.

FIGURE 9

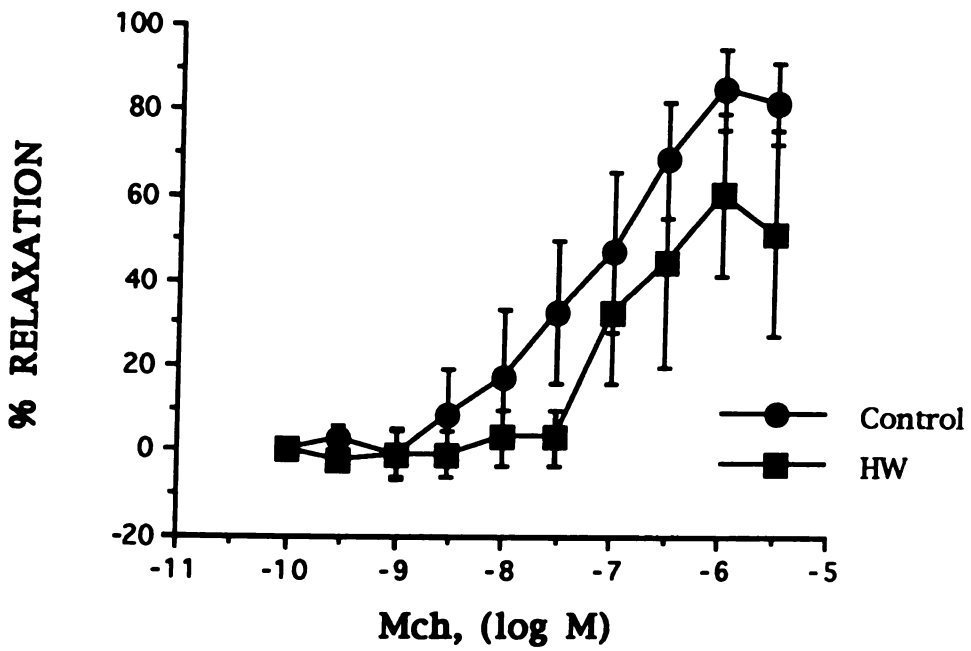

FIGURE 9: Methacholine (Mch) relaxation in $PGF_{2\alpha}$ preconstricted strips of femoral vein from control (●; n=3) and heartworm infected dogs (HW; ■; n=3) studied in spring. Relaxation in control and heartworm infected dogs was not significantly different.

FIGURE 10

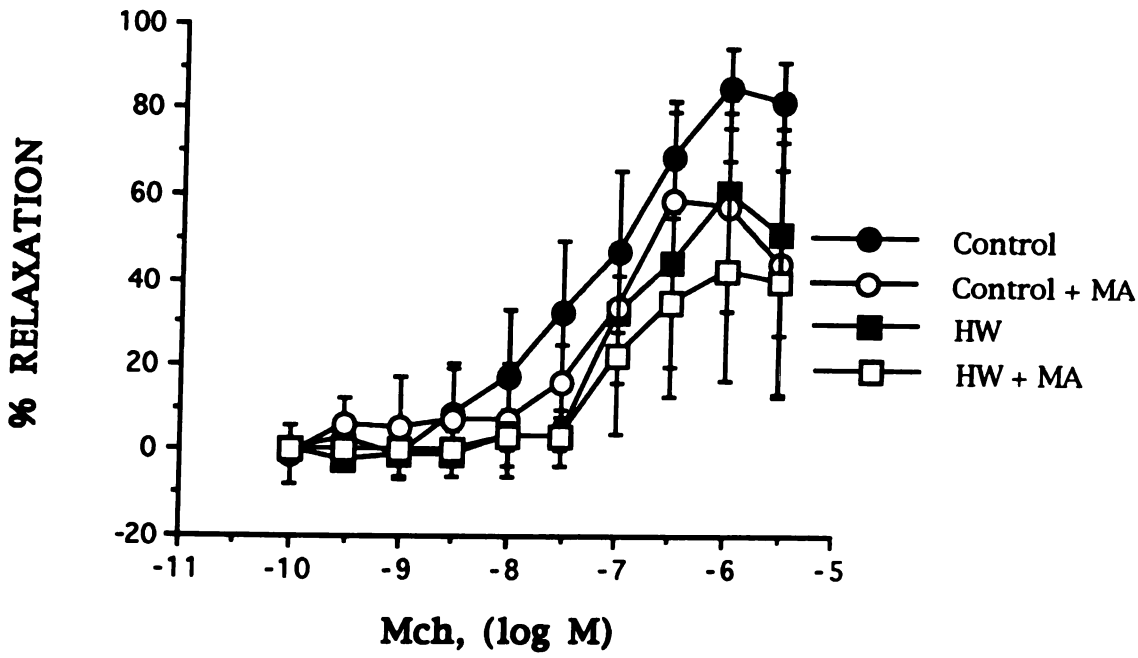

FIGURE 10: Dose-response relationships to methacholine (Mch) in strips of femoral vein from control (●; n=3) and heartworm infected dogs (HW; ■; n=3) in the presence (open symbols) and absence (solid symbols) of mefenamic acid (MA; 10^{-5} M). Mefenamic acid did not alter methacholine relaxation in control or heartworm femoral vein.

FIGURE 11

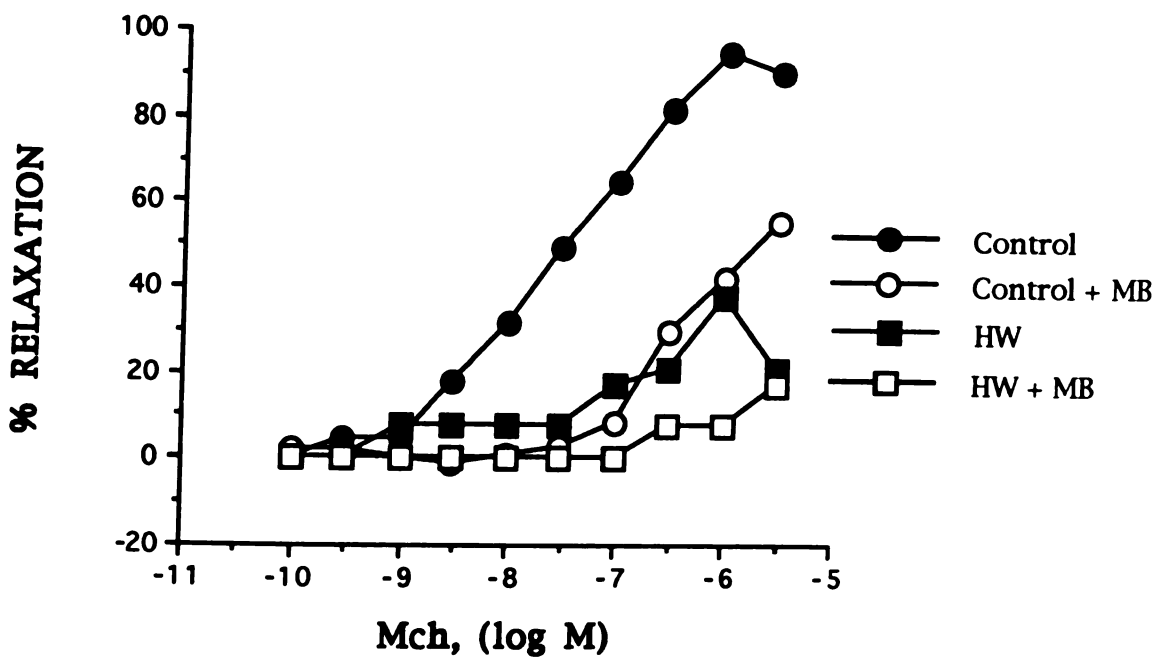

FIGURE 11: Dose-response relationships to methacholine (Mch) in paired strips of femoral vein from control (●; n=2) and heartworm infected dogs (■; n=1) in the presence (open symbols) and absence (solid symbols) of methylene blue (MB; 3 x 10^{-5} M). Experiments were done in the spring in strips of femoral vein preconstricted with $PGF2\alpha$. These preliminary studies suggest that methylene blue depresses methacholine relaxation in control and heartworm femoral vein.

FIGURE 12

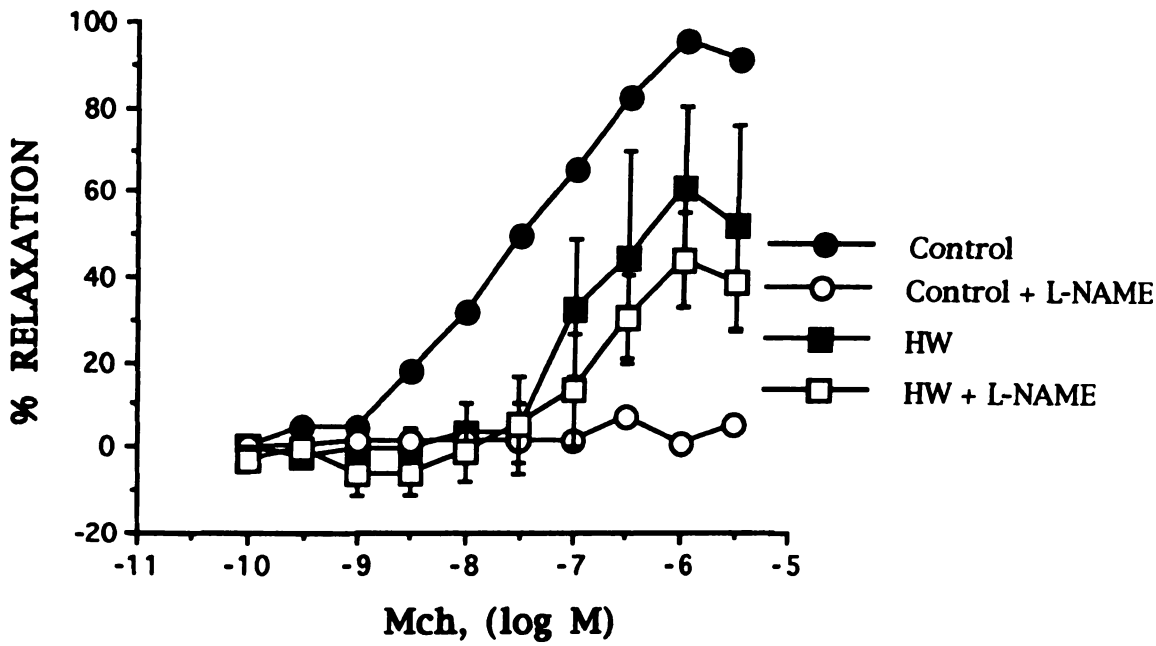

FIGURE 12: Dose-response relationships to methacholine (Mch) in paired strips of femoral vein from control (●; n=2) and heartworm infected dogs (HW; ■; n=3) in the presence (open symbols) and absence (closed symbols) of N^G-nitro-L-arginine methyl ester (L-NAME; 3 x 10^{-5} M). Experiments were done in the spring in strips of femoral vein preconstricted with $PGF_{2\alpha}$. L-NAME almost abolished relaxation in control, but did not significantly alter relaxation in heartworm femoral vein.

3. BOVINE MESENTERIC LYMPHATICS

A. The effect of endothelial cell removal on phasic contractions: Isolated rings of mesenteric lymphatics exhibited phasic contractions that were regular in frequency and amplitude and stable over time (Figure 13). Before removal of endothelial cells, contraction frequency (donor=3.37 ± 0..57 vs. detector=3.96 ± 0.68 contractions/min) and amplitude (donor=2.0 ± 0.21 gm vs. detector=1.3±.23 gm) were not different in donor when compared to detector rings (n=7).

Spontaneous contractions were unaffected by removing and resuspending rings in organ chambers (Figure 14). Spontaneous contractions were abolished, however, in rings from which endothelial cells were removed by rubbing the intimal surface (Figure 14; n=5) or by treatment with collagenase (Figure 15; n=5). Contractile responses to norepinephrine, however, were not different in endothelium-denuded rings before and after removal of endothelial cells (Figure 16) or in endothelium-denuded rings (220 ± 83 mg) when compared to endothelium-intact rings (154 ± 64 mg).

B. Endothelium-intact/endothelium-denuded sandwich: Endothelium-denuded (detector) rings spontaneously contracted when sandwiched with endothelium-intact (donor) rings (Figure 17 & 18). Contraction frequency was not different before and during sandwiching in detector (before=3.96 ± 0.68 vs. during=4.97 ± 1.1 contractions/min; n=7) or donor rings (before=3.37 ± 0.57 vs. during=3.9 ± 1.28 contractions/min; n=7). Contraction amplitude was also unaffected by sandwiching in donor rings (before=2.04 ± 0.28 gm vs. during=1.51 ±0.19 gm; n=7), however, contraction amplitude was significantly depressed in sandwiched detector rings (before=1.33 ± 0.23 gm vs. during=0.42 ± 0.24 gm; n=7). After separation of

donor and detector rings, contractions continued in donor rings with a frequency of 2.99 ± 0.67 contractions/min. and an amplitude of 1.9 ± 0.24 gm. Neither the frequency nor the amplitude of contractions were different in donor rings before and after sandwiching (n=7). Spontaneous contractions stopped in detector rings within 4.9 ± 2.3 minutes after separation from donor rings (n=7). After separation from donor rings, spontaneous contractions did not return in denuded rings suspended alone for times ranging from 3 minutes to 12 hours (n=4).

C. Endothelium-denuded/denuded sandwich: Contractions were not observed in either ring when two endothelium-denuded rings were sandwiched together (Figure 19) for times ranging from 15 minutes to 12 hours (n=4).

D. Histopathology: Evaluation of lymphatics for factor VIII revealed that the majority of endothelial cells were gone from endothelium-denuded preparations (a ranking of 2 was assigned to all endothelium-denuded rings) whereas, the majority of endothelial cells were present in endothelium-intact rings (a ranking of 4 was assigned to all endothelium-intact rings with the exception of one ring which was given a ranking of 3).

4. PRELIMINARY DATA-BOVINE MESENTERIC LYMPHATICS

A. Effects of inhibitors of cyclooxygenase, guanylate cyclase and NO on phasic contractile activity.

Aspirin (n=1), indomethacin (n=2) and mefenamic acid (Figure 20) abolished spontaneous contractions in rings of bovine mesenteric lymphatics. Similar results were obtained with methylene blue (Figure 21) and L-NAME (Figure 22). The effect of L-NAME was reversed by L-arginine (Figure 22), but not D-arginine (n=1). L-arginine did not alter spontaneous contractions when added alone (in the absence of L-NAME; n=1).

B. Effect of heartworm-conditioned medium on phasic contractile activity.

Heartworm-conditioned, but not control medium, inhibited spontaneous contractile activity. Contractions were restored by washing with warm PSS (Figure 23).

C. Effect of Prostaglandin D_2 on phasic contractions

Prostaglandin D_2 ($3x10^{-12}$ to $3x10^{-5}$M) caused a dose-dependent inhibition of spontaneous contractions and an increase in basal tone in isolated rings of mesenteric lymphatics (Figure 24).

FIGURE 13

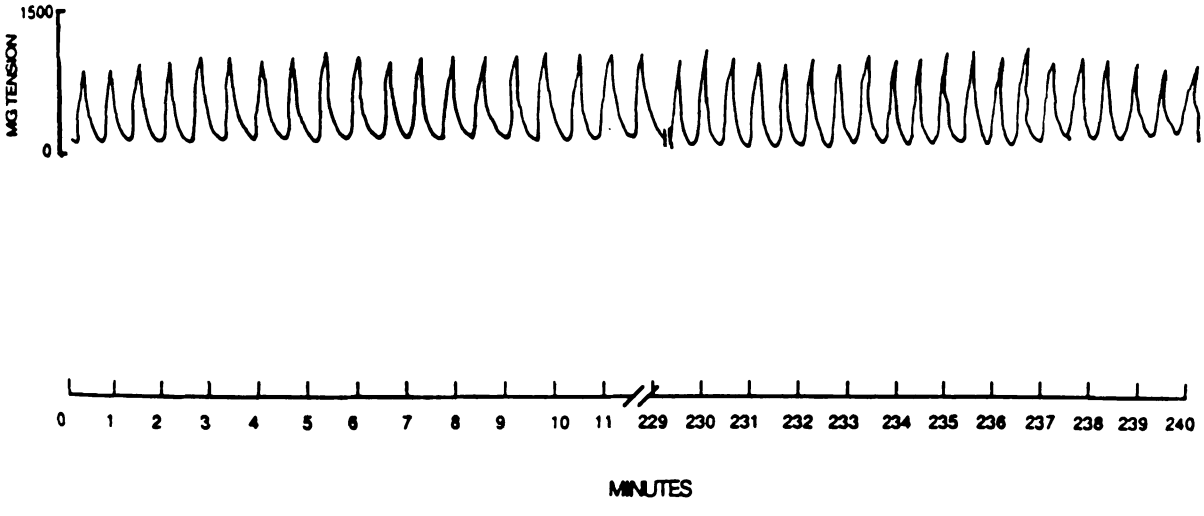

FIGURE 13: Representative trace of phasic contractions of an isolated ring of bovine mesenteric lymphatic. Contractions are stable over time and regular in frequency and amplitude.

FIGURE 14

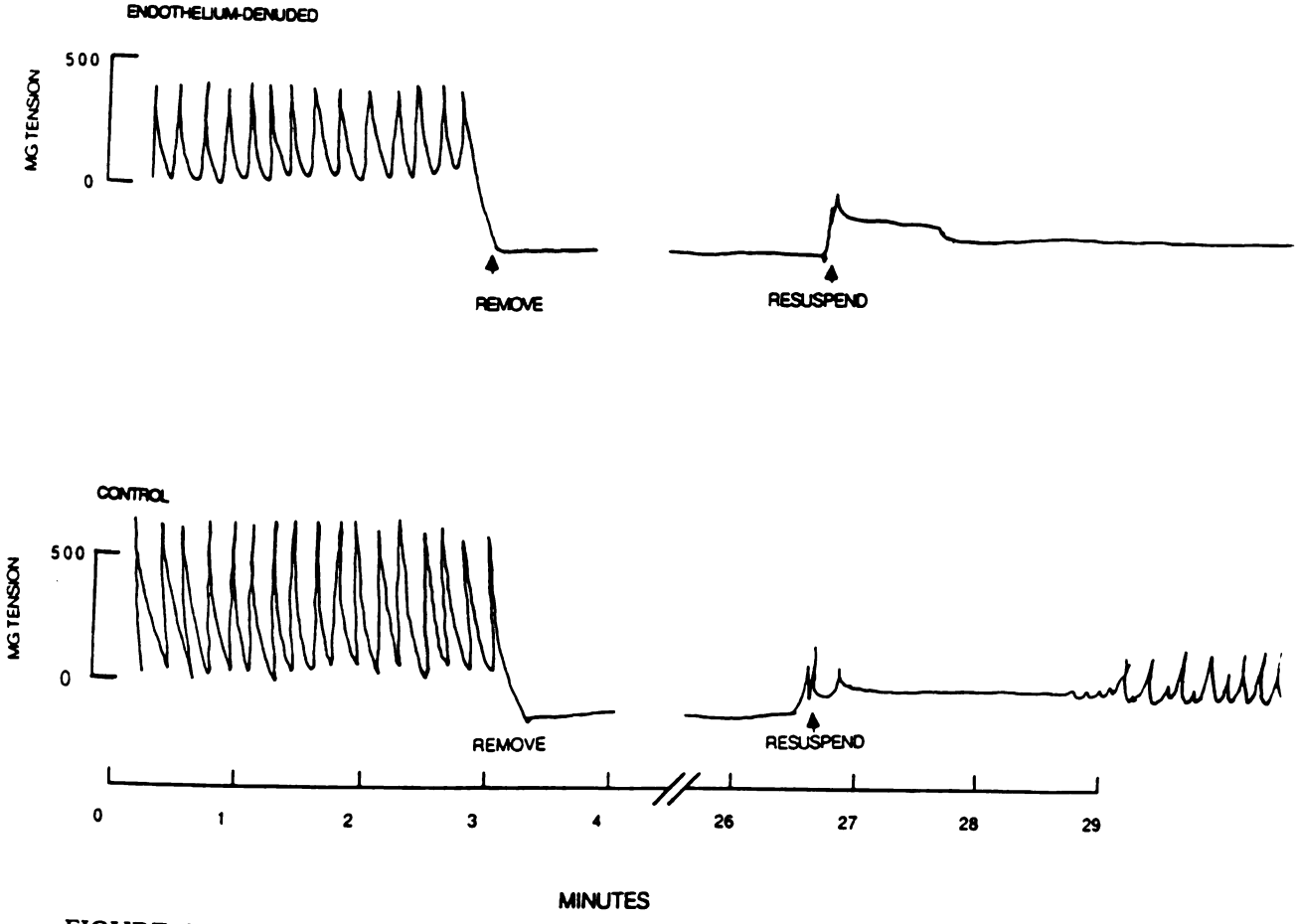

FIGURE 14: Representative trace showing the effect of mechanical removal of endothelial cells on spontaneous contractions of bovine mesenteric lymphatics. Spontaneous contractions returned in control rings (bottom trace) after they were removed (first arrow) and resuspended in organ chambers (second arrow). Spontaneous contractions did not return in rings from which endothelial cells were removed mechanically (top trace; n=3)

FIGURE 15

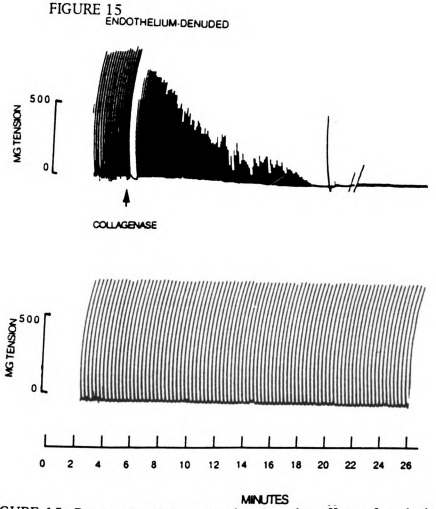

FIGURE 15: Representative trace showing the effect of endothelial cell removal by collagenase. Addition of collagenase (500 U/ml PSS) to the bath abolished spontaneous contractions of bovine mesenteric lymphatics within 30 minutes (top trace; n=5).

FIGURE 16

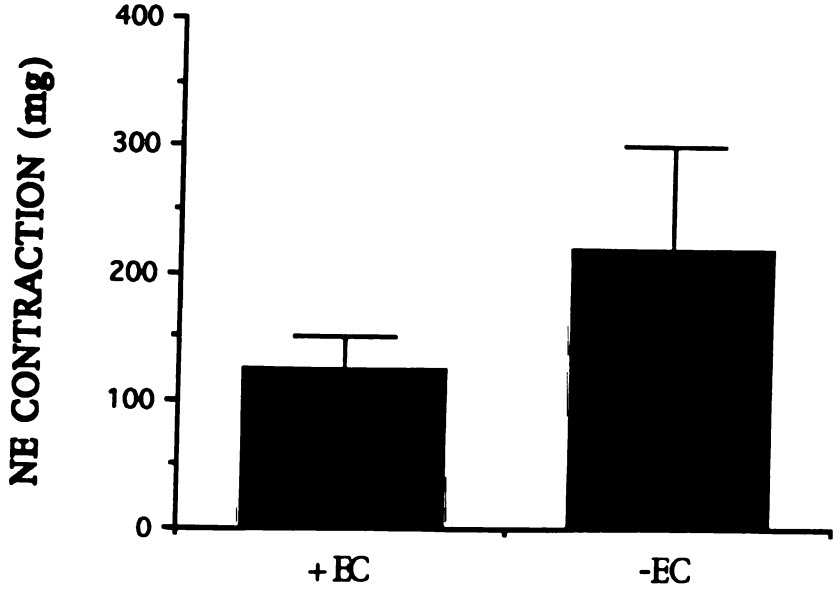

FIGURE 16: Norepinephrine (NE) constriction in paired rings of bovine mesenteric lymphatics before (+EC) and after (-EC) removal of endothelial cells. Endothelial cells were removed by collagenase (n=3) or by rubbing the intimal surface with filter paper (n=3). Endothelial cell removal did not depress norepinephrine constriction (n=6).

FIGURE 17

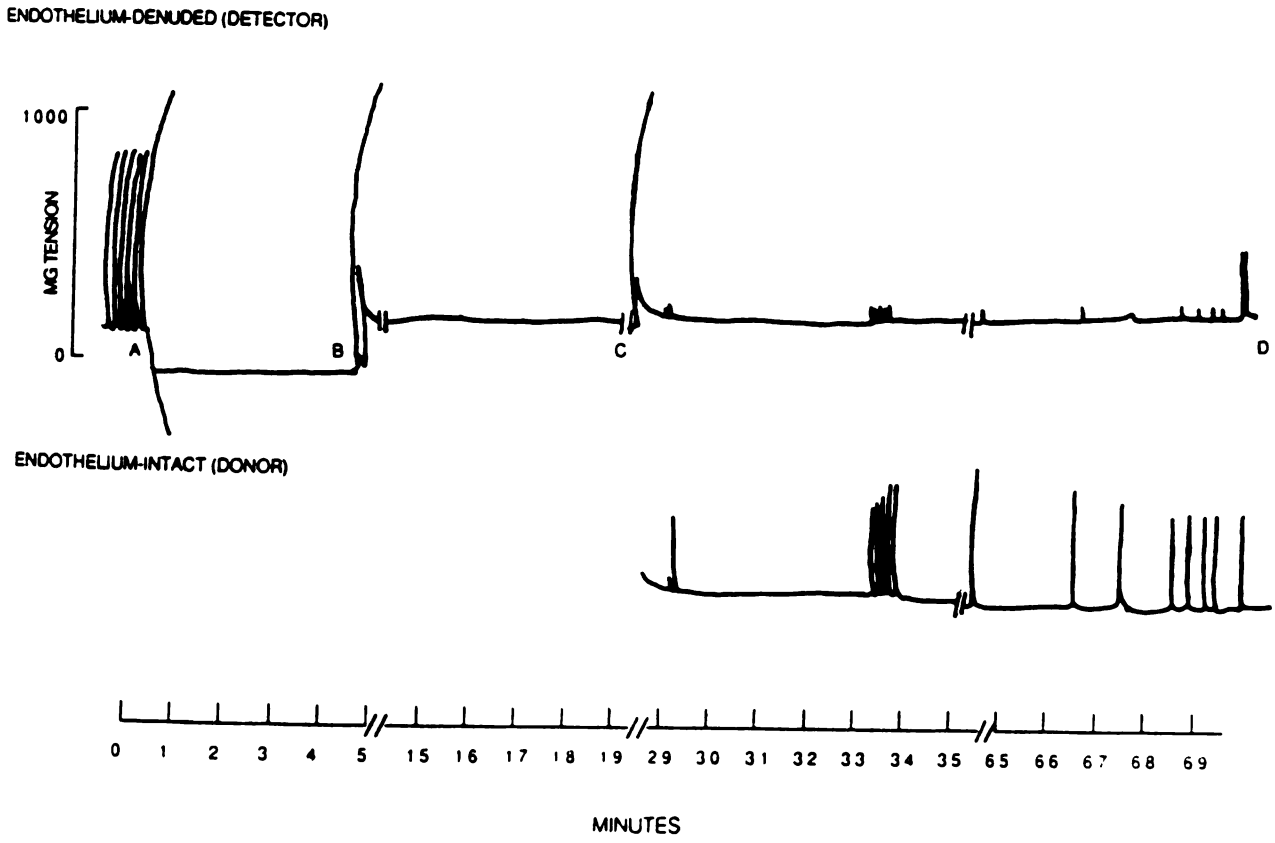

FIGURE 17: Representative trace of an endothelium-denuded detector ring (top trace) sandwiched with an endothelium-intact donor ring (bottom trace). A spontaneously contracting detector ring was removed from the bath at point A, the ring was everted and the endothelial cells removed by rubbing the intimal surface with moistened filter paper. When the ring was resuspended alone in an organ chamber no contractions were observed (point B to point C). However, when the ring was "sandwiched" with an endothelium-intact donor ring, spontaneous contractions returned in the detector ring (Point C to Point D; n=4). Spontaneous contractions stopped in detector rings after separation from donor rings.

FIGURE 18

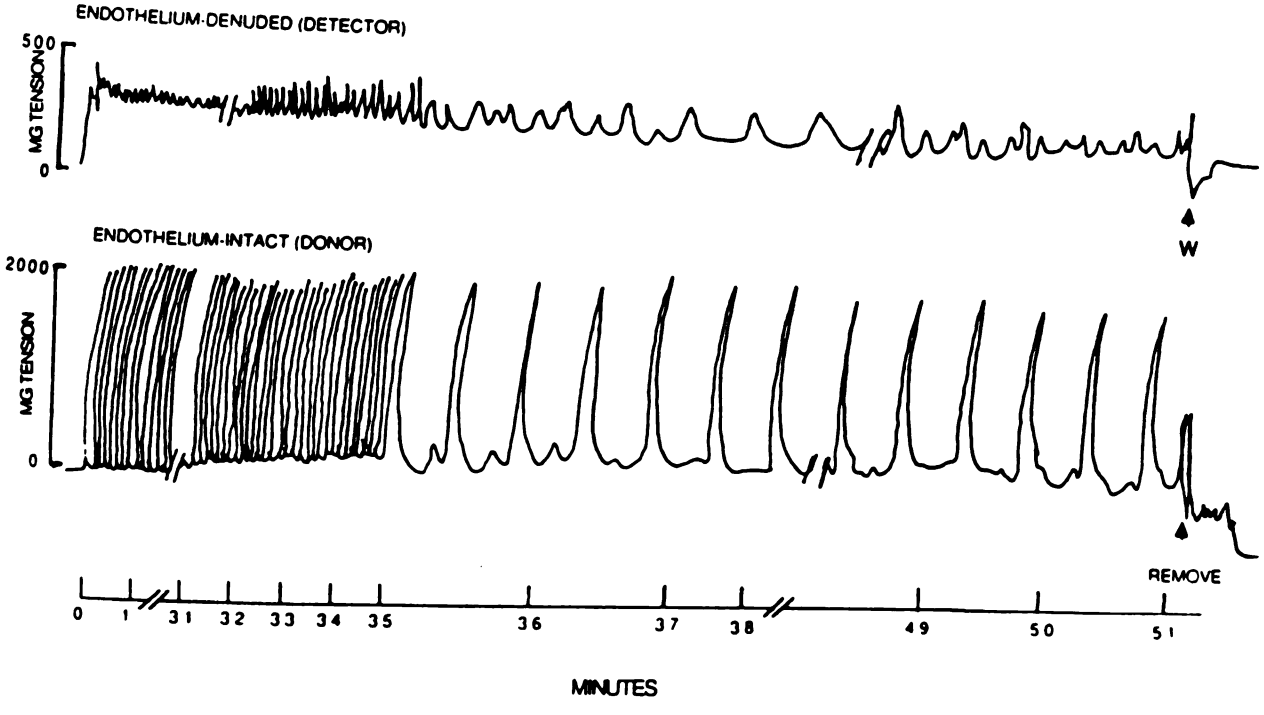

FIGURE 18: Representative trace of an endothelium-denuded detector ring (top trace) sandwiched with an endothelium-intact donor ring (bottom trace). Endothelial cells were removed from detector rings by collagenase (500 U/ml PSS). The detector ring contracted phasically when "sandwiched" with a donor ring. Removal of the donor ring from the bath (at arrow) caused contractions to stop in the detector ring (n=3).

FIGURE 19

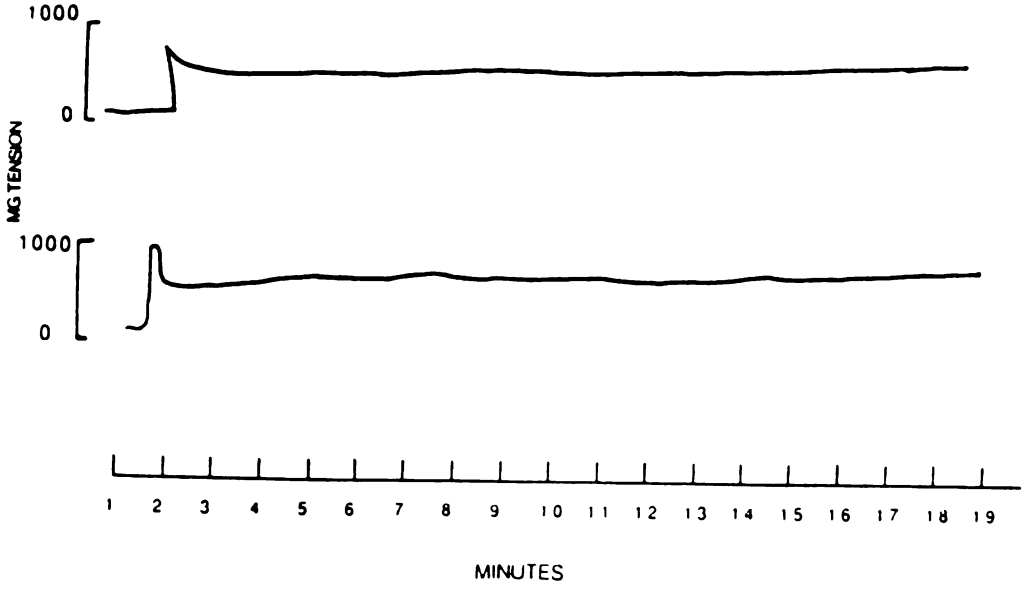

FIGURE 19: Representative trace of two endothelium-denuded rings "sandwiched" together. Spontaneous contractions were not observed in either ring (n=4).

FIGURE 20

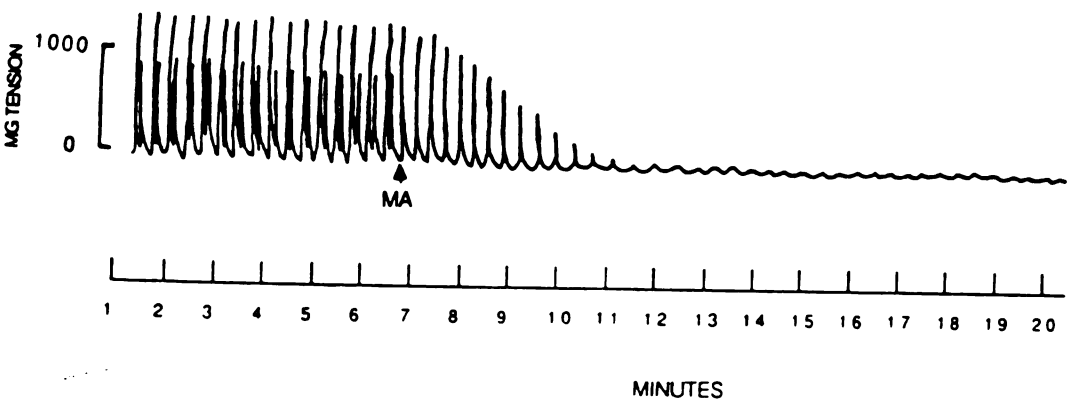

FIGURE 20: Representative trace showing the effect of mefenamic acid (MA;10^{-5} M) on phasic contractions of bovine mesenteric lymphatics. Mefenamic acid abolished phasic contractions in preliminary experiments (n=2).

FIGURE 21

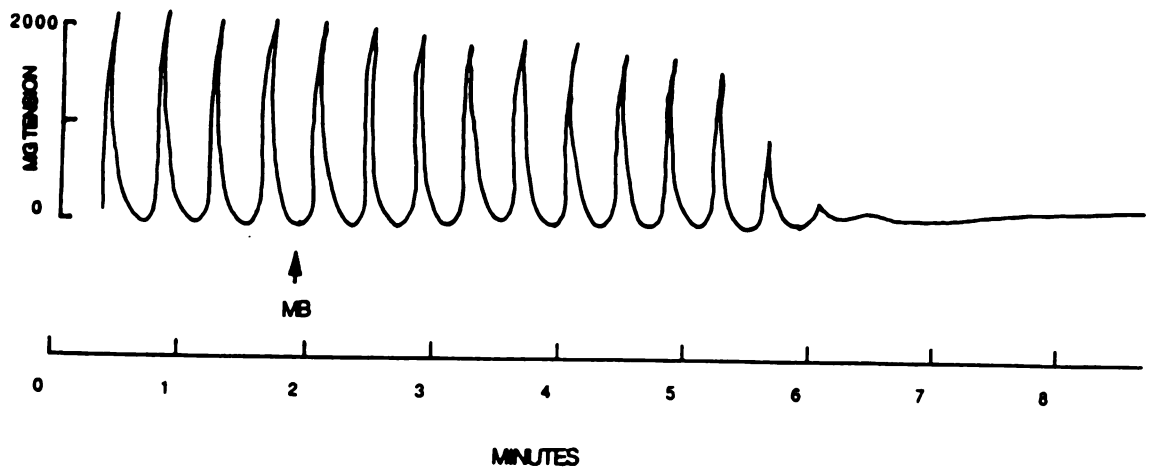

FIGURE 21: Representative trace showing the effect of methylene blue (MB; 10 uM) on phasic contractions of bovine mesenteric lymphatics. Methylene blue abolished phasic contractions in preliminary experiments (n=3).

FIGURE 22

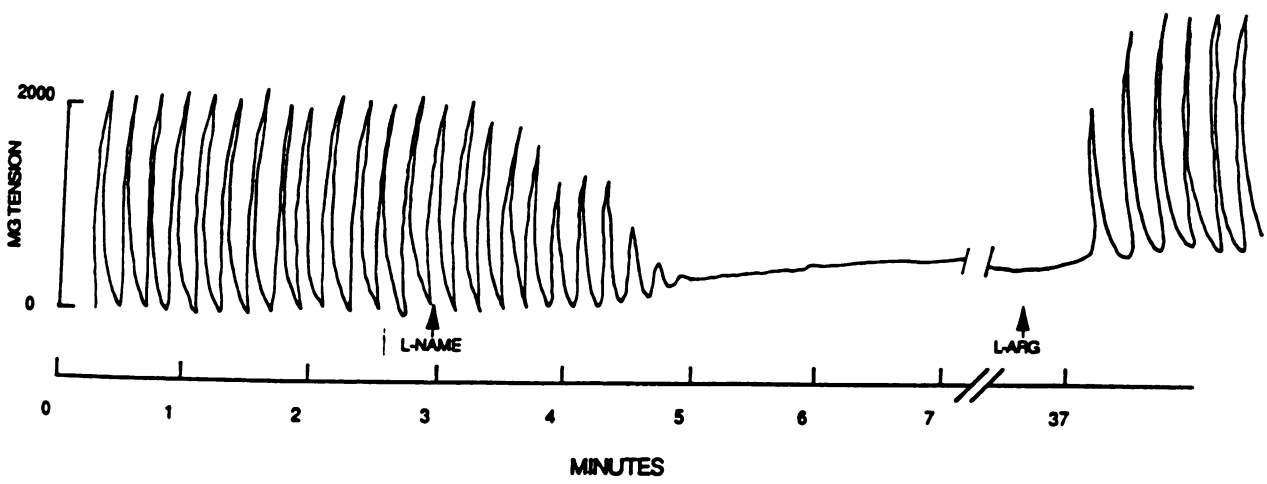

FIGURE 22: Representative trace showing the effect of N^G-nitro-L-arginine methyl ester (L-NAME; 3 x 10^{-5} M) on phasic contractions of isolated rings of bovine mesenteric lymphatics. L-NAME abolished spontaneous contractions in preliminary experiments and increased basal tone (n=2). The effect of L-NAME was reversed by L-arginine (9 x 10^{-4} M).

FIGURE 23

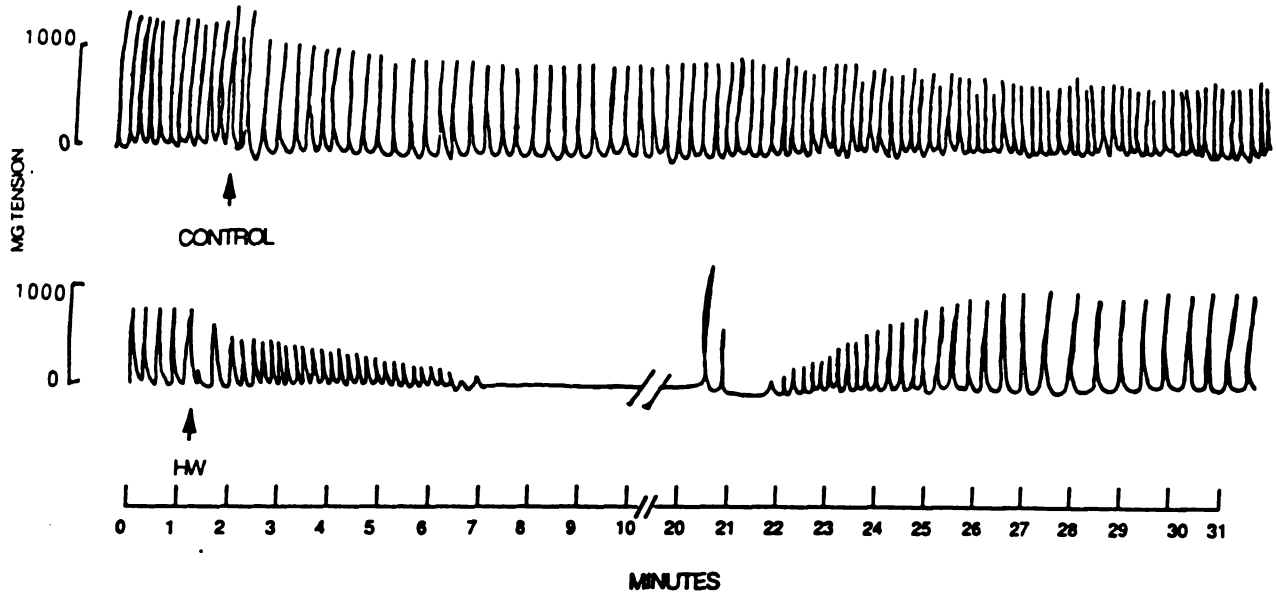

FIGURE 23: Representative trace showing the effect of heartworm-conditioned (HW; n=2; bottom trace) and control medium (control; n=1; top trace) on phasic contractions of bovine mesenteric lymphatics. In preliminary experiments, heartworm-conditioned medium, but not control medium, abolished contractions. Contractions were restored by washing (W) the ring. Arrows show time of addition of conditioned medium to the bath.

FIGURE 24

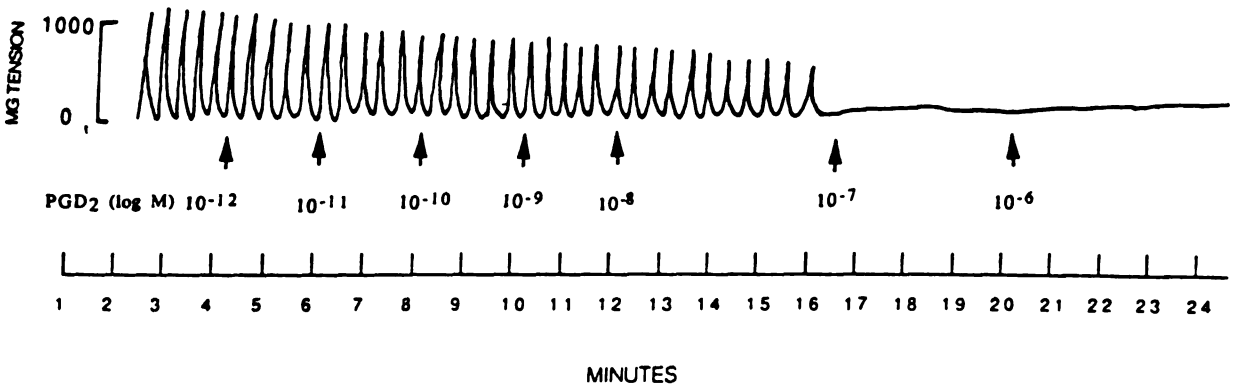

FIGURE 24: Representative trace showing the effect of Prostaglandin D_2 (PGD_2) on phasic contractions of an isolated ring of bovine mesenteric lymphatic. PGD_2 caused a dose-dependent inhibition of spontaneous contractions and an increase in active tone at higher concentrations (n=1).

5. THORACIC DUCT-PRELIMINARY DATA

A. Effects of heartworm-conditioned medium on endothelium-dependent relaxation.

(1) Acetylcholine relaxation: Endothelium-intact, but not endothelium-denuded rings relaxed to acetylcholine. Acetylcholine relaxation, assessed before addition of heartworm conditioned or control medium, was not different between rings that received control medium (48.6 ± 10.7%; n=5) and rings that received heartworm-conditioned medium (55.4 ± 9.4%; n=5).

(2) Norepinephrine constriction: Norepinephrine (10^{-5} M) caused constriction in all rings. There were no significant differences in norepinephrine constriction between rings exposed to heartworm-conditioned medium (506 ± 194 mg; n=5) and rings that received control medium (530 ± 104 mg; n=5). Norepinephrine constriction was not different before and after exposure to control medium (before=536 ± 126.4 mg vs. after=506 ± 194 mg) or heartworm-conditioned medium (before=567 ± 94 mg vs. after=530 ± 104 mg).

(3) Methacholine dose-response relationships: Methacholine caused a dose-dependent relaxation response in all endothelium-intact rings. Methacholine relaxation was significantly depressed in rings exposed to heartworm-conditioned medium when compared to control (Figure 25). Removal of endothelial cells abolished relaxation in both groups (control n=2; HW n=2).

(4) ***Sodium nitroprusside dose-response relationships:*** Sodium nitroprusside caused a dose-dependent relaxation response in all rings. Sodium nitroprusside relaxation was not depressed by exposure to heartworm-conditioned medium (Figure 26).

FIGURE 25

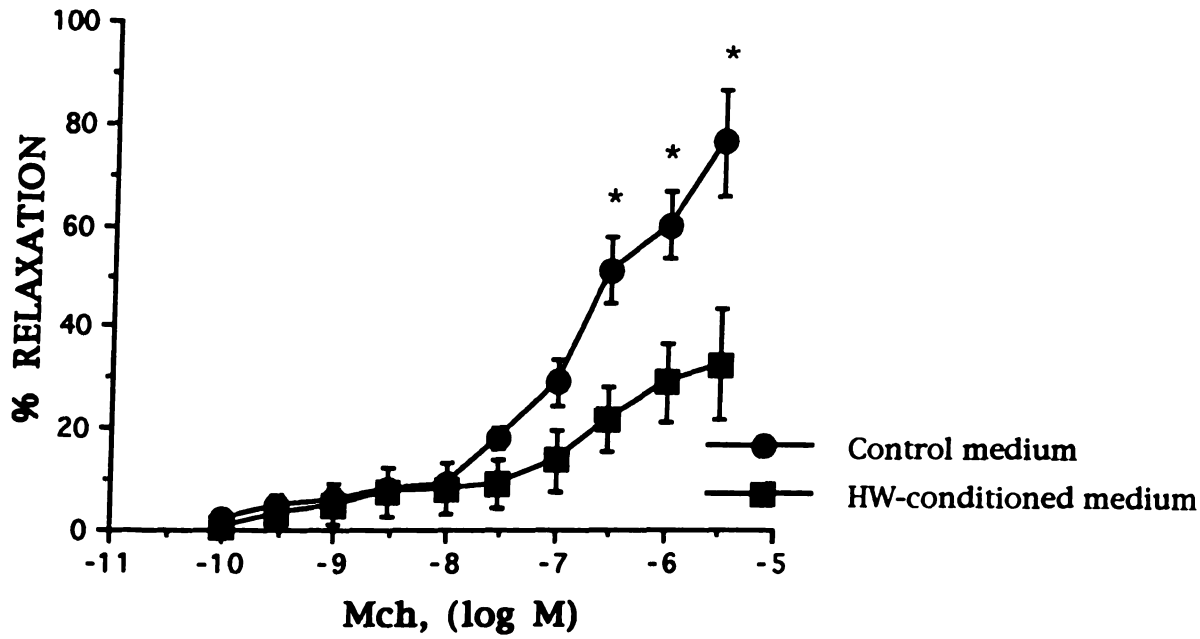

FIGURE 25: Methacholine (Mch) relaxation in paired rings of thoracic duct in the presence of heartworm (HW)-conditioned medium (■; n=5) or control medium (●; n=5). Heartworm-conditioned medium significantly depressed relaxation. * statistically significant difference; $p<0.05$.

FIGURE 26

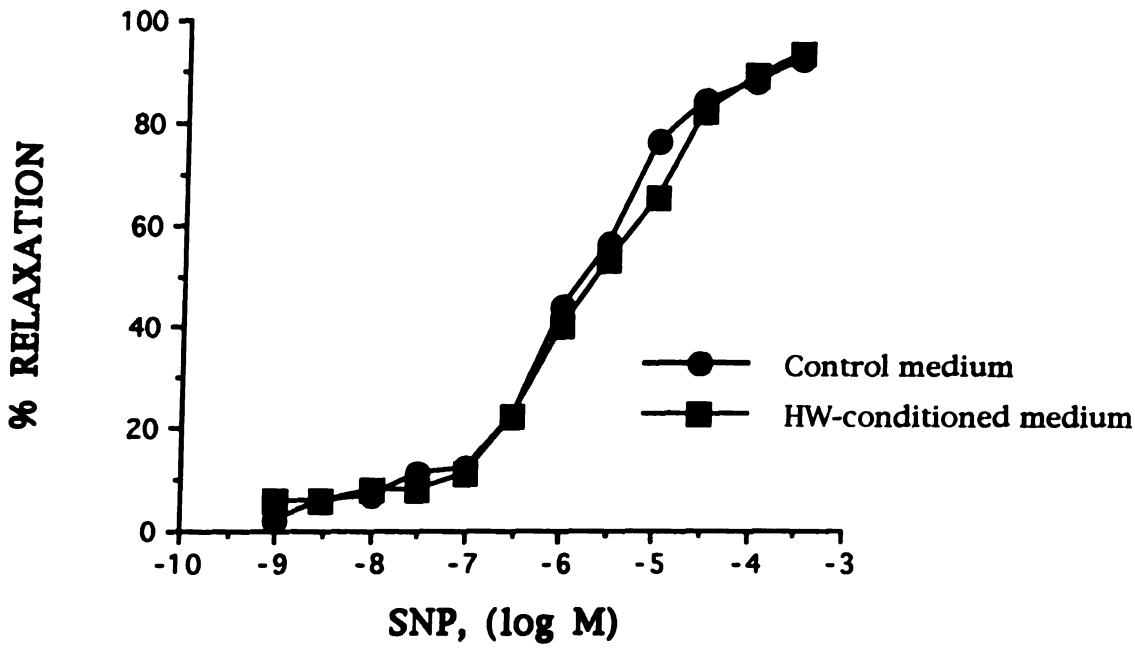

FIGURE 26: Sodium nitroprusside (SNP) relaxation in paired rings of thoracic duct in the presence of heartworm (HW)-conditioned (■; n=2) or control medium (●; n=2). Heartworm-conditioned medium did not depress sodium nitroprusside relaxation.

B. Effect of in vivo exposure to D. immitis on endothelium-dependent relaxation of thoracic duct in vitro.

(1) Norepinephrine dose-response relationships: Norepinephrine caused a dose-dependent constriction response in thoracic duct from heartworm infected and control dogs. Preliminary studies suggest that norepinephrine constriction is not different in heartworm when compared to control rings (Figure 27).

(2) Methacholine dose-response relationships: Methacholine caused a dose-dependent relaxation in rings from control dogs. Methacholine relaxation was significantly depressed in rings of thoracic duct from heartworm infected dogs when compared to control (Figure 28).

(3) Methacholine dose-response relationships +/- mefenamic acid: Mefenamic acid did not alter methacholine relaxation in thoracic duct from control or heartworm infected dogs (Figure 29).

(4) Methacholine dose-response relationships +/- methylene blue: In preliminary studies, methylene blue abolished the relaxation response to methacholine in thoracic duct from control dogs (n=1). However, relaxation was not abolished by methylene blue in thoracic duct from heartworm infected dogs (Figure 30).

(6) Methacholine dose-response relationships +/- L-NAME: L-NAME significantly depressed methacholine relaxation in control, but not in heartworm thoracic duct (Figure 31).

(7) The effect of D- and L-arginine on methacholine response in L-NAME-treated rings: D-arginine did not cause relaxation in thoracic duct from heartworm or control dogs. L-arginine, however, caused significant relaxation in L-NAME-treated rings from heartworm (22±14.7%) and control dogs (19%; n=2).

FIGURE 27

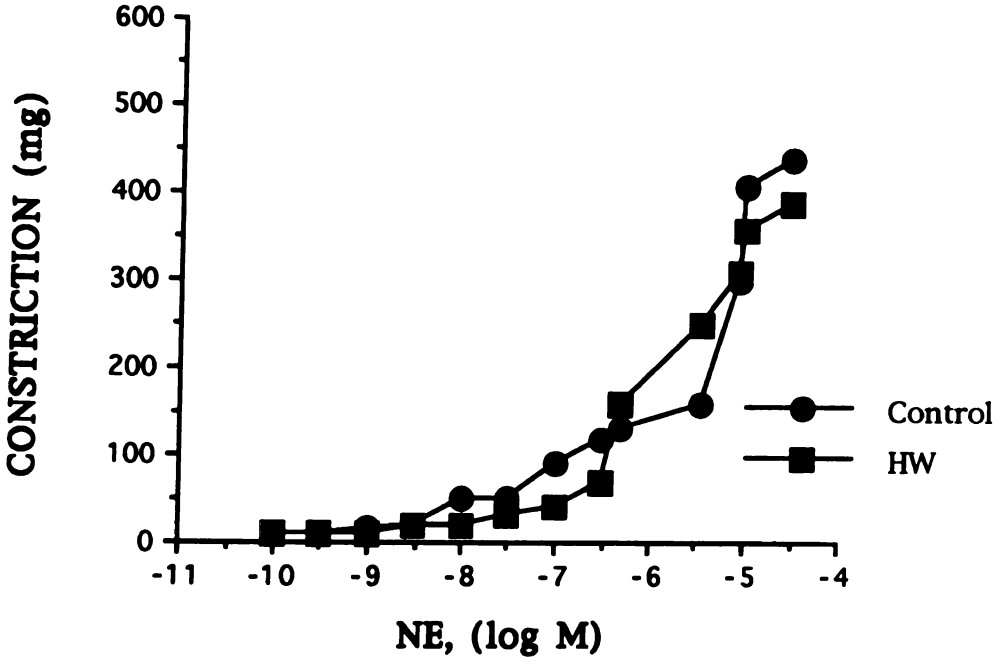

FIGURE 27: Norepinephrine (NE) constriction in rings of thoracic duct from heartworm infected (HW; ■; n=2) and control dogs (●; n=2). Preliminary studies suggest that norepinephrine constriction is not depressed in rings from heartworm when compared to control.

FIGURE 28

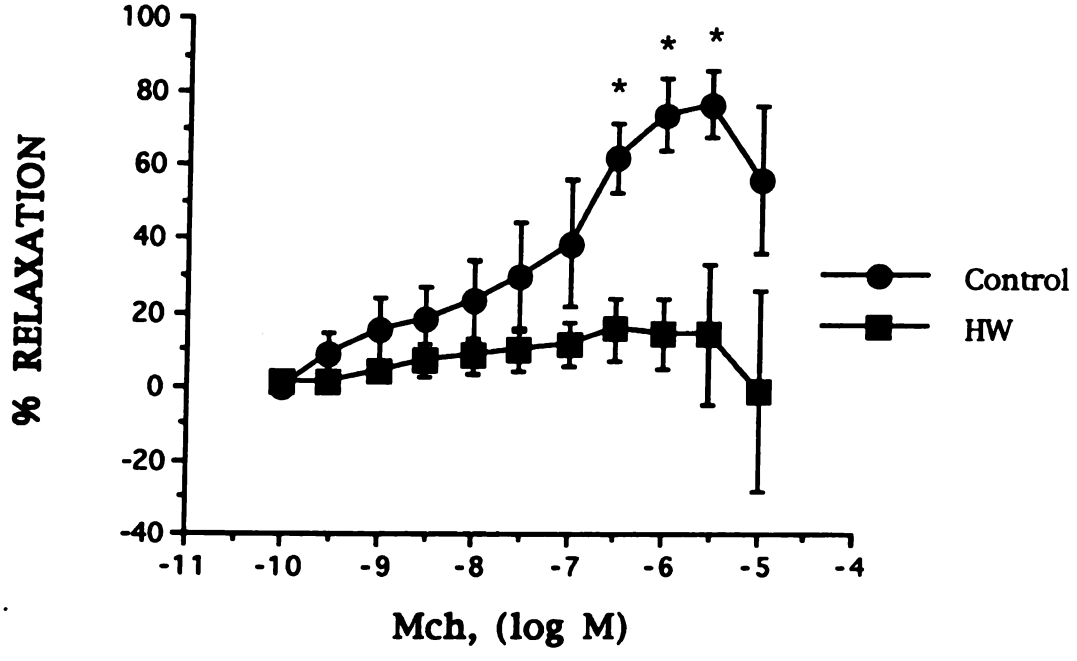

FIGURE 28: Methacholine (Mch) dose-response relationships in thoracic duct from heartworm infected dogs (HW; ■; n=4) and control dogs (●; n=4). Methacholine relaxation was significantly depressed in rings from heartworm infected dogs when compared to control.

FIGURE 29

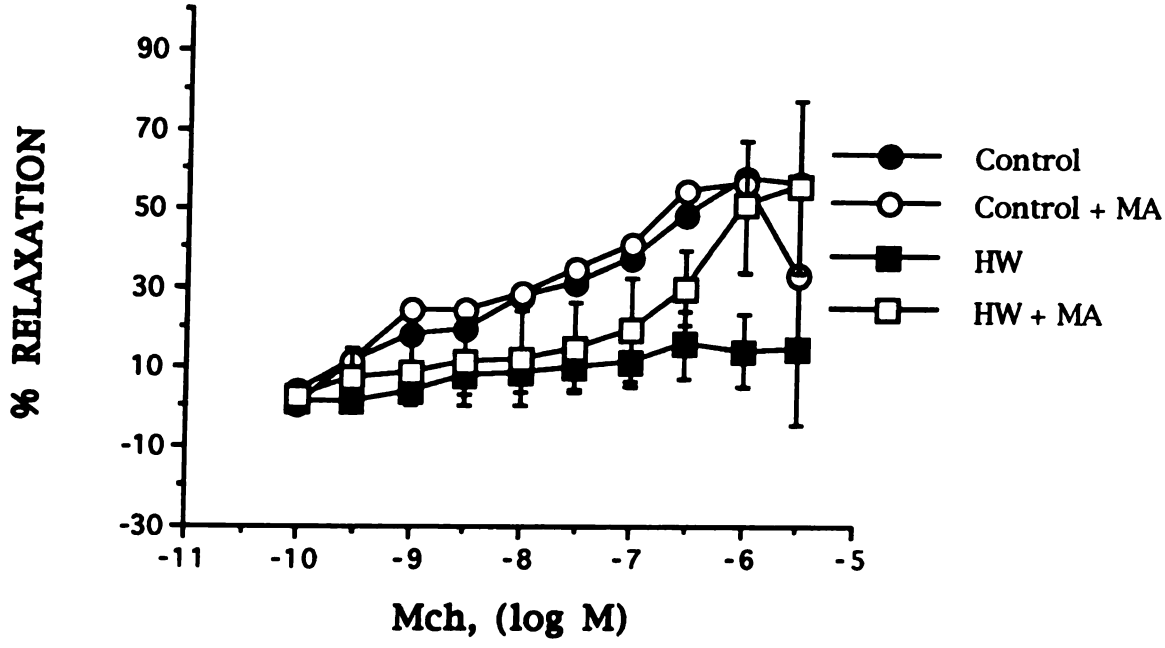

FIGURE 29: Dose-response relationships to methacholine (Mch) in paired rings of thoracic duct from heartworm infected (HW; ■; n=3) and control dogs (●; n=3) in the presence (open symbols) and absence (closed symbols) of mefenamic acid (MA; 10^{-5} M). Mefenamic acid did not alter methacholine relaxation in thoracic duct from control or heartworm infected dogs. Error bars are not shown with control responses for clarity.

FIGURE 30

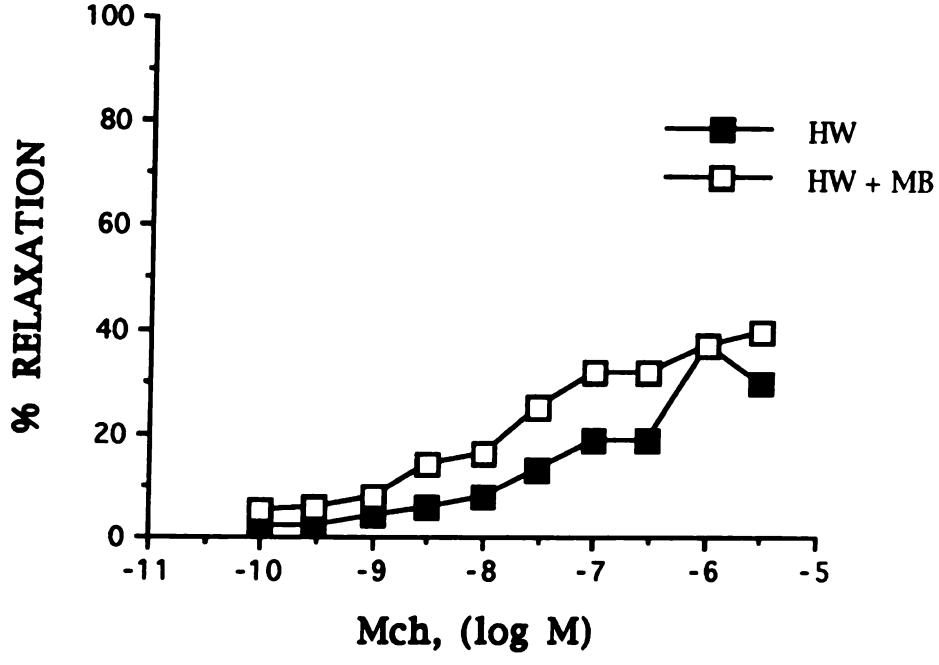

FIGURE 30: Methacholine (Mch) dose-response relationships in paired rings of thoracic duct (n=2) from heartworm infected dogs in the presence (❑) and absence (■) of methylene blue (MB; 10^{-5} M). Methylene blue did not abolish relaxation in thoracic duct from heartworm infected dogs in preliminary experiments.

FIGURE 31

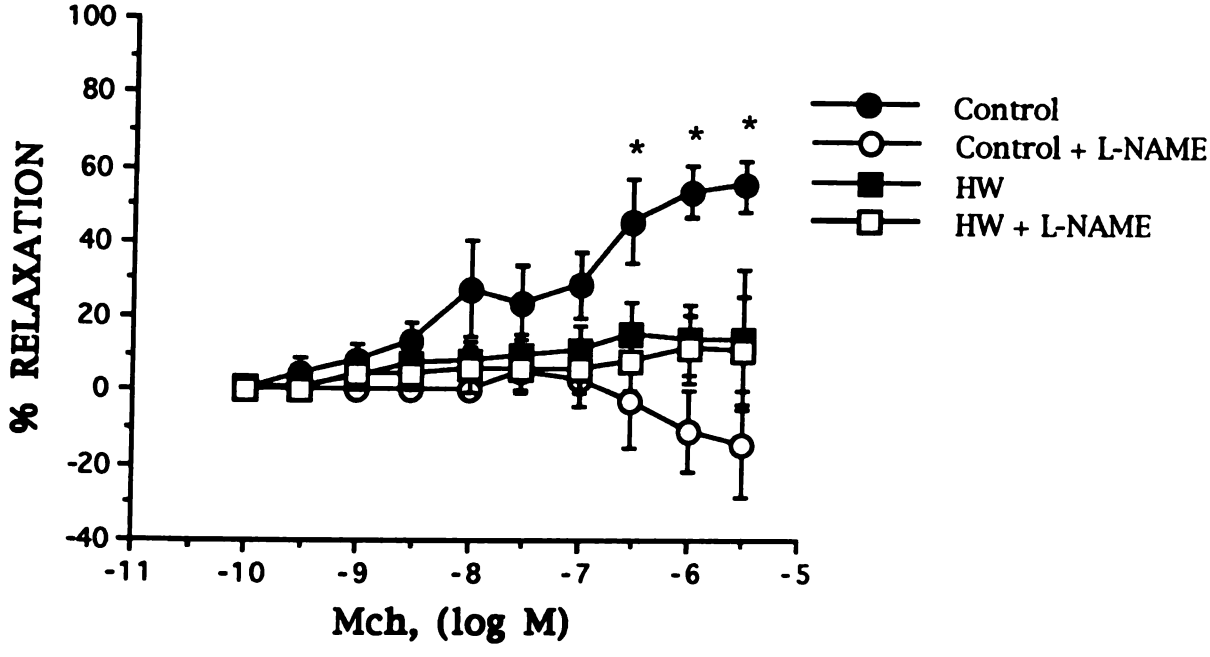

FIGURE 31: Methacholine dose-response relationships in paired rings of thoracic duct from heartworm infected (■; n=3) and control dogs (●; n=3) in the presence (open symbols) and absence (solid symbols) of N^G-nitro-L-arginine methyl ester (L-NAME; 1.5 x 10^{-5} M). L-NAME significantly depressed relaxation in control (*), but not heartworm thoracic duct; $p<0.05$.

Discussion

The Effect of in vivo Exposure to D. immitis on Endothelium-Dependent Relaxation of the in vitro Femoral Artery and Vein

Endothelium-dependent relaxation is not depressed in the in vitro femoral artery of heartworm infected dogs when compared to control. In preliminary studies, relaxation of the in vitro femoral vein also appears to be unaffected by chronic exposure to D. immitis. Therefore, seasonal depression of endothelium-dependent relaxation observed in the in vivo femoral artery of heartworm infected dogs is not seen in vitro in the absence of the parasites. These results suggest that infection with D. immitis does not permanently alter the behavior of systemic vascular endothelial cells, thus depression of endothelium-dependent relaxation may require continuous exposure to filarial factors in systemic blood vessels.

Endothelium-dependent relaxation is depressed in the isolated femoral artery from heartworm infected dogs studied in the spring when compared to responses obtained in the fall. Since relaxation was also depressed in femoral artery from control dogs in spring when compared to fall, the depression is unlikely due to an effect of D. immitis and may reflect seasonal variations in endothelium-dependent relaxation in canine blood vessels. Although there are no previous reports concerning the effect of season on endothelium-dependent relaxation in canine blood vessels, seasonal variations have been observed in other species (Kaiser et al., 1990).

In vivo, indomethacin attenuates acetylcholine dilation in the femoral artery from heartworm infected, but not control dogs (Kaiser et al., 1987, 1989a). These data suggest that the mechanism of acetylcholine dilation is

different in the in vivo femoral artery from heartworm infected dogs when compared to control and involves a cyclooxygenase product. In vitro, however, neither indomethacin nor mefenamic acid depress methacholine relaxation in heartworm femoral artery thus indicating that the alteration of mechanism of acetylcholine dilation observed in vivo is not seen in vitro. Several cell types are present in vivo which are capable of metabolizing arachidonic acid. Although mammalian cells may be the source of arachidonic acid metabolites, these products may also arise from the adult parasites or the microfilariae. Kaiser et al., (1992) has previously demonstrated that adult D. immitis release cyclooxygenase product(s) which depress endothelium-dependent relaxation of the in vitro rat aorta (Kaiser et al., 1992). Liu et al., (1990) demonstrated that microfilariae of Brugia malayi, a closely related filarial parasite, metabolize exogenous and endogenous arachidonic acid into vasodilator prostanoids including prostacyclin, prostaglandin E_2, and PGD_2. Thus arachidonic acid metabolites derived from adult parasites or microfilariae may account for the indomethacin-induced depression of acetylcholine dilation in vivo. The absence of the parasites in vitro may account for the lack of effect of cyclooxygenase inhibitors in vitro.

In femoral artery from heartworm infected dogs in vivo, methylene blue causes a biphasic response; enhanced dilation at low concentrations and depressed dilation at high concentrations of acetylcholine, whereas in control dogs, methylene blue almost abolishes acetylcholine dilation (Kaiser et al., 1989). These data suggest that in vivo, the mechanism of acetylcholine dilation is different in femoral artery from heartworm infected and control dogs. However, in vitro, the mechanism of relaxation is not different in femoral artery from heartworm infected dogs when compared to control; methylene blue and L-NAME depress methacholine relaxation in both groups. These data

suggest that the alteration of mechanism of endothelium-dependent relaxation observed in vivo is not demonstrable in vitro.

The inhibition of methacholine relaxation caused by methylene blue and L-NAME in control femoral artery agrees with previous in vivo (Kaiser et al., 1986; Kirkeboen et al., 1991) and in vitro studies (Miller, 1991; Vidal et al., 1991). L-arginine, the substrate for synthesis of nitric oxide, relaxes L-NAME-treated rings in the presence of methacholine, while D-arginine does not. These data suggest that inhibition of methacholine relaxation by L-NAME is due, at least in part, to competitive inhibition of NO synthesis (Moore et al., 1990). However, L-NAME is also a muscarinic receptor antagonist (Buxton et al., 1993), therefore the effect of this agent on methacholine relaxation may be due to antagonism of muscarinic receptors rather than inhibition of NO synthesis. Several factors, however, argue against a significant effect of L-NAME on muscarinic receptors in femoral artery. Methylene blue, which inhibits guanylate cyclase (Gruetter et al., 1981) and directly inactivates NO (Wolin et al., 1990; Furchgott, 1984) causes a similar degree of inhibition of methacholine relaxation as L-NAME. Methylene blue, which is structurally dissimilar to muscarinic agonists, is unlikely to behave as a muscarinic receptor antagonist. Also, L-arginine partially reverses the inhibition of relaxation caused by L-NAME and L-arginine does not reverse the effects of a muscarinic antagonist. Finally, previous studies indicate that N-nitro-L-arginine, which does not appear to be a muscarinic receptor antagonist (Buxton et al., 1993), also depresses acetylcholine dilation in femoral artery in vitro (Miller, 1991). Thus, it is likely that the effect of L-NAME in this study is due to inhibition of NO synthesis, and not antagonism of muscarinic receptors.

The seasonal depression of endothelium-dependent relaxation seen in the in vivo femoral artery from dogs with patent (microfilariae positive) heartworm infection is also seen in dogs with occult (microfilariae negative) dirofilariasis (Kaiser et al., 1987), suggesting that the adults, rather than the microfilariae, are responsible for depression of endothelium-dependent relaxation. Since the adults are in a site distal to the femoral artery it is likely that circulating filarial factors are responsible for depression of relaxation.

Exposure of isolated rat aorta to adult D. immitis, or D. immitis-conditioned medium mimics the depression of acetylcholine dilation seen in the in vivo femoral artery of heartworm infected dogs (Kaiser et al., 1992). Pretreatment of the parasites with cyclooxygenase inhibitors reverses the filarial-induced depression of relaxation, suggesting that a filarial cyclooxygenase product is responsible for the effect of heartworm. Analysis of chloroform-extracted heartworm-conditioned medium with Gas Chromatography-Mass Spectrometry reveals a peak in the biologically active medium which is not present in control or aspirin-treated heartworm-conditioned medium. This peak has a retention time that is consistent with mammalian PGD_2 standard. Finally, in the in vitro rat aorta, exogenous PGD_2 mimics the filarial-induced depression of relaxation at low concentrations of acetylcholine. These results suggest that filarial PGD_2 is responsible, at least in part, for depression of endothelium-dependent. relaxation in the isolated rat aorta exposed to D. immitis. Thus, circulating PGD_2 may be one filarial factor responsible for depression of acetylcholine dilation in the in vivo femoral artery from heartworm infected dogs. In addition to depressing endothelium-dependent dilation, filarial PGD_2 may alter the mechanism of dilation so that an endothelial cell cyclooxygenase product is involved in the response to acetylcholine and this may account for the

indomethacin-induced depression of acetylcholine dilation in vivo. Although there is no direct evidence that PGD_2 alters endothelial cell cyclooxygenase activity, several studies are suggestive. Arachidonic acid metabolites from one cell type can alter arachidonic acid metabolism in other cells. For example, both leukotriene C_4 (Muller et al., 1987; Pologe et al.,1984) and thromboxane (Clemmons et al., 1986) stimulate endothelial cells to release prostacyclin. Although there is no direct evidence that PGD_2 stimulates endothelial cell prostacyclin production, previous studies indicate that arachidonic acid metabolism can be altered by exogenous PGD_2. Infusion of PGD_2 causes vasoconstriction in the in vivo pulmonary vasculature of newborn pigs. This constriction is attenuated by indomethacin and the thromboxane synthesis inhibitor OKY 1581, suggesting that PGD_2 causes smooth muscle contraction indirectly by stimulating the release of a cyclooxygenase product which may be thromboxane A_2 (Perreault et al., 1990). Thus, the indomethacin-induced depression of acetylcholine dialtion seen in the in vivo femoral artery of heartworm infected dogs may result from an effect of filarial PGD_2 on endothelial cell cyclooxygenase activity.

Studies suggest that the hemodynamic effects of PGD_2 are short-lived. Infusion of a single concentration of PGD_2 (340 ng/g dry lung) into the isolated perfused porcine lung causes an increase in pulmonary vascular resistance that subsides within 2-3 minutes (Perreault et al., 1990). Also, PGD_2, like other prostanoids, is metabolized quickly in biological medium (Fitzpatrick & Wynalda, 1983). It spontaneously degrades in plasma to four metabolites which are present within 5 minutes (Fitzpatrick & Wynalda, 1983). Therefore, the effect of PGD_2 may be short-lived and this may explain why D. immitis depresses relaxation of femoral artery in vivo, but not in vitro. In vivo, circulating PGD_2 may be high, however, when the blood vessel is studied in

vitro, the effect of this circulating factor may be lost. Further studies are necessary to determine the role of PGD_2 in depression of relaxation of canine femoral artery. If PGD_2 is responsible for depression of femoral artery relaxation, then the longevity of this depression can be determined by examining endothelium-dependent relaxation at various times after incubating isolated rings with authentic PGD_2.

The effect of D. immitis on the isolated femoral artery appears to differ from the response of pulmonary blood vessels to heartworm. Previous in vivo exposure to heartworm depresses endothelium-dependent relaxation in the in vitro pulmonary artery and vein (Mupanomunda et al., 1992; Schwartz et al., 1993), thus suggesting that D. immitis causes a more permanent alteration of endothelial cell function in pulmonary blood vessels. A more longterm effect of D. immitis on the pulmonary vasculature is indicated by the fact that depression of relaxation in the in vitro pulmonary artery is present in both fall and spring (Mupanomunda et al., 1992) whereas, the depression observed in the in vivo femoral artery occurs only in spring (Kaiser et al.,1987). There are several possible reasons why D. immitis infection depresses endothelium-dependent relaxation in pulmonary, but not systemic blood vessels in vitro.

Depression of relaxation in pulmonary, but not systemic vessels may be due to the proximity of the pulmonary vasculature to the adult parasites and their products. The adults rather than the microfilariae are responsible for depression of endothelium-dependent relaxation (Kaiser et al., 1987). Since the adults reside in the pulmonary vasculature, these vessels are more proximal than systemic vessels to the adult parasites. A similar regional depression of endothelium-dependent relaxation is seen in rats with Brugia pahangi infection. Endothelium-dependent relaxation is depressed and the mechanism

of relaxation is altered in the in vitro abdominal aorta, but not the thoracic aorta of Brugia infected rats when compared to control. Since the adult B. pahangi reside in the lymphatics particularly in the abdomen, the regional depression of endothelium-dependent relaxation in abdominal aorta may result from the proximity of this blood vessel to the adult parasites. Because the abdominal aorta is more proximal than the thoracic aorta to adult parasites, the abdominal aorta is potentially exposed to higher concentrations of filarial factors and this may account for the depression of relaxation in abdominal, but not thoracic aorta. Alternatively, an inflammatory response in the region of the abdominal aorta may account for depression of endothelium-dependent relaxation in this blood vessel (Kaiser et al., 1991). Inflammatory changes are observed in the abdominal aorta of Brugia infected rats and are characterized by a 3-6 fold increase in mast cells, eosinophils, and other mononuclear cells. This response is not seen in the abdominal aorta of control rats or in the thoracic aorta of rats infected with Brugia. The distribution of inflammatory cells in the region of the abdominal, but not the thoracic aorta, and the depression of relaxation only in the abdominal aorta suggests that inflammatory cells may be responsible, at least in part, for depression of endothelium-dependent relaxation associated with chronic Brugia infection (Kaiser et al., 1991).

In dogs infected with D. immitis, the adult parasites rather than the microfilariae are primarily responsible for inflammatory reactions to heartworm (Liu et al., 1966; Schaub & Rawlings, 1980; Keith et al., 1983). Thus infection with D. immitis causes histopathological changes that are most evident in the pulmonary vasculature. This inflammatory response is present primarily in the pulmonary arteries, which can be in direct contact with adult filaria, but has also been described in the pulmonary veins, which are

downstream from the parasites (Schaub and Rawlings, 1980). The inflammatory response is characterized by endothelial cell damage, myointimal proliferation and accumulation of inflammatory cells within the vessel wall, particularly eosinophils, histiocytes and activated macrophages (Hirth et al., 1966; Keith et al., 1983; Schaub & Rawlings, 1980). This inflammatory response is seen infrequently in systemic vessels, and then only in association with ectopic adult parasites (Liu et al.,1966). Depression of relaxation observed in the in vitro pulmonary vessels from heartworm infected dogs may be the result of this inflammatory reaction. Inflammatory cells either present within the blood vessel wall or in surrounding tissues may produce locally active factors which modulate endothelial cell function.

A similar inflammatory response is observed in human filarial infections. In patients infected with Wuchereria bancrofti or Brugia malayi, vascular and lymphatic lesions are seen in the region of the adult parasites and include intimal thickening, endothelial hyperplasia and perivascular infiltration of inflammatory cells (Case et al., 1991).

Inflammatory cells release several biologically active mediators which may act locally to alter endothelial cell function. Prostaglandin D_2, which has been shown to depress endothelium-dependent relaxation (Kaiser et al., 1992) is the major cyclooxygenase product released from canine mast cells (Thomas et al., 1992), human alveolar macrophages (Giles and Leff, 1988) and IgE-stimulated human and rat mast cells (Lewis et al., 1982). Superfusion of canine trachea in vivo with PGD_2 causes accumulation of eosinophils within the tracheal lumen suggesting that PGD_2 is chemotactic for eosinophils (Emery et al., 1989). Furthermore, eosinophils take up PGD_2 and metabolize it to another product, 9α, 11β-PGF_2, which has multiple actions and may contribute to the overall biological profile of PGD_2 (Parsons & Roberts, 1988). PGD_2 has

been implicated in the pathogenesis of several diseases that are associated with mast cell degranulation and accumulation of eosinophils. These include allergic hypersensitivity, asthma, angioedema and systemic mastocytosis (Beasley et al., 1988; Emery et al., 1989). PGD_2 may be important in the pathogenesis of filariasis which is also characterized by accumulation of eosinophils and mast cells (Case et al., 1991).

In filarial infections, PGD_2 may arise from two sources, the parasites as well as inflammatory cells present within the blood vessel wall or in adjacent tissues. The presence of these inflammatory cells within the wall of pulmonary vessels may cause depression of endothelium-dependent relaxation which is demonstrable in vitro. The lack of an inflammatory response in systemic vessels may account for the lack of depression of relaxation in these vessels in vitro. Further studies are necessary to determine the role of inflammatory cells in depression of endothelium-dependent relaxation in the pulmonary artery and vein.

Infection with heartworm causes other morphological changes in the pulmonary vasculature which are not seen in systemic vessels, and these changes may also account for the in vitro depression of endothelium-dependent relaxation in pulmonary blood vessels. The pulmonary vessels of heartworm infected dogs may have thickened walls as a result of edema, myointimal proliferation and fibrous endarteritis (Atwell, 1980; Keith et al., 1983). Increased wall thickness may create a barrier to diffusion of EDRF to vascular smooth muscle and thus inhibit endothelium-dependent relaxation. Dinh-Xuan et al., (1991) showed an inverse correlation between endothelium-dependent relaxation to acetylcholine and ADP and wall thickness in isolated pulmonary arteries from patients with chronic obstructive lung disease. The depression of endothelium-dependent relaxation

was attributed to a barrier to diffusion of EDRF from endothelial cells to vascular smooth muscle. Depression of endothelium-dependent relaxation in atherosclerotic vessels has also been attributed, in part, to increased wall thickness creating a barrier to diffusion of EDRF (Verbeuren et al., 1986). Although this mechanism may, in part, explain the depression of endothelium-dependent relaxation in pulmonary arteries, it is unlikely to explain the effect of heartworm on pulmonary veins, in which myointimal proliferative lesions are infrequent (Schaub & Rawlings, 1980).

An alternative explanation for the more permanent effect of D. immitis on pulmonary vessels may be due to the exposure of pulmonary vessels to a higher concentration of filarial factors than the concentration to which systemic vessels are exposed. Since the adult parasites reside in the pulmonary arteries and right heart, and the adult parasites are responsible for depression of endothelium-dependent relaxation (Kaiser et al., 1987, 1990), pulmonary vessels are potentially exposed to the highest concentrations of filarial products. These products may undergo significant metabolism before reaching the systemic circulation, therefore, femoral vessels may be exposed to a significantly lower concentration of filarial factors. The ability of parasite products to permanently alter endothelial cell function may be dependent on the concentration of filarial factors to which the endothelial cells are exposed.

Although the way in which filarial PGD_2 is metabolized by mammalian cells is unknown, it may be metabolized by mammalian tissues in a manner similar to the metabolism of mammalian PGD_2 which is metabolized in liver and plasma to several products, some with and some without biological activity (Koizumi et al., 1991; Beasley et al., 1988). In the liver, PGD_2 is metabolized primarily to 9α, 11β-Prostaglandin F_2 (9α 11β-PGF_2). Some of the effects of 9α, 11β-PGF_2 are similar to that of PGD_2. For example, 9α, 11β-PGF_2 inhibits platelet

aggregation (Pugliese et al., 1985), and contracts human bronchial smooth muscle equipotent with PGD_2 (Beasley et al., 1987). However, this metabolite also has actions distinct from that of PGD_2 (Perreault et al., 1990). In general, 9α, 11β-PGF_2 is a potent constrictor of systemic and pulmonary blood vessels, whereas PGD_2 constricts the pulmonary vasculature, but frequently causes vasodilation of systemic vessels (Ito et al., 1989). In the rat, PGD_2 decreases blood pressure when given intravenously, whereas 9α, 11β-PGF_2 infusion is associated with hypertension (Liston & Roberts, 1985). Another biologically active metabolic product of PGD_2 is 9-deoxy-Δ^{12}-PGD_2 (PGJ_2) which is the major metabolic product in plasma. This compound has similar effects but is less potent than PGD_2 (Kikawa et al., 1984).

In contrast, in the isolated perfused rat lung, PGD_2 is very slowly metabolized with the major product after 15 minutes of incubation being 13,14-dihydro-15-keto-PGD_2, a stable metabolite without biological activity (Hayashi et al., 1987). If PGD_2 is metabolized as slowly in canine lung, then its concentration in the pulmonary vasculature may be high. In systemic vessels, however, the concentration of PGD_2 may be significantly lower due to metabolism in plasma and liver. Further investigation is necessary to determine if differences in concentration of filarial factors are responsible for the more longterm effect of D. immitis on endothelium-dependent relaxation in the pulmonary artery and vein when compared to the effects on femoral artery.

An unexpected finding in this study is that endothelial cell removal enhances relaxation to sodium nitroprusside in femoral artery from both control and heartworm infected dogs. Augmentation of sodium nitroprusside relaxation by endothelial cell removal has been reported in the in vitro rat aorta (Shirasaki et al., 1985), but this may be the first report of this effect in

canine blood vessels. The mechanism whereby removal of endothelial cells enhances relaxation to sodium nitroprusside is unknown. One possibility is that, in endothelium-intact preparations, sodium nitroprusside stimulates the release of an endothelium-derived constrictor which partially counteracts the relaxant effect of the nitrovasodilator on vascular smooth muscle. Further studies are required to determine the mechanism whereby endothelial cell removal augments sodium nitroprusside relaxation in canine femoral artery.

Previous studies by Miller et al., (1989, 1991) showed that the mechanism of endothelium-dependent relaxation varies with the preconstricting agent in the isolated canine femoral vein. Relaxation to acetylcholine and the calcium ionophore, A23187, is not inhibited by methylene blue in indomethacin-treated rings of femoral vein preconstricted with norepinephrine (Miller & Vanhoutte, 1989; Miller, 1991), however, in rings preconstricted with $PGF_{2\alpha}$, methylene blue attenuates acetylcholine and A23187 relaxation (Miller & Vanhoutte, 1989). Preliminary results from the present study are in agreement with these data. Methylene blue and L-NAME significantly depress methacholine relaxation in strips of femoral vein from control dogs when $PGF_{2\alpha}$ is the preconstrictor. When the preconstricting agent is norepinephrine, however, L-NAME does not inhibit methacholine relaxation. Thus, the endothelium-derived factor responsible for methacholine relaxation in strips preconstricted with $PGF_{2\alpha}$ may be NO; whereas the factor responsible for relaxation in strips preconstricted with norepinephrine is unknown.

Preliminary studies suggest that in vivo exposure to heartworm does not depress endothelium-dependent relaxation in the isolated femoral vein. These results are in agreement with studies with the in vitro femoral artery and suggest that D. immitis does not cause a permanent change in behavior of

endothelial cells from systemic blood vessels. However, preliminary studies suggest that the mechanism of methacholine relaxation may be different in the femoral vein from heartworm infected dogs when compared to control L-NAME, which almost abolishes relaxation in control, does not signficantly depress the response to methacholine in heartworm femoral vein. Similarly, methylene blue causes less depression in heartworm than in control femoral vein. If this trend continues, then the mechanism of relaxation may be altered in the in vitro femoral vein from heartworm infected dogs such that the NO/guanylate cyclase/cGMP system does not play an important role in relaxation to methacholine. Further experiments are needed to determine if in vivo exposure to D. immitis alters the mechanism of methacholine relaxation in the isolated femoral vein.

The Role of Endothelial Cells in Control of Lymphatic Smooth Muscle Tone

Rings of bovine mesenteric lymphatics spontaneously contract in a rhythmic fashion when studied in vitro. Mechanical or chemical removal of endothelial cells abolished rhythmic contractions. Since norepinephrine constriction was not different before and after endothelial cell removal, loss of phasic activity was unlikely due to injury to lymphatic smooth muscle. Immunohistochemical examination of endothelium-denuded rings for Factor VIII confirmed the absence of intact endothelial cells in these preparations. These data provide evidence that endothelial cell removal abolishes spontaneous contractions of isolated bovine mesenteric lymphatics.

To test the hypothesis that an endothelium-derived diffusible factor is responsible for spontaneous contractions experiments were performed using a modification of the "sandwich" preparation described by Furchgott and

Zawadzki (1980). When endothelium-denuded detector rings were placed in close apposition or "sandwiched" with endothelium-intact donor rings, spontaneous contractions returned in the detector rings. Shortly after separation of donor and detector rings, spontaneous contractions stopped in detector rings. Because the detector and donor rings were not in direct contact, the contractions in detector rings were not the result of simple mechanical displacement of the detector ring by contractions in the donor ring. Spontaneous contractions did not return in detector rings when suspended alone or "sandwiched" with endothelium-denuded donor rings for up to 12 hours, thus suggesting that the return of contractions in detector rings was not due to recovery of injured smooth muscle over time. Therefore, a diffusible factor released by endothelial cells is likely responsible for spontaneous contractions of isolated rings of bovine mesenteric lymphatics.

The amplitude of spontaneous contractions was significantly decreased in denuded preparations during "sandwiching" when compared to the amplitude before removal of endothelial cells. There are two possible explanations for this. Removal of endothelial cells may have damaged lymphatic smooth muscle. However, because the contractile response to norepinephrine was not different before and after endothelial cell removal, this possibility seems unlikely. Another possible explanation is that the diffusion distance between endothelial cells in the intact preparation and smooth muscle in the denuded preparation was sufficient to allow significant degradation and loss of the endothelium-derived contracting factor. Several endothelium-derived vasoactive factors are unstable; EDRF has a half-life of 3-5 seconds (Ignarro et al., 1987; Palmer et al., 1987), that for thromboxane is 30 seconds (Moncada & Vane, 1979) while prostacyclin has a half-life of 3 minutes (Moncada & Vane, 1979). Endothelial cells and smooth muscle are in

intimate contact and may even communicate directly via myoendothelial junctions (Davies et al., 1985). Thus under normal conditions, vasoactive factors released from the endothelial cells diffuse a very short distance to reach smooth muscle. However, in these experiments, there was a distance of about 5 mm between endothelial cells of the donor ring and smooth muscle of the detector ring. If the endothelium-derived factor has a short half-life then it may undergo significant degradation before reaching the detector smooth muscle and this may account for the decreased amplitude of contractions in detector rings.

These results disagree with a previous study by Hanley et al., (1992) who reported that removal of endothelial cells does not alter transmural pressure-induced pumping in isolated perfused 5-lymphangion segments of bovine mesenteric lymphatics. In the study by Hanley, endothelial cells were removed by 3-0 suture threaded unidirectionally through the lumen of 2 cm segments of lymphatics. Endothelium-denuded and endothelium-intact vessels were examined using silver nitrate staining and electron micropscopy and the authors reported that greater than 90% of endothelial cells were removed from denuded preparations.. In preliminary studies in our laboratory, 3-0 suture threaded through the lumen of much shorter segments of lymphatic (5 mm) did not adequately remove endothelial cells. Therefore, in future studies, endothelial cells were removed by everting the ring and rubbing the intimal surface with filter paper according to the method described for canine thoracic duct (Ohhashi et al.,1991). This suggests that suture threaded through the lumen is not the best technique for endothelial cell removal in lymphatics. Even if regions between valves were successfully denuded with suture in the study by Hanley et al., (1992), it is unlikely that endothelial cells were removed completely from valvular sections. Lymphatic valves are bicuspid or tricuspid

leaflets with two surfaces covered by endothelial cells (Takada, 1971). Thus suture threaded in only one direction through the lumen would not remove endothelial cells from both surfaces of the valves. Since the results of silver nitrate staining and electron microscopy were reported only for segments of lymphatics between valves, it is uncertain whether valve regions were examined. Therefore, without information about the effect of endothelial cell removal on valvular regions, it is impossible to conclude that endothelium-denuded lymphatics were indeed devoid of greater than 90% of endothelial cells.

Endothelial cells present only in the valvular regions may be sufficient for pumping activity of lymphatics in response to changes in transmural pressure. Ohhashi et al., (1980) reported that the pacemaker for contractile activity in isolated one-lymphangion preparations of bovine mesenteric lymphatics is in the region of the inlet valve. The fact that the valve region is composed predominantly of endothelial cells and is devoid of smooth muscle (Takada, 1971) suggests that the pacemaker may be valvular endothelium. Coordinated contractile activity in cardiac and smooth muscle results from intercellular communication via gap junctions (Spray & Burt, 1990). In cardiac muscle, gap junctional conductance, as measured by the transfer of Lucifer yellow between cells, is modulated by intracellular Ca^{++}, cAMP and cGMP. It has been suggested that positive and negative inotropes act indirectly to alter the intracellular concentration of second messengers and these second messengers regulate the conductance between gap junctions (Spray & Burt, 1990). Jackson et al., (1991) suggested that the mechanism whereby the NO/guanylate cyclase/cGMP system induces rhythmic contractile activity of hamster aorta is by increasing gap junctional conductance between vascular smooth muscle cells. Since gap junctions are important in the propogation of

contractile activity in the vasculature, they may serve a similar function in lymphatics. The existence of gap junctions between lymphatic smooth muscle cells has previously been reported (Roddie et al., 1980). Evidence for an involvement of these junctions in propogation of lymphatic contractile activity is provided by Zawieja et al., (1990) who reported that n-heptanol, an agent which decreases gap junctional conductance (Spray & Burt, 1990), decreases rhythmicity in rat mesenteric lymphatics. Endothelial cells in the valvular region of lymphatics may initiate coordinated smooth muscle contractions by releasing a factor which acts by increasing gap junctional conductance between smooth muscle cells. Thus, only valvular endothelial cells may be required for rhythmic contractions, therefore, incomplete removal of endothelial cells from the valvular region in the study by Hanley et al., (1992) may account for the persistence of lymphatic pumping after removal of endothelial cells with suture.

Endothelial cell removal by intraluminal infusion of chemicals is more effective than mechanical denudation in small arteries (Furchgott et al., 1987; Tesfamarian et al., 1985; Eskinder et al., 1990). Bovine mesenteric lymphatics are extremely small with an internal diameter less than 0.5 mm. Thus, in 2 cm long 5-lymphangion segments, chemical rather than mechanical denudation may be a more effective means of removing endothelial cells. Since the effect of chemical denudation on pumping activity was not reported in the study by Hanley et al., (1992), the conclusion that endothelial cells are not required for lymphatic pumping is unconvincing.

The nature of the endothelium-derived factor responsible for spontaneous contractile activity of bovine mesenteric lymphatics is unknown. Preliminary studies with mefenamic acid, indomethacin and aspirin agree with the results of previous in vivo and in vitro studies which indicate that

cyclooxygenase inhibitors abolish contractile activity (Johnston & Gordon, 1981; Johnston & Feuer, 1983; Allen et al., 1984). These results suggest that cyclooxygenase products derived from the lymphatic wall are responsible for generation of rhythmic contractions. The inhibitors of thromboxane synthase, imidazole and UK37248 inhibit spontaneous activity, while the stable PGH_2 endoperoxide analogue U46619 stimulates rhythmic contractions in quiescent lymphatic rings. These data suggest that thromboxane A_2 is responsible for lymphatic contractions and that prostaglandin endoperoxide may also have contractile activity (Johnston & Gordon, 1981). However, because cyclooxygenase products were not directly measured, the involvement of arachidonic acid metabolites in the generation of phasic contractile activity is not conclusive.

In preliminary studies, the inhibitor of guanylate cyclase, methylene blue and the inhibitor of NO synthesis, L-NAME, abolish spontaneous contractions in isolated rings of bovine mesenteric lymphatics. The effect of L-NAME is reversed by L-arginine suggesting that the effect of L-NAME is due to inhibition of nitric oxide synthesis. Although the NO/guanylate cyclase/cGMP system is usually associated with relaxation in the vasculature, Jackson et al., (1991) demonstrated that this system is responsible for agonist-induced phasic contractions of isolated hamster aorta. Bohlen and Lash (1992) demonstrated that spontaneously active rat mesenteric lymphatics contract in response to acetylcholine. This contractile response is inhibited by L-nMMA suggesting that the NO/guanylate cyclase/cGMP system contracts rather than relaxes rat lymphatics (Bohlen & Lash, 1992). Thus, it is not unreasonable to postulate that the NO/guanylate cyclase/cGMP system is involved in spontaneous contractions of bovine mesenteric lymphatics.

Further evidence that NO is important in the contractile activity of bovine mesenteric lymphatics is provided by Elias et al., (1992) who reported that intraluminal exposure to oxyhemoglobin abolishes lymphatic pumping in isolated perfused five-lymphangion segments of bovine mesenteric lymphatics. In the vasculature, oxyhemoglobin inhibits endothelium-mediated responses by binding and inactivating NO (Ignarro et al., 1987a & b; Palmer et al., 1987; Hutchinson et al., 1987). Although oxyhemoglobin may have different effects on lymphatics, the inhibition of pumping may be due to inactivation of NO.

To determine the role of the NO/guanylate cyclase/cGMP system in spontaneous activity of bovine mesenteric lymphatics it is necessary to compare NO and the endothelium-derived factor that causes lymphatic contractions in terms of half-life and response to pharmacological inhibitors. This can be done with a cascade bioassay system similar to that described for characterization of EDRF. It is also necessary to measure NO release from isolated rings of bovine mesenteric lymphatics, and to demonstrate that authentic NO restores contractions in endothelium-denuded preparations.

It is of interest that both inhibitors of cyclooxygenase and inhibitors of NO/guanylate cyclase/cGMP abolish spontaneous contractions of bovine mesenteric lymphatics in preliminary studies. There are several possible interpretations of these results. It is possible that both endothelium-derived thromboxane and NO are required for spontaneous contractile activity and inhibition of either product abolishes contractions. Phasic contractions of vascular smooth muscle has been attributed to oscillations in cytoplasmic calcium concentration, (Myers et al., 1985; Desilet et al., 1989; Lamb et al., 1985). Although oscillations in calcium concentration have not been demonstrated in lymphatic smooth muscle, a role for extracellular calcium in contractile

activity has been shown. McHale and Allen reported that contractile activity of isolated lymphatics is totally abolished by replacement of normal Kreb's solution with calcium-free medium (McHale & Allen, 1983). Thromboxane may act by increasing intracellular calcium (Folger et al., 1985) whereas guanylate cyclase/cGMP causes relaxation by decreasing intracellular calcium (Rapoport, 1986). Thus it is possible that alternate release of NO and thromboxane by endothelial cells results in oscillations in smooth muscle calcium concentration which are responsible for rhythmic smooth muscle activity. This possibility seem unlikely, however, because there was not a one-to-one correspondance between contractions in detector and donor rings.

An alternative explanation is that the guanylate cyclase/cGMP system in lymphatic smooth muscle is responsible for rhythmic contractions. A role for cGMP in phasic contractions has previously been demonstrated in vascular tissue. Jackson et al. (1991) demonstrated that basally released endothelium-derived nitric oxide stimulates vascular smooth muscle guanylate cyclase/cGMP and this transduction mechanism is responsible for agonist-induced phasic contractions in hamster aorta. A similar mechanism may be responsible for spontaneous contractions of bovine mesenteric lymphatics. Although the mechanisms of guanylate cyclase activation in lymphatic smooth muscle are unknown, arachidonic acid metabolites have been shown to increase soluble guanylate cyclase activity in other cell types. Guanylate cyclase activity is increased 2.5 to 5-fold by PGG_2 and PGH_2 in guinea pig splenic cells (Graff et al., 1978) and Hidaka and Asano (1977) demonstrated that oxidized unsaturated fatty acids including arachidonic acid stimulate guanylate cyclase activity in platelets. Thus inhibition of spontaneous contractions by both cyclooxygenase and NO/guanylate

cyclase/cGMP inhibitors may result from inhibition of a final common pathway.

Another possibility is that all three inhibitors abolish spontaneous contractions by the common inhibition of an unidentified endothelium-derived factor. This possibility is likely since nonspecific effects are associated with all these inhibitors (Peterson et al., 1992; Martin et al., 1989; Kennedy et al., 1990; Rosenblum, 1982). In early studies concerning the biochemical nature of EDRF, several agents with diverse actions were shown to depress endothelium-dependent relaxation. Because lipoxygenase inhibitors and phospholipase A_2 inhibitors depressed endothelium-dependent responses, investigators postulated that EDRF was a lipoxygenase product of arachidonic acid (Furchgott & Zawadzki 1980; Van de Voorde & Leusen, 1983), while other studies with antioxidants suggested that EDRF may be an oxygen radical (Furchgott, 1983). It is now known that depression of endothelium-dependent relaxation by these various inhibitors was due to an effect other than their intended action (Furchgott & Vanhoutte, 1989).

The common inhibition of phasic contractions by methylene blue, cyclooxygenase inhibitors and L-NAME may be due to an effect of these inhibitors on free radicals. Methylene blue generates superoxide anion and this is mechanism is thought to be important in the inhibition of endothelium-dependent relaxation by methylene blue (Wolin et al.,1990). Since superoxide anion can interact with and subsequently inactivate other radical species besides NO (Rubanyi, 1988b), the inhibitory effect of methylene blue on spontaneous contractions of lymphatics may be due to inactivation of free radicals by superoxide anion. Also, since free radicals can activate guanylate cyclase (White et al., 1976; Mittal & Murad, 1977), methylene blue

may inhibit spontaneous contractions by preventing free radical-induced activation of guanylate cyclase.

The inhibition of spontaneous contractile activity by L-NAME may also be due to an effect on free radicals. L-NAME contains functional groups which can interact with iron to form chelates. Since NO synthase and soluble guanylate cyclase both contain iron, it has been postulated that L-NAME inhibits endothelium-dependent relaxation by binding iron centers contained within these enzymes. Peterson et al., (1992) demonstrated that L-NAME inhibits the transfer of electrons from ferrous iron to ferric cytochrome C thus suggesting that L-NAME may interact nonspecifically with other enzymes which contain iron. (Peterson et al., 1992). These results suggest that L-NAME can potentially inhibit any process that involves the transfer of electrons from ferrous to ferric iron. An important source of oxygen radicals is the Haber-Weiss reaction which involves the interaction of iron-containing molecules with hydrogen peroxide, the reduction of ferrous iron and the formation of hydroxyl radical (Rubanyi, 1988). L-NAME may inhibit this reaction by binding iron and preventing the transfer of electrons; this effect may have potential importance in the inhibitory action of L-NAME on spontaneous contractile activity. The mechanism whereby L-NAME inhibits the transfer of electrons is unknown. If L-NAME interacts with iron-containing groups in a competitive fashion, it is possible that this effect can be reversed with L-arginine which also contains iron-binding groups. Thus the inhibition of spontaneous contractile activity by L-NAME and its reversal with L-arginine may be due to an effect on formation of oxygen free radicals rather than on NO synthesis.

Free radicals are also produced during the metabolism of arachidonic acid (Kukreja et al., 1986; Mason et al., 1980; Sing et al., 1981). Some

cyclooxygenase inhibitors also act as iron chelators (Kennedy et al., 1990), therefore, as is true for L-NAME, cyclooxygenase inhibitors may abolish spontaneous contractions by decreasing the production of free radicals.

Although there is no direct evidence for a role of oxygen radicals in spontaneous contractions, several investigators have shown that endothelium-derived free radicals can modulate vascular smooth muscle tone (Kontos et al., 1984; Rosenblum, 1987; Katusic & Vanhoutte, 1989). Hydroxyl radical derived from endothelial cells mediates the relaxation response to bradykinin in cerebral arterioles of the mouse and cat (Rosenblum, 1987; Kontos et al., 1984). Katusic and Vanhoutte (1989) demonstrated that indomethacin abolishes endothelium-dependent contractions in canine basilar artery. Since the contractile response is also inhibited by superoxide dismutase, the author suggested that superoxide anions produced during metabolism of arachidonic acid is responsible for endothelium-dependent contractions in canine basilar artery. Furthermore, free radicals can activate guanylate cyclase. Hydrogen peroxide stimulates soluble guanylate cyclase in rat lung (White et al., 1976) and human platelet guanylate cyclase is activated by unsaturated fatty acid peroxides (Hidaka & Asano, 1977). Also, Mittal and Murad (1977) showed that superoxide dismutase and hydroxyl radical cause an increase in cGMP. Thus inhibition of free radical generation by cyclooxygenase inhibitors, methylene blue and L-NAME may account for the effect of these agents on spontaneous contractions. This hypothesis can be tested by investigating the response of isolated lymphatics to free radical scavengers. Also a cascade bioassay system similar to that used for characterization of EDRF would be useful to determine the chemical nature of the endothelium-derived factor which is responsible for phasic contractions of bovine mesenteric lymphatics.

The Effect of Dirofilaria immitis on Endothelium-Dependent Responses of Lymphatics

Preliminary experiments with isolated canine thoracic duct agree with previous studies by Ohhashi et al., (1991) and demonstrate that endothelial cells are required for the relaxation response to muscarinic agonists in the in vitro canine thoracic duct. Ohhashi et al., (1991) showed that L-nMMA and oxyhemoglobin, but not indomethacin, attenuate acetylcholine relaxation in thoracic duct. Preliminary data from the present study suggest that methylene blue and L-NAME, but not mefenamic acid, inhibit methacholine relaxation in thoracic duct from control dogs. These data agree with Ohhashi et al., (1991) and suggest that NO is involved in endothelium-dependent relaxation to muscarinic agonists in canine thoracic duct whereas cyclooxygenase products are not.

In preliminary experiments, the relaxation response to methacholine is significantly depressed in thoracic duct from dogs infected with D. immitis when compared to control. Because methacholine relaxation is depressed by L-NAME and methylene blue to a lesser extent in heartworm thoracic duct than the effect of these inhibitors in control, the NO/guanylate cyclase/cGMP system may be less important in the relaxation of heartworm thoracic duct to methacholine. These preliminary studies suggest that previous in vivo exposure to D. immitis depresses endothelium-dependent relaxation in canine thoracic duct. Because the effect of D. immitis is demonstrable in vitro in the absence of the parasites, exposure to heartworms may cause a more permanent alteration of function in this lymphatic.

Endothelium-dependent relaxation is depressed in vitro in the pulmonary artery (Mupanomunda et al., 1992) and vein (Schwartz et al., 1993), but not in the femoral artery of heartworm infected dogs when compared to control. Depression of relaxation in isolated pulmonary, but not systemic vessels may be due to the proximity of the pulmonary vasculature to the adult parasites and parasite products. Since the thoracic duct is located in the chest cavity, it is also closer than systemic vessels to the adult parasites. Vessels that are proximal to the adult parasites may exhibit more permanent changes in endothelium-dependent relaxation because of a regional inflammatory response or because of chronic exposure to high concentrations of filarial factors. Vasoactive substances released from inflammatory cells may be responsible for the more permanent effect of D. immitis on this lymphatic. Although there are no reports concerning the effect of D. immitis on thoracic duct morphology, this vessel, because of its close proximity to the adult parasites, may exhibit inflammatory changes. Alternatively, the depression of endothelium-dependent relaxation of isolated thoracic duct may result from chronic exposure of thoracic duct to high concentrations of filarial factors. Since the thoracic duct communicates directly with the lymphatics that drain the lungs (Warren & Drinker, 1942), it is potentially exposed to a higher concentration of parasitic products than the systemic circulation.

Depression of endothelium-dependent relaxation is also observed in the in vitro thoracic duct from control dogs upon shortterm exposure to heartworm-conditioned medium. Because norepinephrine constriction is not different in rings exposed to heartworm-conditioned and control medium, an effect of D. immitis on lymphatic smooth muscle function is unlikely. Since relaxation to sodium nitroprusside is not depressed, it is unlikely that the effect of D. immitis is due to alteration in the guanylate cyclase/cGMP system.

These preliminary studies provide evidence that the depression of endothelium-dependent relaxation observed in the in vitro thoracic duct from heartworm infected dogs is due to biologically active factors released by the adult parasites.

Preliminary experiments suggest that heartworm-conditioned medium abolishes endothelium-dependent spontaneous contractions of isolated bovine mesenteric lymphatics. The response to heartworm-conditioned medium is immediate and is reversed by washing the lymphatic. Because filarial PGD_2 is responsible, in part, for the depression of endothelium-dependent relaxation of isolated rat aorta, filarial PGD_2 may also be responsible for the effect of heartworm-conditioned medium on spontaneous lymphatic contractions. In preliminary experiments, PGD_2 abolishes contractile activity of bovine mesenteric lymphatics. If further experiments substantiate these results, then filarial PGD_2 may be important in the inhibitory effect of D. immitis on lymphatic contractile activity.

These data suggest that biologically active filarial factors alter the behavior of endothelial cells in both the vascular and the lymphatic system. Alteration of endothelial cell function may be important in the pathogenesis of human filariasis. This study and the study by Ohhashi et al., (1991) indicate that lymphatic endothelial cells play an important role in controlling the tone of underlying smooth muscle. Alteration of this control by biologically active filarial factors may be important in the pathogenesis of lymphedema and elephantiasis associated with human filarial infection.

A major function of the lymphatic system is the return of fluid and protein from the interstitial space back to the circulation. Under normal circumstances an equilibrium exists between the transport of lymph and the rate of lymph production. Edema results if lymph production increases beyond

the transport capacity of the lymphatic system, thus either an increase in lymph production or a decrease in lymphatic transport capacity can cause edema. Several studies indicate that an increase in lymph production causes the amplitude and frequency of lymphatic pulsations to increase. These results provide indirect evidence that lymphatic contractile activity plays an important role in maintaining the equilibrium between lymph production and lymphatic transport capacity (Hall et al., 1965; McHale & Roddie, 1976; Roddie et al., 1980; Hargens & Zweifach, 1977; Benoit et al., 1989). If spontaneous contractions are important in this equilibrium, then a decrease in contractile activity may result in edema. Thus, if filarial factors inhibit contractile activity of peripheral lymphatics, as the preliminary results of this study indicate, then this inhibitory effect may be important in the pathogenesis of lymphedema and elephantiasis in humans infected with filarial parasites.

Summary and Conclusions

The depression of endothelium-dependent relaxation seen in the in vivo femoral artery from heartworm infected dogs is not demonstrable in vitro in the absence of the parasites. Similar results are obtained in preliminary studies with femoral vein, thus suggesting that infection with D. immitis does not cause a longterm change in endothelium-dependent relaxation of systemic vessels. Preliminary studies, however, suggest that endothelium-dependent relaxation is depressed in isolated thoracic duct from heartworm infected dogs when compared to control. This depression is likely due to biologically active factors released by D. immitis because shortterm exposure to heartworm-conditioned medium depresses methacholine relaxation in thoracic duct from control dogs.

Based on these experiments and the results of previous studies, a mechanism can be proposed whereby D. immitis alters endothelium-dependent

relaxation (Figure 32). Exposure to filarial PGD_2 results in a shift in endothelial cell metabolism of relaxing factors such that the NO/guanylate cyclase/cGMP pathway is depressed and the cyclooxygenase pathway is activated. The result is an overall depression and alteration in the mechanism of endothelium-dependent relaxation. In sytemic blood vessels this effect is not permanent and requires the continuous presence of filarial factors.

Figure 32

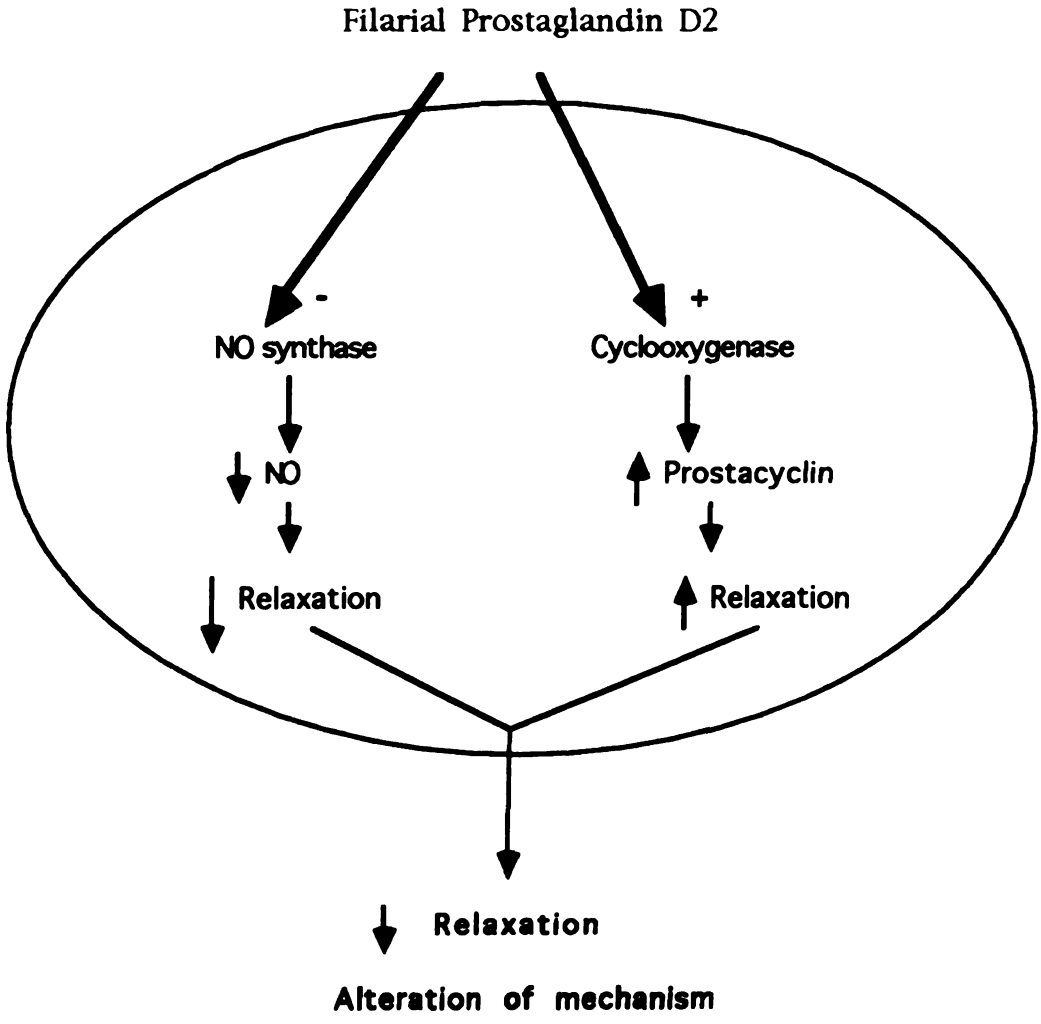

FIGURE 32: Schematic of potential mechanism of action of filarial factors on endothelium-dependent relaxation. Endothelial cell nitric oxide synthase activity is depressed and cyclooxygenase activity is enhanced in the presence of filarial prostaglandin D_2. The decrease in nitric oxide synthase activity results in decreased relaxation via the NO/guanylate cyclase/cGMP pathway. The increase in cyclooxygenase activity results in enhanced relaxation via the cyclooxygenase pathway. The overall effect of filarial prostaglandin D_2 is to depress and alter the mechanism of endothelium-dependent relaxation. In systemic blood vessels this effect is not permanent and occurs only in the presence of filarial PGD_2.

Spontaneous contractions are important in the propulsion of lymph in peripheral lymphatics. The results of this study suggest that endothelial cells release a diffusible substance which is responsible for spontaneous contractile activity in isolated rings of bovine mesenteric lymphatics. Preliminary studies suggest that this factor may be a cyclooxygenase product, NO or an unidentified substance, however, further experiments are needed to conclusively identify the endothelium-derived factor that is responsible for spontaneous contractions of bovine mesenteric lymphatics.

Preliminary studies suggest that biologically active factors released by adult D. immitis depress endothelium-mediated contractions of bovine mesenteric lymphatics. If contractions are important in the propulsion of lymph, then filarial-induced inhibition of contractile activity may be important in the pathogenesis of lymphedema seen in people infected with filarial disease.

Over 250 million people worldwide are infected with filarial parasites. Despite the prevalence of these diseases, the pathogenesis is poorly understood. Furthermore, treatment, eradication or prevention of filarial infection has generally been unsuccessful. A novel hypothesis is that alteration of endothelial cell function by biologically active parasite products is important in the pathogenesis of filariasis. The development of pharmacological agents which counteract the effect of parasite products on endothelial cell function may prove useful in the treatment of human and animal filarial disease.

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