Assessment of the impact of acute and chronic nitric oxide synthase blockade on functional and structural components of the cardiovascular system.

By

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"Imagination is but a free thinking. The imaginative are blessed with a facility in association of facts. High latent force develops when such a man is faced with a perplexing problem and wide spark gaps are bridged."

- Dr. F. G. Banting, 1935

Abstract

The development of enhanced cardiac and vascular structural indices represent significant risk factors with respect to the morbidity and mortality associated with essential human hypertension. To date the majority of research efforts have focused on the contribution of neuro-humoral systems and not on local systems (such as nitric oxide and endothelin) to the development of cardiac and vascular structural alterations, most noteably the sympathetic nervous system and the renin-angiotensin system. The present studies characterized the functional and structural alterations that initiate and maintain the hypertension following the blockade of a local system, nitric oxide (NO).

In terms of functional mechanisms, endothelin-mediated vasoconstriction was demonstrated to almost completely prevent (or reverse) the hypertension that follows acute NO synthase blockade with N^{ω}-nitro-L-arginine methyl ester (L-NAME). In contrast, it was also demonstrated that the sympathetic nervous system, renin-angiotensin system, arginine vasopressin system and the heart rate-baroflex system do not contribute to the pressor response following NO synthase blockade. These studies also revealed that the contribution of endothelin-mediated vasoconstriction to the resting level of mean arterial pressure was minimal, if any. As such, the removal of NO resulted in a specific up-regulation of endothelin-mediated vasoconstriction as opposed to a 'generalized' increased sensitivity to all other endogenous vasoconstrictor systems. Taken together,

these results led to the concept that NO does not function as a chronic vasodilator *per se*, but rather functions to inhibit endothelin-mediated vasoconstriction.

Endothelin-mediated vasoconstriction was demonstrated to play an important role in the maintenance of chronic L-NAME induced hypertension both directly and indirectly. In terms of a direct contribution, endothelin-mediated vasoconstriction was playing a significant role in the hypertension, but the overall direct involvement was diminished in comparison to the acute phase. Indirectly, endothelin-mediated vasoconstriction resulted in a marked increase in sensitivity to α_1-adrenoceptor activation following chronic L-NAME treatment. Similarly, in the isolated perfused pudendal vasculature, it was demonstrated that both sub-pressor concentrations of endothelin-1 or L-NAME resulted in a marked increased sensitivity to α_1-adrenoceptor activation. This enhanced sensitivity to α_1-adrenoceptor activation was completely reversed in the L-NAME treatment group by the administration of an ET_A/ET_B receptor antagonist. These results taken together with previous findings provides strong evidence in support of the concept that NO does not function as a chronic vasodilator, but rather a chronic suppresser of endothelin-mediate vasoconstriction.

Despite the prolonged hypertension induced by the imbalance between NO and endothelin that occurs in L-NAME treated rats, cardiac and structurally-based vascular resistance properties were not enhanced to a significant extent. The overall lack of cardiovascular structural up-regulation was consistent with the lack of prolonged

activation of the growth related enzyme ornithine decarboxylase in comparison to the activation profile observed with equipressor angiotensin II infusion. The lack of enhanced cardiovascular growth following L-NAME treatment may indicate that a sustained increase in mean arterial pressure alone is not a sufficient stimulus to induce cardiovascular growth processes or that L-NAME may non-specifically inhibit cardiovascular growth processes.

Overall the present series of studies demonstrated the important contribution(s) local systems can make to the homeostasis of mean arterial pressure. The studies also indicated that a sustained increased in the level of mean arterial pressure alone, may not provide a sufficient stimulus to induce the development of cardiovascular structural alterations. In that, although a local vasoactive systems can play an important role in the regulation of mean arterial pressure, the development of cardiovascular hypertrophy likely requires that activation of a more potent growth stimulus, such as the sympathetic nervous system or the renin-angiotensin system. The imbalance of NO and endothelin are of importance in several peripheral vascular clinical pathologies including: renal failure, cyclosporin A induced hypertension and male erectile dysfunction, where therapies aimed at restoring the NO-endothelin balance have and will , at least in part, reverse these conditions.

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Dedication

To Donna:

With all my love.

Thank you does not say enough for all your support throughout the completion of this degree. I look forward to all of the love, life and laughs we will be spending together.

Statement of Co-Authorship

The following thesis was performed and written by Mr. James Banting with the following co-authorships and assistance:

- Chapter 1: one section was co-authored by Dr. Michael Adams (Adams MA, Madigan M, Banting JD, Friberg P, Blood Pressure,1994)

- Chapter 2: was co-authored by Dr. Michael Adams and Dr. Peter Friberg. (Banting JD, Friberg P and Adams MA, Journal of Hypertension, 1996)

- Chapter 3: was co-authored by Dr. Michael Adams.

- Chapter 4: was co-authored by Dr. Michael Adams and Ms. Shari Wiseman (Banting JD, Wiseman SL, Adams MA, Journal of Hypertension, 1996)

- Chapter 5: was co-authored by Dr. Michael Adams, Dr. Peter Friberg and Dr. Karen Thompson.
  (Banting JD, Thompson KE, Friberg P, Adams MA, Hypertension, 1997)

- Chapter 6: some technical assistance was provided by Dr. Nabil Khouri, and was co-authored by Dr. Michael Adams, Dr. Stephen Pang and Dr. Nabil Khouri.

- Chapter 7: the experiments were designed based on the results of Chapters 2 & 3 by Mr. James Banting and Dr. Michael Adams. The data analysis was performed by Mr. James Banting. The technical aspects of this study were performed by Dr. Kazuchi Manabe and Dr. Sunping Ge, and was co-authored by Dr. Michael Adams, Dr. Jeremy Heaton and Dr. Kazuchi Manabe.

- Chapter 8: was co-authored by Dr. Michael Adams.

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List of Abbreviations

ACE inhibitor ..... angiotensin converting enzyme inhibitor
ACF ..... aorto-caval fistula
Ang II ..... angiotensin II
ANP ..... atrial natriuretic peptide
AVP ..... arginine vasopressin
CNS ..... central nervous system
CsA ..... cyclosporin A
DOCA ..... deoxycorticosterone acetate
ECE ..... endothelin converting enzyme
ED ..... erectile dysfunction
ET ..... endothelin
FCS ..... fetal calf serum
GTN ..... glyceryl trinitrate
HR ..... heart rate
L-NAME ..... N^{ω}nitro-L-arginine methyl ester
LV ..... left ventricle
MAP ..... mean arterial pressure
MXA ..... methoxamine
NO ..... nitric oxide
O_2 ..... oxygen
ODC ..... ornithine decarboxylase
RAS ..... renin-angiotensin system
RVR ..... renal vascular resistance
SHR ..... spontaneously hypertensive rat
SNP ..... sodium nitroprusside
SNS ..... sympathetic nervous system
TPR ..... total peripheral resistance
VIP ..... vasoactive intestinal polypeptide
IGF-1 ..... insulin-like growth factor-1

Chapter 1: General Introduction

Preamble

A well recognized theme in research on the etiology of hypertension relates to the concept that accelerated smooth muscle growth is a key feature of the structural changes in blood vessels in hypertension. The resultant vascular hypertrophy (i.e. medial thickening and luminal narrowing) impacts directly on vascular resistance and therefore is an important determinant of the long term level of arterial pressure. The roles played by different factors including mechanical forces, vasoactive substances, growth factors and endocrine/autocrine hormones in mediating vascular smooth muscle growth responses *in vivo* have not been clearly identified, particularly with respect to potency and contribution to the overall growth response in acute, short term and chronic conditions.

Appropriate assessment of the capacity of neuro-humoral and local systems to induce trophic changes will help determine the rational choice of antihypertensive drugs to block induction of growth processes by these systems. Recent advances have recognized that endogenous vasodilator systems can act in antitrophic capacities on cardiovascular growth processes (Dzau and Gibbons 1987). The concept that vasoactive agents may modulate vascular growth *in vivo* has received substantial attention in part because it suggests a mechanism whereby the mass of vascular tissue is regulated by these factors to match the workload (Owens 1991). However, despite the interest in direct trophic effects of contractile agonists there is considerable circumstantial evidence implicating a role for various mechanical factors (blood pressure, wall stress, etc.) in the vascular growth response (Krieger and Dzau 1991). Accordingly, an additional concept

that requires validation is whether the trophic changes induced via neuro-humoral and local systems as well as the antitrophic activity of vasodilator systems (e.g. nitric oxide (NO), ANF, prostaglandins, kinins) are also dependent on changes in pressure as opposed to direct effects, independent of pressure.

Lever's (1986, 1992) reviews on the mechanisms involved in the pathogenesis of hypertension indicated that not only have the causes of primary hypertension not been fully elucidated, but the mechanisms that directly produce blood pressure elevation in secondary hypertension are also not generally agreed upon even though the 'initial cause' of the changes is often obvious. Research has pointed to causes/mechanisms which are 'quick-acting' whereas the rise in pressure they produce is slow and ultimately larger than can be accounted for by the 'initiating mechanism' (Lever 1986, Lever and Harrap 1992). More recently, there has been an increased recognition of the prominence of these 'slow pressor mechanisms' involving a role for hypertrophy of resistance vessels (Korner *et al.* 1987). Folkow and his colleagues (Folkow *et al.* 1970, Folkow *et al.* 1972, Folkow *et al.* 1984) were the first to demonstrate the critical role that vascular structural hypertrophy (↑ wall thickness:lumen ratio) plays in maintaining elevated pressure in the established hypertensive circulation. In combination with others (Folkow *et al.* 1972; Lee *et al.* 1983, Lee *et al.* 1983a, Mulvany *et al.* 1978, Anderson *et al.*, 1981) their work revealed the consequences that even small changes in structurally-based resistance are hemodynamically amplified by a 4^{th} power relationship. It is not surprising, therefore, that in the last few years it has become apparent that the quantitative contribution these

structural alterations make in chronic elevation of blood pressure is probably greater than the basic initiating cause or stimulus (Lever 1986, Anderson *et al.* 1981, Korner 1982). An example comes from renal hypertension (2K1C) studies in which the basic cause accounts for less than 30% of the pressure during the established phase of hypertension (Korner and Anderson 1983) and activation of the renin-angiotensin system (RAS) accounts for the pressure rise only in the first few days. It appears that the pressure increase initially induced by the RAS is replaced by cardiovascular structural mechanisms. In addition Adams *et al.* (1989, 1989a) and others (Lee *et al.* 1983, Lee *et al.* 1983a) have shown that in genetic models of hypertension, vascular hypertrophy may be 'primary' and therefore be a major contributor to the development of hypertension. There have been two main themes proposed to account for the sequence of events involved in the development of hypertension in relation to cardiovascular structural changes (Figure 1-1).

Folkow and co-workers (Folkow *et al.* 1970, Folkow 1989, Folkow 1986, Folkow 1982) suggested that vascular hypertrophy and blood pressure are related through a positive feedback loop (Figure 1-1). In this situation, vasoconstrictor hyperactivity increases arterial pressure which is then subsequently reinforced by the development of pressure-dependent vascular hypertrophy, slowly leading to a more substantial level of hypertension. This hypothesis suggest that the development of cardiac and vascular hypertrophy would further increase the level of hypertension.

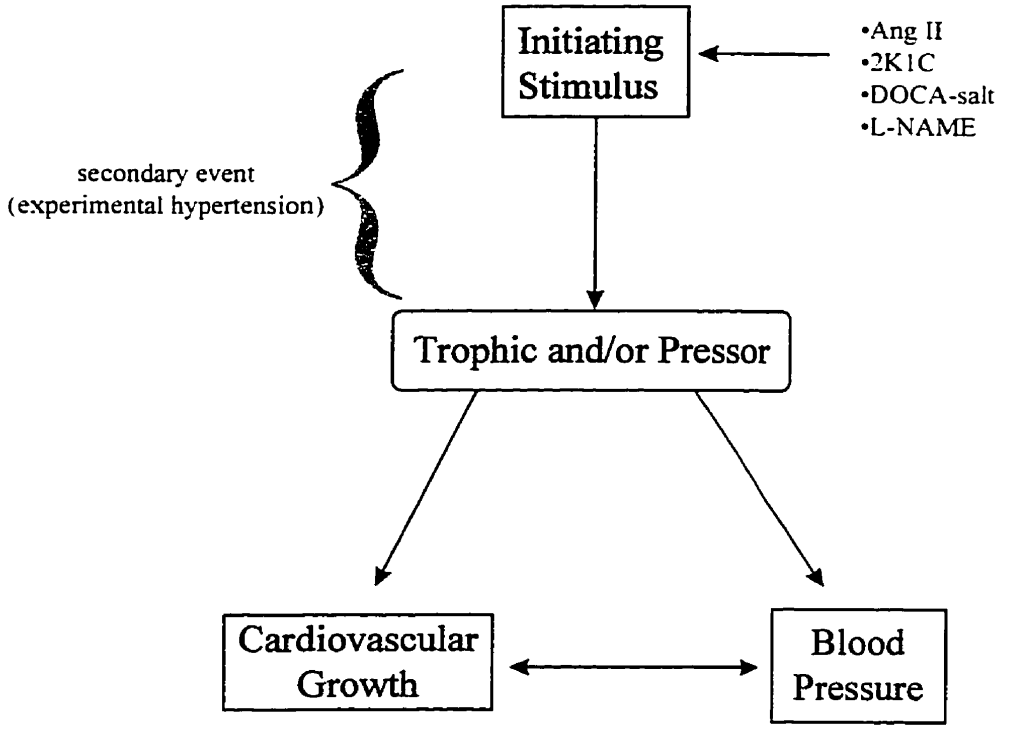

Figure 1-1: Modified from Folkow *et al.*(1970) outlines the series of events involved in the development and maintenance of hypertension that reveals the potential the relationship between pressure-independent and pressure-dependent changes in cardiovascular structural alterations.

Another sequence leading to cardiovascular structural changes and hypertension would involve a trophic mechanism producing hypertrophy directly. An alteration that then produces blood pressure elevation by increasing resistance based on a structural mechanism (Adams *et al.* 1990, Folkow *et al.* 1972, Adams *et al.* 1989, Griffin *et al.* 1991, Plunkett and Overbeck 1985, Sen *et al.* 1986). This process would occur in the different experimental hypertensive models although structural changes would be expected to occur concomitantly with increase in blood pressure thus obscuring the causal relationship.

Folkow *et al* (1972) suggested that a small change in the activity of pressor and/or trophic mechanism, for example the renin-angiotensin system (RAS) or the sympathetic nervous system, leads to a slight increase in MAP. They proposed that subsequently the small increase in MAP creates a positive feedback loop between cardiovascular hypertrophy and blood pressure; i.e. prolonged low level vasoconstrictor hyperactivity would increase pressure which is in turn reinforced by pressure-dependent cardiovascular hypertrophy, leading to a slow progression towards a higher level of hypertension. To complicate matters, it has been shown that these trophic or pressor mechanisms may also play a role in cardiovascular hypertrophy in a manner independent of the changes in blood pressure (Folkow *et al.* 1970). Taken together, it appears that cardiac and vascular hypertrophy, blood pressure and trophic/pressor factors all interact in a manner not fully understood, to increase MAP over the long term level of arterial pressure (Lever 1986).

In both processes, sustained long term pressure elevation appears to be dependent on the vascular structural changes. However, the unequivocal demonstration that the level of structural hypertrophy of the vasculature is directly reflected in blood pressure elevation has yet to be made. Identifying initiating mechanisms which produce hypertrophic cardiovascular structural changes either directly or through pressure-dependent effects will help elucidate some of the 'causes' of hypertension.

Evidence for a role for vasoconstrictor systems in the development of cardiovascular structural upregulation

Cardiac hypertrophy

Catecholamines have long been thought to play a role in the development of cardiac hypertrophy (Korner 1982, Thyberg and Fredholm 1987, Thyberg and Fredhom 1987a, Johnson *et al* 1983, Majesky *et al* 1985, Adams and Hirst 1986) in a manner that does not appear to be dependent on changes in pressure. In fact, the SNS has been termed as a final common pathway by which trophic changes in the heart become manifest (Tsoporis *et al* 1991, Thyberg and Fredholm 1987, Adams and Hirst 1982). More recently, the RAS, originally described as a circulating endocrine system, has been shown to have activity in the heart at the level of endocrine, intracrine, autocrine and/or paracrine regulatory processes involving growth, metabolism and function (Tsoporis *et al* 1991, Thyberg and Fredholm 1987, Adams and Hirst 1982). Demonstration of RAS involvement has come from evidence of direct inotropic and chronotropic effects,

positive interactive effects with the cardiac sympathetic nerve activity, and because of direct growth-promoting effects (Sen and Tarazi 1983).

Increased blood vessel wall mass (tissue hypertrophy) occurs in vessels of all sizes, ranging from conduit arteries to resistance arterioles. The enhanced muscle mass may be due to an increase in cell number (hyperplasia) or an increase in cell size (cellular hypertrophy). Vascular smooth muscle cell hyperplasia appears to predominate in small arteries and arterioles whereas hypertrophy or hypertrophy plus DNA replication (polyploidy) occurs in conduit vessels (Kreiger and Dzau 1991). A wide variety of vasoactive agents appear to be able to modulate this vascular growth (i.e. vasoconstrictors are growth promoters and vasodilators possess growth-inhibiting properties).

Vascular Structural Changes

The concept that the SNS can cause hypertrophic structural changes in the vasculature is supported by evidence from a number of studies (Yamori *et al* 1984, Yamori *et al* 1980, Womble *et al* 1980, King *et al* 1987). In cultured vascular smooth muscle cells catecholaminergic stimulation has been shown to increase protein synthesis, enhance the rate of cell proliferation and increase the number of hyperploid cells (Heagerty 1991, Dzau 1988, Owens 1991). Thompson *et al* (1994) have shown acutely *in vivo* that there is a direct relationship between α_1-adrenoceptor activation and vascular trophic responses measured as activation of an obligatory growth related enzyme, ornithine decarboxylase (ODC). This relationship was characterized by a logistic

function stimulus-response relationship in both the aorta and mesenteric arterial vasculature, a tissue preparation that includes 3rd and 4th order resistance vessels. Thadani and Schanberg (1979) and Johnson *et al* (1983) similarly demonstrated *in vivo* . in the aorta only, that prolonged adrenoceptor stimulation activated ODC for the duration of time in which there was ongoing development of cardiac and aortic hypertrophy. Chronic experiments have shown that neonatal sympathectomy in the SHR can attenuate the subsequent development of cardiovascular structural changes and the elevation of blood pressure in adults (King *et al* 1987, Lee *et al* 1987). Plunkett and Overbeck (1985), using an aortic constriction model of hypertension showed that adrenoceptor mediated hypertrophy of the heart and vasculature *in vivo* can occur independently of changes in pressure (i.e. growth also occurs in the low pressure portion of the vasculature distal to the aortic constriction).

The studies from the Lever (1991) and Kanbe *et al* (1983) have revealed that *in vivo* Ang II infusions similar to catecholamines, can directly promote increased vascular smooth muscle expression of oncogenes (c-myc and c-fos), provoke growth (hypertrophy and hyperploidy), and enhance neointimal proliferation of vascular myocytes after endothelial denudation in a manner, at least in part, that is independent of changes in blood pressure. Harrap *et al* (1986) have also demonstrated a trophic role for Ang II in vascular growth during growth and development which can lead to persistent effects on blood pressure. Taken together the various studies have indicated that both the SNS and RAS can promote vascular structural changes but the studies (i) have not quantified what

capacity the systems have for directly stimulating trophic responses in blood vessels *in vivo* independently of blood pressure elevation, (ii) they have not demonstrated this trophic capacity in small arterial vessels which contribute more to vascular resistance and (iii) they have not demonstrated the hemodynamic importance of the structural changes.

Recent studies have addressed this issue, in part, using ornithine decarboxylase (ODC) activation as a marker of trophic stimulation (Thompson *et al* 1992, Thompson and Adams 1994). The experiments demonstrated that acute α_1-adrenoceptor-induced growth responses were completely blocked by either hydralazine (a vasodilator agent), felodipine (a calcium channel blocker) or ethanol suggesting that vaso-relaxing pharmacological agents either interfere with both growth processes as well as contractile processes or that the responses are in part pressure-dependent. These data revealed that it would be difficult, using pharmacological agents, to discriminate between direct acting trophic stimulation and pressure-dependent processes which have mechanisms convergent with contractile processes.

In addition to neural-humoral mediated vascular hypertrophy, local systems may also play an important role in the development of structural upregulation in certain conditions. Endothelins, a local vasoconstrictor system, are a family of 21-amino acid sequence peptides that are powerful vasoconstrictors. They include endothelin-1 (ET-1), ET-2, ET-3 and their respective precursors and metabolites. Currently ET-1 and ET-3 are known to be produced ubiquitously (Hanyes *et al* 1993, Luscher *et al* 1993). *In vitro* ET-

1 has been shown to stimulate mitogen-activated protein kinases (42- and 44- kDa) in rat aortic smooth muscle cells (Weber *et al* 1994). The induction of immediate-early genes by ET-1 has been demonstrated in adult rat cardiomyocytes independently of changes in blood pressure (Neyes *et al* 1993). The growth induction of proto-oncogenes, such as c-myc, with ET-1 treatment has been demonstrated in both cardiomyocyte and rat aortic smooth muscle cell lines (Neyes *et al* 1993, Komuro *et al* 1988). The growth inducing effects reported in the above studies were blocked with the co-administration of an endothelin receptor antagonists. The recent findings, in particular from Schiffrin's group (Schiffrin 1995), in DOCA-salt hypertension also point to a trophic role for endogenous endothelin in the development of vascular structural changes. They found increased pre-pro ET-1 mRNA and ET-1 immunoreactivity in blood vessels, but not in the plasma. There was a substantial development of vascular hypertrophy in the DOCA-salt treated rats compared to controls. Concomitant treatment of DOCA-salt rats with the ET_A/ET_B receptor antagonist, bosentan, markedly attenuated the development of vascular hypertrophy. The concept that ET-1 is a vascular trophic factor is further supported by findings in cultured vascular smooth muscle cells showing the addition of ET-1 produces a mitogenic response (Weber 1994) as well as in other *in vivo* studies indicating a role in structural changes associated with pulmonary hypertension (Eddinger and Murphy 1991).

A system that can be negatively impacted by the upregulation of cardiovascular structure: The baroreflex-heart rate relationship

Baroreceptor reflex re-setting occurs in response to sustained hypertension

both in human beings (Zanchetti and Mancia 1991) and in animals (Segar *et al.* 1994, Moreira *et al.* 1989, Moreira *et al* 1990). This resetting is usually associated with an overall reduced sensitivity of the baroreceptor reflex control of heart rate (Zanchetti and Mancia 1991, Sleight *et al.* 1977, Struyker-Boudier *et al.* 1982, Korner *et al.* 1972). The mechanisms which have been proposed to explain this deficit have not been clearly elucidated, although a number of causative factors have been postulated including, the development of left ventricular hypertrophy (Head and Minami *et al* 1992), alterations in the central nervous system (Gonzales *et al.* 1983) and vascular hypertrophy (Andresen and Yang 1989).

However, since these changes in cardiac hypertrophy and hypertension occur in parallel their causal relationship to the baroreflex deficit remains obscured. According to Folkow's hypothesis (Figure 1-1), it remains impossible to resolve pressure-independent versus pressure dependent effects on the development of a baroreflex-heart rate deficit. The ideal model to study the development of a baroreflex-heart rate relationship deficit would allow an investigator to assess baroreflex function either under conditions of (i) increased mean arterial pressure alone or (ii) left ventricular hypertrophy in the absence of any increase in mean arterial pressure.

The vascular endothelium

While much of the research into the etiology of cardiovascular structural changes has focused on neural-humoral systems, more and more evidence indicates that local

vasoactive systems may also have an impact on the development of cardiovascular structural alterations. The vascular endothelium, historically, was considered to be "inert wall paper lining the inside of a blood vessel" (Tolins *et al.* 1991). It is now well understood that the endothelium facilitates/regulates numerous diverse and complex physiological functions such as the synthesis and release of vasoactive substances that play a role in dictating the level of vascular tone. (Figure 1-2). Many compounds, such as acetylcholine, ATP, histamine, vasoactive intestinal peptide and leukotrienes, have been shown to induce vasodilation in a manner dependent on the presence of the vascular endothelium (Luscher and Noll 1995). The vasodilation produced by these compounds, and others, is believed to be funnelled down to a common mediator, nitric oxide (NO) (Palmer *et al* 1988).

Inhibition of NO synthase: a new model of hypertension

A new model of arterial hypertension, as mentioned briefly above, may provide a useful model to investigate the contribution of local systems, such as nitric oxide, to the cardiovascular structural up-regulation that represents a hallmark of many other models of hypertension. Several L-arginine analogs have been developed that competitively inhibit the nitric oxide synthase enzyme, leading to decreased NO production. Investigation of the specific function(s) that NO mediates in normal physiology has been relatively recent (Tolins *et al* 1991). L-arginine analogs, acting as precursor substrates for NO synthase, provide tools for researchers in attempts to assess the physiological roles of NO. For example, in rat and rabbit, a single dose of N^{ω}-nitro-L-arginine methyl ester (L-

NAME) (100 mg/kg, i.p.) which blocks the NO synthase produces a significant and long lasting increase in arterial pressure (Arnal *et al* 1993, Pollock *et al* 1993, Morton *et al* 1993). Infusion of L-arginine rapidly and completely reverses the rise in blood pressure (Morton *et al* 1993). Arnal *et al* (1993) showed that a 4-week L-NAME treatment (100 mg/kg per day) induced a marked hypertension was associated with inhibition of cGMP generation in the vasculature (Figure 1-3). They suggested that significant decreases in intracellular cGMP, resulting from the L-NAME treatment, creates an imbalance amongst the factors regulating vascular tone leading to a predominance of the vasoconstrictor response.

This imbalance among the factors regulating vascular tone has also been implicated as a potential causative mechanism underlying many models of hypertension. As discussed above, in addition to NO's proposed role as a regulator of vascular tone, NO may regulate cardiovascular growth processes. Several studies have demonstrated that different NO-generating vasodilators have resulted in the inhibition of vascular smooth muscle growth in culture (Gard and Hassid 1991, Garg and Hassid 1989, Assender *et al* 1991, Griffin *et al* 1991). It may be that NO *in vivo* acts to inhibit vascular growth responses in a number of conditions in which there is the potential for trophic stimulation. For example, recent evidence reveals that there is likely an anti-trophic role for NO, via the kallikrein-kinin system, in the actions of angiotensin-converting enzyme inhibitors in blunting neointimal proliferation of smooth muscle cells (Morgan *et al* 1990).

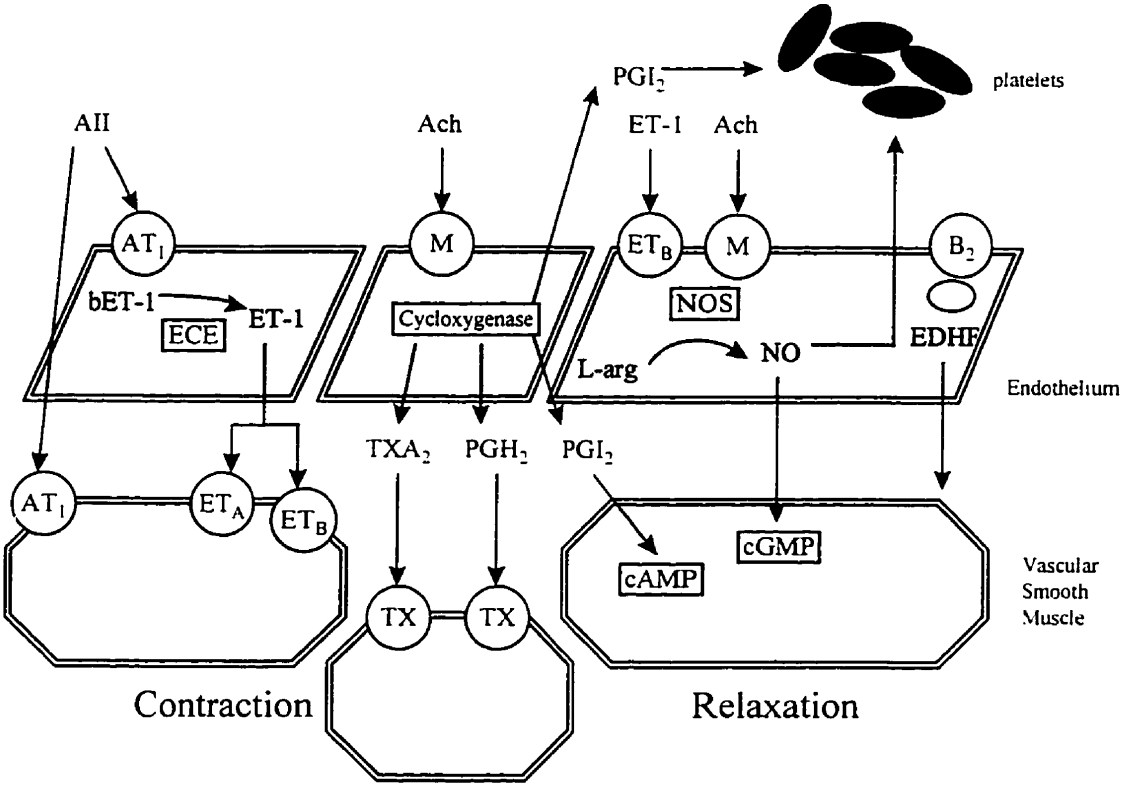

Figure 1-2: The multiple and overlapping systems that can influence the level of vascular tone at the local level, modified from Luscher *et al* (1995). Acetylycholine (Ach), endothelin (ET), angiotensin II (AT), thromboxane (TX), endothelin convertring enzyme (ECE), nitric oxide synthase (NOS).

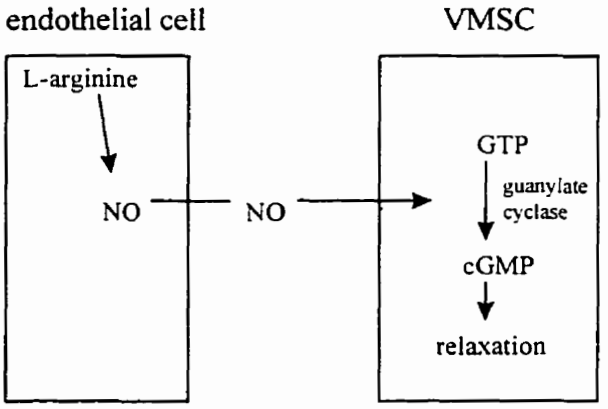

Figure 1-3: The proposed physiological pathway nitric oxide, or a closely related compound leads to the tonic vasodilator signal, via the activation of guanylate cyclase in vascular smooth muscle cells.

Functional assessments of the vasoactive system(s) mediating the development and maintenance of hypertension.

As discussed previously, the development and maintenance of hypertension requires an initiating event, followed by the upregulation of mechanisms involved in the maintenance of the hypertension (Figure 1-1). Characterization of the functional mechanism(s) involved in the initiation and maintenance of the hypertension represents an important factor with respect to assigning causative relationships between the development of cardiovascular hypertrophy and a specific physiological system. Since all classes of vasoconstrictor systems (neuro-humoral and local) have been demonstrated to have the capacity to induce cardiovascular growth processes, the assessment of the relative contribution(s) that each of these of class systems make to the hypertension will help to target the investigation of the mechanisms involved in the development of enhanced cardiovascular growth responses.

One approach to quantitate the contribution of a vasoactive system, to a given model of hypertension, would entail taking measurements of the vasoconstrictor levels in the plasma or tissue. These measurements, however, do not necessarily reflect the absolute contribution a system is making to the level of mean arterial pressure. For instance, Boulanger and Luscher (1991) has suggested that measurements of plasma levels of endothelin may not reflect the 'true' contribution of endothelin *in vivo* since endothelin has been demonstrated to be preferentially released towards the vascular smooth muscle cell layer, as opposed to towards the general circulation. As well,

measurements of plasma or tissue concentrations do not take into account the potential interaction(s) that may be occurring with other vasoactive systems. Since the majority of the vasoconstrictor systems utilize the phosphatidyl inositol/ protein kinase C signal transduction pathway, small changes in one system may serve to 'prime' or amplify the contribution of another system. As such the measurements of a vasoconstrictor system that demonstrate plasma levels that are identical to controls, may in fact be playing an enhanced at the receptor activation level. Taken together, the relative contributions that the functional control systems make to a particular model of hypertension using plasma or tissue concentrations alone may lead to inaccurate or, at the very least, result in an incomplete assessment of the causative mechanisms.

In contrast, utilizing a cumulative sequential pharmacological receptor antagonist approach may afford the ability to assign the contribution(s) of the endogenous vasoconstrictor systems are making to the level of hypertension (utilized by Banting *et al* 1996). This approach involves the administration of a receptor antagonist to each of the major vasoactive systems, such as the SNS, RAS and endothelin, in control and treated rats. By sequentially removing each vasoconstrictor system in each rat, one should eventually 'normalize' the mean arterial pressure in the treated rats, to that of the controls. Also, altering the order of receptor antagonist administration will provide useful information in regards to the specific interactions that occur amongst the vasoactive systems in the treated state and allow comparison to the normal interactions that occur under control conditions. While this approach relies on the development and specificity

of receptor antagonists, the cumulative sequential technique of assessing the contribution each of the neuro-humoral and local vasoconstrictor systems makes to a given level of hypertension may provide much more information than measurements of tissue or plasma concentrations.

Biochemical markers of cardiovascular growth induction

While the measurement of end-point structural changes provides valuable information as to the outcomes of sustained hypertension, following the activity profile of biochemical markers of growth induction allows for the targeting of specific time points where changes occur, allowing investigators to focus in on the 'critical' events that start the cascade of cardiovascular growth.

The exact mechanism(s) that initiate cardiovascular growth processes, *in vivo*, via trophic factors (such as the endogenous vasoconstrictor systems) are not completely clear. It is generally agreed upon, however, that these trophic factors are likely mediating the signaling of growth processes by occupying specific receptors and leading to receptor-mediated activation of intracellular signal transduction cascades. The binding of these vasoconstrictors to specific receptors, leads to the enhanced activation of the phosphatidyl inositol second messenger system (Owen 1986, Owens 1989). Increased rates of phosphatidyl inositol turnover results in alterations in the transmembrane fluxes and the release of calcium from intracellular stores. Enhanced intracellular levels of calcium leads to the activation of protein kinase C and the sodium-hydrogen antiporter (Owen

1986, Owens 1989). Overall these events lead to altered levels of cellular pH which, at least in part, has been proposed to promote DNA synthesis leading to cellular growth. These early changes in the fluxes of ions are closely followed by the induction of several regulatory genes such as c-fos and c-myc, as well as ornithine decarboxylase, which leads to DNA and protein synthesis and ultimately overall cell growth. As such, the initiation of cardiovascular growth processes can be measured via several potential markers, one of which is being the activation of ornithine decarboxylase (ODC), the rate limiting enzyme in the biosyntheses of polyamines. For several decades the role of polyamines (putrescine, spermine and spermidine) in the control of cellular growth and differentiation has been the subject of much interest (Thyberg *et al* 1987, Johnson *et al.* 1983, Majesky *et al.* 1985). The biosynthesis of these polyamines is rate limited by the activity of the enzyme ODC. This enzyme has a remarkably short half-life of 10-20 minutes and has been shown to preferentially stimulated by various trophic factors. Various studies have shown that in blood vessels and in the heart, synthesis of new ODC is required to sustain basal activity. The involvement of polyamines in the regulation of DNA, RNA and protein metabolism in response to trophic agents is directly related to the level of ODC activity (Thyberg *et al* 1987, Johnson *et al.* 1983, Majesky *et al.* 1985) in that sustained elevation of ODC activity is characteristic of tissues undergoing rapid growth and/or differentiation. This concept has been extended to reveal that ODC activation is an obligatory event in a number of growth processes (Thyberg *et al* 1987, Johnson *et al.* 1983, Majesky *et al.* 1985). Johnson *et al.* (1983) demonstrated that in response to catecholamine stimulation temporal increases in ODC activity were closely correlated

with, but preceded, the development of cardiac and aortic vascular hypertrophy. The trophic stimulus, an infusion of noradrenaline or adrenaline, produced maximum trophic effects within 5 days were also associated with marked blood pressure elevation. Further, it was found that ODC activity returned to normal levels once the growth response had stopped and tissue mass had reached a new steady-state (i.e. ODC was not elevated when the tissue was not in a dynamic state of growth). The possibility that there was some pressure-dependent induction of aortic and cardiac growth processes confounded interpretation of direct growth effects of catecholamines with these data.

In summary, the level of activation of ODC above control levels is a direct marker of an ongoing growth process and a return towards normal levels represents a return towards steady-state. It is likely that the assessment of the time course of ODC activation will predict the time course and magnitude of cellular growth responses after different durations of trophic stimulation in cardiac, aortic and mesenteric vascular tissue. A return of ODC activity to normal levels will indicate a return towards a differentiated state. Full differentiation is marked by a matching of contractile function appropriate to the level of change in overall tissue mass (i.e. the amount of contractile machinery is proportionately increased with the overall change in tissue mass) (Malmqvist and Arnér 1990, Malmqvist and Arnér 1988). Accordingly, to accurately assess the impact of cardiovascular growth responses it is important that hemodynamically significant vascular structural changes in fully differentiated tissue be established definitively.

Measurement of end-point cardiovascular structural alterations

While the development of vascular structural hypertrophy is widely agreed to be a strong mortality factor in essential human hypertension., the methods used to quantitate the nature and level of structural hypertrophy are generally not agreed upon. The 'ideal' method for assessing vascular structure would be an approach that could measure the structural changes that have occurred in all orders of vessels, in a given vascular bed. Also, the 'ideal' technique would provide detailed information regarding branching patterns as well as dimensions of the vessel media and media:lumen ratios, as this would provide much more information as to the 'type' of vascular structural change occuring (ie hypertrophy, hyperplasia or remodelling). The 'ideal' method should provide all of this information with the assumption that the conditions under which the various structural parameters are measured reflect the *in vivo* situation (i.e. appropriate flow and perfusion pressure).

Each methodological approach has inherent advantages and disadvantages. Two general approaches are utilized to quantitate the structural changes that occur in experimental models of vascular hypertrophy: (i) functional assessments or (ii) morphological assessments. Functional assessments involve artificially perfused isolated vascular beds, *in situ*, and deduce changes in structurally based vascular resistance based on changes in perfusion pressure are compared in treated versus control conditions.

Morphological assessments generally entail isolation and extraction of the vascular bed of interest followed by histology and/or myography studies. Although the latter type of analysis does include a functional assessment of a small vessel segment. The major limitation of morphological studies is, first off, the size of vessel that can be assessed. The various myographic techniques wire, pressurized video etc. can only use vessels that can be tested under *in vitro* conditions, where a 'wire' must be passed through the vessel and the various dimensional measurements taken (i.e. limiting the size of vessels to greater than 100 μm). The second source of variability stems from the decision regarding (i) how much to arbitrarily stretch a vessel (length) and (ii) what level of pressurization to use during the measurement of the various parameters. Since a blood vessel is a long elastic tube, changes in the length of the vessel will impact both the lumen diameter and the media thickness (Folkow 1990).

Another disadvantage of using direct histological techniqures stems from the potential of sampling bias. Since there is extensive anatomical heterogeneity amongst different animals from the same species, direct comparisons between control and treated animals can often be difficult. The second variable arises from taking measurements of a blood vessel that has been fixed, where it has been questionable as to whether or not these measurements are reflect of *in vivo* changes (Folkow 1990). Various analysis can be employed to overcome these potential discrepancies. For instance, determinations of cross-sectional area allow controls for both the degree of stretch (length) and the level of fixation pressure (Folkow 1990).

The utilization of an isolated perfusion technique can provide several advantages over other methods of assessing the contribution of structure in a given experimental model. The perfusion study approach takes into account the overall changes in vascular resistance properties throughout an entire vessel, rather than just a small segment. This provides a higher degree of physiological relevance as it relates to the resistance of an entire vascular bed. Also, the structure of the vascular bed being investigated is 'preserved' in the *in vivo* state, where the surrounding connective tissue that normally dictates the 'stretch' of the vessel remains intact. Although vascular perfusion does not directly measure media thickness or lumen diameter, it provides a sensitive assessment of vascular resistance properties based on vascular structure set at maximum dilation and at various levels of constrictor tone. In addition, vascular perfusion can also be utilized to test the sensitivity to various pharmacological agonist, in attempts to determine the changes in vascular sensitivity to various vasoactive system end-points.

Research Hypothesis

Since much of the attention with regard to the development of cardiovascular functional and structural changes has focused on neuro-humoral systems, I thought it would be of interest in the present thesis to focus on characterizing the cardiovascular functional and structural changes that occur with the removal of a local endogenous vasodilator system, nitric oxide. The initial phases of the present thesis were designed to

elucidate the contribution of neuro-humoral and local vasoconstrictor systems to the hypertension following NO synthase blockade both acutely (1 hour) and chronically (12 days). To account for the involvement of these systems, the approach used to assess the contribution of the neuro-humoral and local vasoactive systems involved using a hemodynamic index of vasoconstrictor function, where each of the vasoconstrictor systems were removed in a cumulative and sequential manner, to determine each system's absolute and proportional contribution to the NO synthase blockade induced hypertension. Since baroreflex function has been demonstrated to play an important role in mean arterial pressure homeostasis, the present thesis also investigated the alterations in the heart-rate baroreflex relationship as a potential contributor to the hypertension associated with NO synthase blockade. Taken together, the elucidation of the mechanism(s) involved in the initiation and development of NO synthase induced hypertension will allow for the targeting of key vasoactive systems and critical timepoints with respect to the elucidation of the mechanisms underlying the development of cardiovascular structural alterations.

The research hypotheses that were tested in the present thesis were the following:

1. the removal of a the nitric oxide vasodilator system results in a marked increased sensitivity to a specific vasoconstrictor system, as opposed to an overall generalized increased sensitivity to all opposing vasoactive systems.
2. the development of cardiovascular structural upregulation does not occur as a direct result of increased mean arterial pressure alone.

3. a sustained increase in the level of mean arterial pressure alone (i.e. in the absence of cardiovascular hypertrophy) does not result in a heart-rate baroreflex relationship deficit.

Research Objectives

Study 1: Acute mechanisms of NO synthase blockade induced hypertension.

1. to determine the time course and peak levels of hypertension induced by acute NO synthase blockade.
2. to assess the contribution of neural, humoral and local systems to the regulation of mean arterial pressure, before and after NO synthase blockade.
3. to determine whether a 3 day pre-treatment with an angiotensin converting enzyme inhibitor alone, or in combination with 1 % salt blunts the acute pressor response induced by acute NO synthase blockade.

Study 2: Chronic mechanisms of NO synthase blockade induced hypertension:

4. to assess the levels of mean arterial pressure with 12 days of NO synthase blockade to determine the contribution of neural, humoral and local vasoconstrictor systems to the hypertension associated with NO synthase blockade.

Study 3: The impact of acute and chronic NO synthase blockade on the blood pressure heart-rate baroreflex.

5. to determine whether a baroreflex deficit plays a role in the development and

maintenance of hypertension induced by NO synthase blockade.

6. to determine whether an increase in mean arterial pressure-alone (ie no cardiac hypertrophy) has the capacity to induced a baroreflex deficit.

Study 4: The lack of induction of cardiovascular growth processes with NO synthase blockade.

7. to determine the activation pattern of the growth related enzyme ornithine decarboxylase with hypertension induced by NO synthase blockade.
8. to assess the impact of 12 days of NO synthase blockade induced hypertension on structurally based vascular resistance.

Study 5: Evidence that the NO synthase inhibitor L-NAME may inhibit cardiovascular growth processes.

9. to compare the activation of the growth related enzyme ornithine decarboxylase with 4 hour L-NAME treatment to that with 4 hour angiotensin II treatment
10. to assess the capacity of L-NAME to inhibit the proliferation of cultured aortic vascular smooth muscle cells stimulated with 2.5, 5 and 10% fetal calf serum.
11. to assess the capacity of L-arginine, the NO pre-cursor, to reverse the effects of L-NAME on aortic vascular smooth muscle cell proliferation with 2.5% fetal calf serum.

Study 6: Evidence that the role for NO, in blood vessels, is not as a chronic vasodilator mechanism, but rather a chronic suppresser of endothelin mediated vasoconstriction:

12. to assess the capacity of the administration of NO pro-drug (SNP, GTN) to reverse the hypertension associated with acute and chronic NO synthase blockade, at doses which produce no change in the level of mean arterial pressure in 'control' animals.

Study 7: The increased sensitivity to α-adrenoceptor activation, in blood vessels, following NO synthase can be accounted by a "priming" role of endothelin mediated vasoconstriction.

13. to assess the capacity of an endothelin blocker to reverse the increased sensitivity to α_1-adrenoceptor activation in the perfused pudendal vasculature following NO synthase blockade.

Chapter 2: Acute Hypertension Following Nitric Oxide Synthase Inhibition is Mediated Primarily by Increased Endothelin Vasoconstriction.

Abstract:

Objective: To determine, in conscious rats, the quantitative role of the different vasoconstrictor systems to the acute pressor response before and after inhibition of NO synthase with N^{ω}-nitro-L-arginine-methyl ester (L-NAME).

Methods: In conscious male Sprague-Dawley rats, previously instrumented with aortic and venous catheters, the contribution of the different systems were assessed by maximal cumulative pharmacological blockade of α_1-adrenoceptors (prazosin, 1 mg/kg, i.p), AT_1 receptors (losartan 30 mg/kg, i.p.) and V_1 /V_2 receptors ((O-Et)VAVP 10 mg/kg per min i.v.). In addition, the contribution of endothelin-1 (ET-1) induced vasoconstriction to L-NAME (100 mg/kg, i.p.) hypertension was assessed by administering the ET_A/ ET_B receptor antagonist PD 145065 (5-100 mg/kg, i.v.) in three different conditions (i) as the last step of a series of antagonists in the cumulative pharmacological blockade subsequent to inducing the L-NAME pressor response, (ii) alone prior to L-NAME treatment and (iii) alone following the full development of the L-NAME pressor response. A separate group of rats were treated with losartan, acutely (30 mg/kg, i.p.) or a 3 day pre-treatment with the angiotensin I converting enzyme (ACE) inhibitor, enalapril (30 mg/kg via drinking water) alone or in combination with 1 % salt were used to assess the role of the renin-angiotensin system (RAS) in the L-NAME hypertension.

Results: Short term administration of the combined ET_A/ET_B receptor antagonist (PD

145065) did not change arterial pressure under control conditions. Inhibition of the RAS, α_1-adrenoceptors or vasopressin receptors alone or in combination did not alter the magnitude of the L-NAME pressor response. In contrast, our results show that both treatments prior to and during acute NO synthase blockade hypertension using the ET_A/ET_B receptor antagonist, PD 145065, almost completely abolished the pressor response (≈85%).

Conclusions: These studies indicate that the predominant mechanism of hypertension, at least in the acute phase, following acute NO synthase blockade with L-NAME is associated with a marked increase in ET_A/ET_B receptor activation and not increases in α_1, AT_1 and V_1/V_2 receptor activation. It remains to be determined whether endothelin participates also in the chronic phase of NO deficient hypertension.

Introduction:

Endothelium-derived relaxing factor (EDRF), likely acting as nitric oxide (NO) or a closely related compound, has been widely described as having a predominant role as a chronic vasodilator of vascular smooth muscle (Johnson and Freeman 1992, Tolins *et al.* 1991, Bank *et al.* 1994). Inhibition of the production of NO, via NO synthase inhibition using L-arginine analogues, provokes a rapid and sustained increase in total peripheral resistance, impaired flow to regional circulations and a marked increase in the level of mean arterial pressure (MAP)(Sigmon and Beierwaltes 1994). The hierarchy of circulatory control systems that have the capacity to exert influences on the level of vascular tone can be described as combinations of the following: neuronal (e.g. sympathetic nervous system (SNS), neuropeptide Y), humoral (e.g. Ang II, epinephrine, AVP) and local systems (e.g. NO, endothelin-1 and prostaglandins). Until recently, research has focused mostly on factors governing the level of vascular tone via neural and humoral mechanisms. Fewer studies have considered the quantitative contribution of 'local' systems *in vivo* in the regulation of vascular tone and mean arterial pressure. Even in studies that have used antagonists of 'local' NO production (L-NAME, L-NOLA, L-NMMA) the emphasis has been placed on circulatory control mechanisms other than those mediated by the 'local' systems (Pollock *et al.* 1993, MacLean *et al.* 1994, deNicola *et al.* 1992). However, in studies of hypertension induced by NO synthase inhibition, the activity of neural-humoral systems, such as the SNS (Pucci *et al.* 1992) and the renin-angiotensin system (RAS) (Arnal *et al.* 1993), have been found to remain at the same levels or lower. Based on this indirect evidence (i.e. consequences of decreased

NO) it has been proposed that a key function of NO is as a local regulator of vascular tone (Moncada *et al.* 1991).

The role of NO has been inferred largely from the hemodynamic consequences that occur in its absence and not by its contribution when it is active. The objective of the present study was to determine quantitatively the contribution of each of these vasoconstrictor systems acting alone or in combination at neural, humoral and local levels, to the increased level of mean arterial pressure in rats treated acutely with the NO synthase inhibitor, L-NAME, in comparison to control rats. The emphasis of these studies is on the initiating mechanisms involved in the onset of L-NAME hypertension and not on chronic aspects since the mechanisms involved in maintaining chronic L-NAME hypertension are much more difficult to elucidate. Specifically, assessment of chronic mechanisms would require the characterization of both structural adaptations in resistance vessels and the heart as well as the entire time course of changes in activity of the different vasoactive systems.

Methods:

Animals

Male Sprague-Dawley rats (325-400 g) obtained from Charles River Laboratories (Montreal, Canada) were housed individually under conditions of 12-hour light/dark cycle, with room temperature at 22-24°C, and were provided with Purina rodent chow and tap water *ad libitum* for at least 2 days before any experiments were started.

Measurement of MAP and Short Acting Drug Administration:

The surgical method was based on the technique of Thompson *et al.* (1992). In brief, rats were anaesthetized with ketamine/xylazine (70/5 mg/kg i.p.), and the descending aorta distal to the kidneys was catheterized with small bore Teflon® tubing (0.012-in. i.d., 30 gauge, Cole-Parmer, Laval, Canada) inserted into vinyl tubing (0.02-in. internal diameter, 23 gauge). The inferior vena cava was also catheterized distal to the kidneys with small bore Teflon tubing (0.012-in. internal diameter, 30 gauge, Cole-Parmer) The catheters were filled with heparinized saline (10 IU/ml) and held in place by a small amount of cyanoacrylate glue at the puncture site. The catheters were tunnelled subcutaneously and exteriorized at the back of the neck and sutured in place. Two days after surgery, MAP could be recorded (Narco Physiograph, E & I Instruments, Houston, TX or MacLab DAS, ADInstruments, Milford, MA). After connection, an equilibration period of approximately 30 minutes allowed for the determination of the steady state level of MAP before any recording began. Baseline MAP was determined from readings

averaged over 5 minutes, taken from each rat at 15-minute intervals for at least 1 hour prior to the start of any experiment.

Study 1: Cumulative vasoconstrictor blockade following acute NO synthase inhibition.

After obtaining a baseline MAP, the effects of sequential pharmacological blockade of vasoconstrictor systems in conscious rats was investigated. In preliminary experiments we determined the doses of antagonists required to produce a maximal decrease in MAP (i.e. further increases in dose did not result in further lowering of MAP). MAP was determined at each new level of cumulative blockade as the average MAP over 2-3 minutes following the establishment of a new steady state. In these experiments we assessed the contribution of vasoconstrictor systems at the neural (α_1-adrenoceptor), humoral (AT_1, V_1/V_2 receptors) and local (ET_A/ET_B receptors) level in untreated rats (n=5) and after acute L-NAME treatment (n=8) by determining the depressor response with each level of blockade. NO synthase was inhibited acutely (<3 hours) by a single i.p. injection of N^{ω}-nitro-L-arginine-methyl ester (L-NAME, Sigma, 100 mg/kg; 100 mg/ml 0.9% sterile saline solution, Baxter Corp., Toronto, Ontario). A sequential and cumulative blockade approach was used in order to eventually account for the difference between the L-NAME and control groups, i.e. the objective was to step-wise add the different pharmacological antagonists until MAP's in the two groups were equalized. Further, in pilot experiments we determined that the impact of changing the order of antagonist administration (specifically, prazosin or losartan given first) did not alter the measured contribution of each of these systems to the level of MAP.

Accordingly, we have performed the experiments in both treatment groups using maximal doses of antagonists administered in an order that reflects the rank order of contribution of these systems to the MAP (SNS, RAS, AVP) in controls conditions.

Each rat received the antagonist drugs in the following order: (i) α_1-adrenoceptors were maximally blocked with prazosin (Sigma Chemical Company, St. Louis, MO, U.S.A.) at a dose of 1 mg/kg (5% ethanol [vol/vol] in 0.9% saline, total volume 1 ml/kg) which maximally decreased pressure for this drug, (ii) the non-peptide AT_1 receptor antagonist losartan (Dupont-Merck, 30 mg/kg i.p.) was used to maximally block the effects of Ang II, (iii) the V_1/V_2 receptor antagonist [β-mercapto-β, β-cyclopenta-methylenepropionyl^1, O-Et-Tyr^2-Val^4-Arg^8]-vasopressin (20 ug/kg per min i.v., Sigma) was used to maximally block the effects of vasopressin, (iv) the effect of the endothelin-1 vasoconstrictor system, via both ET_A and ET_B receptors, was blocked with a bolus of PD 145065 (100 mg/kg, i.v., Parke-Davis Pharmaceuticals) and finally (v) the addition of sodium nitroprusside (SNP, 200 ug/kg i.v. 0.3 c.c. bolus, Sigma) at the end of the experimental period was used to transiently but maximally lower the MAP to a level of minimum vascular resistance across all treatment groups.

In this acute study, the SNP-induced lowering of MAP was found to be similar across all treatment groups and enabled the calculation of the total range of MAP pressure lowering (the difference between the operating MAP in control or L-NAME treated rats and the pressure after maximal SNP infusion; $MAP_{range}=MAP-MAP_{min}$). The

maximal lowering of MAP with SNP was used to non-specifically remove the contribution of any "other" vasoconstrictor system not accounted for by the pharmacological receptor blockade. The interval between administration was between 10 and 15 minutes such that a new steady state MAP was reached prior to the next step in the pharmacological blockade.

Study 2: Acute effects of losartan and PD 145065 on the NO synthase blockade pressor response:

Study 2 investigated the capacity of pre-treatment with the AT_1 receptor blocker losartan or PD 145065 to block the pressor response induced by the L-NAME treatment. After obtaining baseline levels of MAP in previously instrumented rats, losartan (30 mg/kg i.p., n=5) or PD 145065 (5 mg/kg per min, i.v., n=6) were administered in two different groups of rats and the impact on the subsequent L-NAME-induced hypertension was recorded. In a separate group of rats (n=5), L-NAME (100 mg/kg i.p.) was given first and the MAP allowed to reach a new steady-state followed 10 minutes later by an infusion of PD 145065 (5 mg/kg per min.).

Study 3: Chronic pre-treatment with enalapril and 1 % salt alone and in combination for 3 days prior to NO synthase blockade:

Study 3 investigated the capacity of a 3-day pre-treatment with the angiotensin converting enzyme (ACE) inhibitor enalapril (30 mg/kg per day p.o., n=4) or 1 % salt in drinking water (n=3) alone or in combination (n=3) or a control treatment (n=4) to block

the pressor response induced by the L-NAME treatment. For each of these groups baseline levels of MAP in conscious rats , previously instrumented with aortic catheters. were recorded for 1 hour, followed by a single L-NAME (100 mg/kg i.p.) injection. The time course of the L-NAME pressor response was recorded for the next hour after NOS blockade.

Data Analysis:

In Study 1, all data values are expressed as a peak MAP lowering as a mean ± S.D. In Study 2, all data values are expressed as mean ± S.D. or S.E.M. as indicated. Comparison of means between groups at each treatment level was done using a Student's T-test with the Bonferroni correction method as required.

Results:

Study 1:

As described in methods in preliminary experiments, we verified that the dose of each antagonist was maximal, based on the finding that there was no further lowering of MAP with repeated administration (data not shown). In addition, we determined that the order of antagonist administration did not alter the determination of the contribution of each vasoconstrictor system i.e. the lowering of MAP for each antagonist was found to be the same whether the drug was administered alone or following administration of other blockers (data not shown).

Figure 2-1 illustrates the step-wise lowering of MAP following the sequential pharmacological blockade of the α_1, AT_1, V_1/V_2 and ET_A/ET_B receptors in the acute L-NAME and saline control groups in Study 1, respectively. The steady-state level of MAP was increased 44% following acute NO synthase blockade with L-NAME treatment. The data revealed that the absolute decrease in MAP (Δ MAP) with maximal α_1, AT_1, V_1/V_2 receptor blockade in the L-NAME group was similar compared to the responses in controls (Table 2-1). In contrast, ET_A/ET_B receptor blockade produced a 35 ± 13 mmHg decrease in MAP only in the L-NAME treatment group as there was no change in the control group. Thus, following maximal pharmacological blockade of α_1, AT_1, V_1/V_2 receptors but not ET_A/ET_B receptors the MAP in the L-NAME group remained markedly increased (84 ● 13 mmHg vs 46 ± 4 mmHg) above controls. Only following PD 145065

treatment did the MAP's become similar in both groups (Fig 1) indicating a enhanced involvement of ET_A/ET_B receptor activation only in the L-NAME treatment group.

To assess the contribution of each system to MAP we also calculated the decrease in MAP as a percentage of the total MAP range (Figure 2-1, Table 2-1). The data demonstrate that the contribution of AT_1 and V_1/V_2 receptors to MAP was similar, and that of α_1 receptor activation diminished after NO synthase blockade (Table 2-1). In contrast, the contribution of ET_A/ET_B receptor activation was enhanced ≈30 fold following NO synthase blockade as compared to control (Table 2-1).

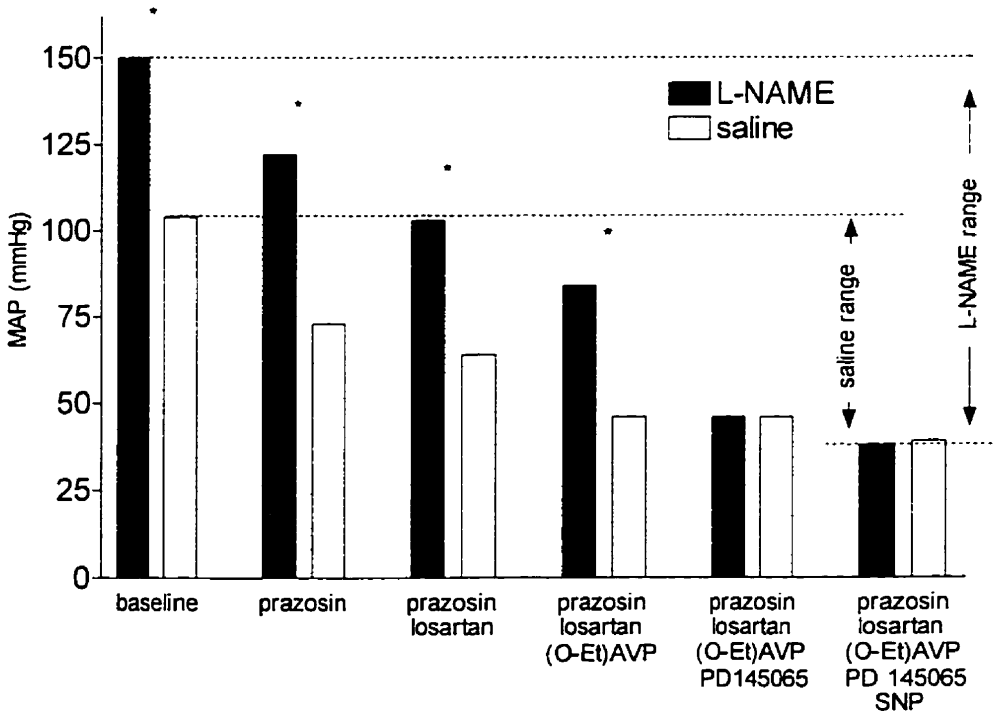

Figure 2-1: The MAP response from baseline (base) with L-NAME or saline treatment with the sequential cumulative addition of: prazosin, losartan, (O-Et)VAVP , PD 145065 and sodium nitroprusside (SNP). Numbers at each level of pharmacological blockade were 8, 8, 8, 8, 7, 8 for control and 8, 8, 8, 8, 7, 8 for L-NAME treatment groups. Values are expressed as means (± S.D.), * denotes significant difference from saline control value ($p<0.05$).

Table 2-1: Contribution of vasoconstrictors to MAP with and without NO synthase blockade

Receptor Antagonist	Δ MAP		% of range		Change
	Saline	L-NAME	Saline	L-NAME	
α_1	↓ 30 ± 14	↓ 28 ± 13	45 ± 15	25 ± 10*	↓
AT_1	↓ 7 ± 6	↓ 19 ± 22	12 ± 10	16 ± 19	↔
V_1/V_2	↓ 20 ± 10	↓ 19 ± 13	30 ± 16	15 ± 9	↓
ET_A/ET_B	↓ 1 ± 2	↓ 35 ± 13*	1 ± 1	32 ± 13*	↑↑
SNP	↓ 7 ± 3	↓ 11 ± 9	11 ± 5	10 ± 8	↔

* denotes significant difference ($p<0.05$) compared to saline control. Blocking agents were administered in a sequential manner, therefore, data reflects decreases from immediately previous baseline.

To account for the contribution of the vasoconstrictors to the L-NAME-induced increase in MAP (≈↑ 46 mmHg) we determined the difference in MAP between groups at each level of pharmacological blockade (Table 2-2). Results previously described for the full range of MAP control (Table 2-1) revealed that the ET signal was enhanced ≈30 fold. Calculations (Table 2-2) revealed that this enhanced activity accounts for 83 % of the L-NAME pressor response (38 out of 46 mmHg) whereas the total "increased" contribution of α_1, AT_1 and V_1/V_2 receptor activation was less than 17% (8 out of 46 mmHg). It is important to note that, in using the four levels of pharmacological blockade, 100% of the difference in MAP between L-NAME and control rats was accounted for.

Reflex decreases in heart rate were found in association with the increases MAP. L-NAME treatment decreased heart rate from 378 ± 16 bpm to 278 ± 10 bpm. This bradycardia was in part reversed (338 ± 40 bpm) during the MAP lowering that occurred with the subsequent pharmacological blockade. In the saline treatment group, in association with the progressive decrease in MAP with the four antagonists, heart rate (368 ± 35 bpm) rose only slightly (408 ± 22 bpm).

Study 2:

Pre-treatment of rats with losartan produced a steady-state decrease in MAP from 100 ± 15 mmHg to 78 ± 13 mmHg (data not shown). In contrast, pre-treatment with the

Table 2-2: Contribution of vasoconstrictors to the acute L-NAME pressor response

Receptor Antagonist	Δ above control MAP (mmHg)	% of L-NAME pressor removed	Change in magnitude of L-NAME pressor
baseline	46 ± 11	0 %	↔
α_1	49 ± 14	0 %	↔
AT_1	39 ± 13	15 %	↓
V_1/V_2	38 ± 13	2 %	↓
ET_A/ET_B	1 ± 7	83 %	↓↓
% of L-NAME tone removed:		100 %	

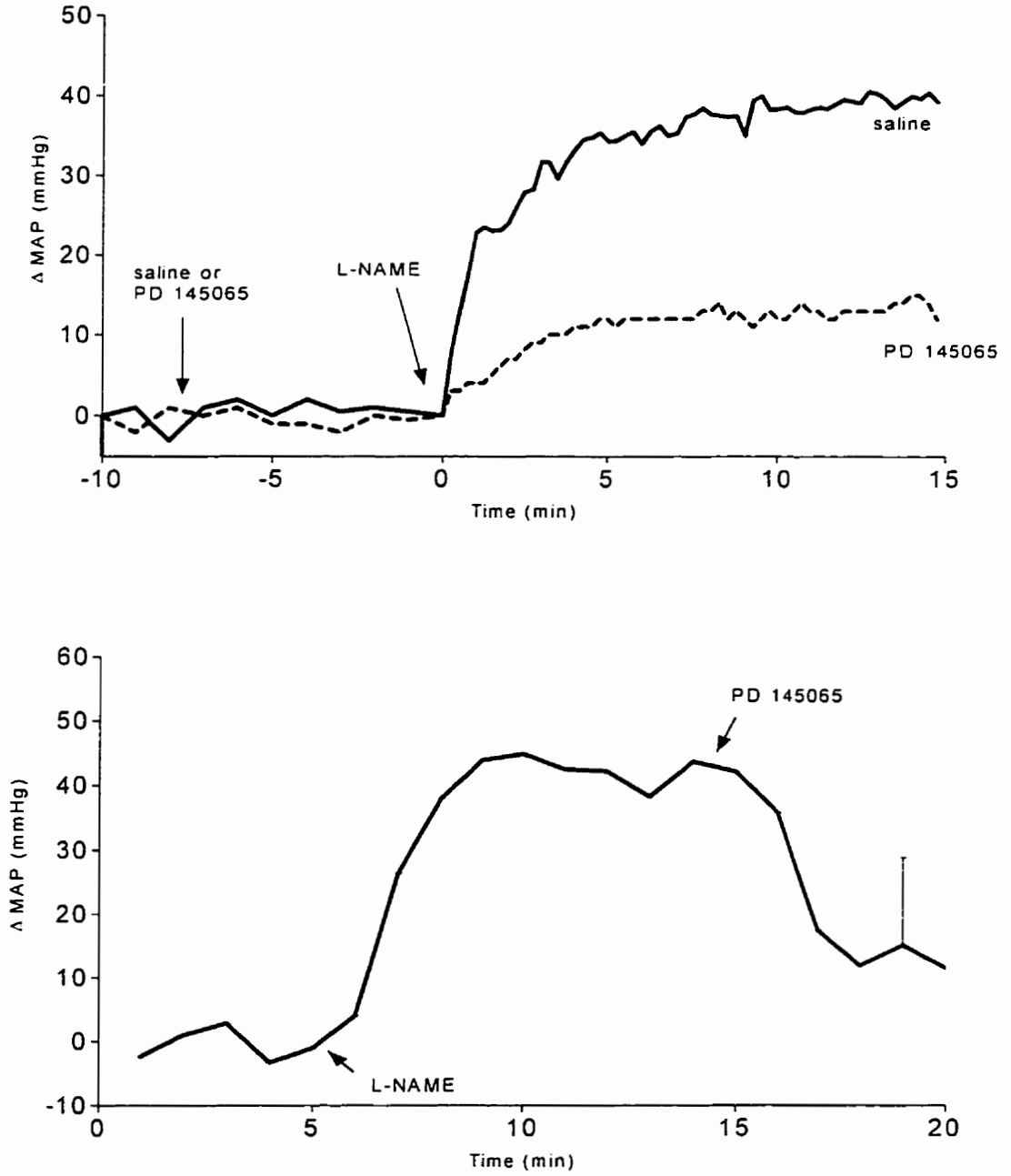

Figure 2-2: Illustrates the Δ mean arterial pressure (MAP) response following NO synthase blockade with prior blockade of endothelin receptors compared to saline control (upper panel, n=6,6) and after NO synthase blockade (lower panel, n=6) for saline and PD 145065 pre-treatment groups, respectively. Values are expressed as mean change in MAP (± SEM at largest value) above steady state baseline.

ET_A/ET_B receptor blocker, PD 145065, did not alter the baseline level of MAP (Figure 2-2). These results demonstrate the significant contribution of angiotensin II in the regulation of arterial pressure under control conditions, confirming both the results of Study 1 and of others. Further, the data provide evidence for minimal, if any, contribution of endothelin in the regulation of arterial pressure in normotensive rats, a finding also consistent with Study 1.

The subsequent administration of L-NAME in the losartan pre-treated group resulted in a rapid pressor response (39 ± 12 mmHg) which was not significantly different from the group treated with L-NAME alone (39 ± 11 mmHg). In contrast, the MAP pressor response to L-NAME was markedly blunted (12 ± 8 mmHg) by the ET-antagonist (Figure 2-2, upper panel). These results reveal a critical contribution of ET_A/ET_B receptor activation, but not of increased AT_1 receptor activation, to the L-NAME pressor response.

Administration of the ET_A/ET_B antagonist PD 145065 following the full development of L-NAME hypertension (i.e. L-NAME alone increased MAP from 108 ● 9 mmHg to 141 ± 11 mmHg) resulted in a rapid, 32 ● 11 mmHg decrease in MAP (Fig. 2 lower panel).

Study 3:

The baseline levels of MAP in Study 3 were similar in the groups given 1 % salt,

1 % salt + enalapril and in the control groups (103 ± 4, 98 ± 9, 109 ± 4 mmHg, respectively). Not surprisingly, the enalapril treatment alone significantly lowered MAP to 78 ± 12 mmHg. Analysis of Δ MAP values following NO synthase blockade in all treatment revealed no differences in the magnitude of the pressor response (Figure 2-3).

Discussion:

In this study we have characterised the vasoconstrictor mechanisms involved in L-NAME hypertension, at least in the acute phase, in conscious rats. The major findings of this study are that: (i) the predominant contributor to the acute L-NAME pressor response was endothelin mediated vasoconstriction, (ii) the contribution of α_1-adrenoceptors, AT_1 receptors and V_1/V_2 receptor activation was unchanged or was lower during the L-NAME pressor response, and (iii) the contribution of endothelin mediated vasoconstriction to arterial pressure in normotensive control rats was shown to be less than 3 mmHg.

In control animals ET_A/ET_B receptor blockade did not alter MAP either under steady-state conditions or following cumulative pharmacological blockade of α_1, AT_1 and V_1 /V_2 receptors. Several other studies have reported similar findings using other endothelin antagonists, such as bosentan, BQ-123 as well as PD 145065 demonstrating that MAP does not significantly change with ET-1 blockade either in normotensive (Wellings *et al.* 1993, McMurdo *et al.* 1993) or in spontaneously hypertensive rats (Li and Schiffrin *et al.* 1995). In contrast, endothelin has been shown to play an enhanced role in abnormal circulatory states such as DOCA-salt hypertension (Schiffrin *et al.* 1995) and cyclosporin nephrotoxicity (Takeda *et al.* 1995).

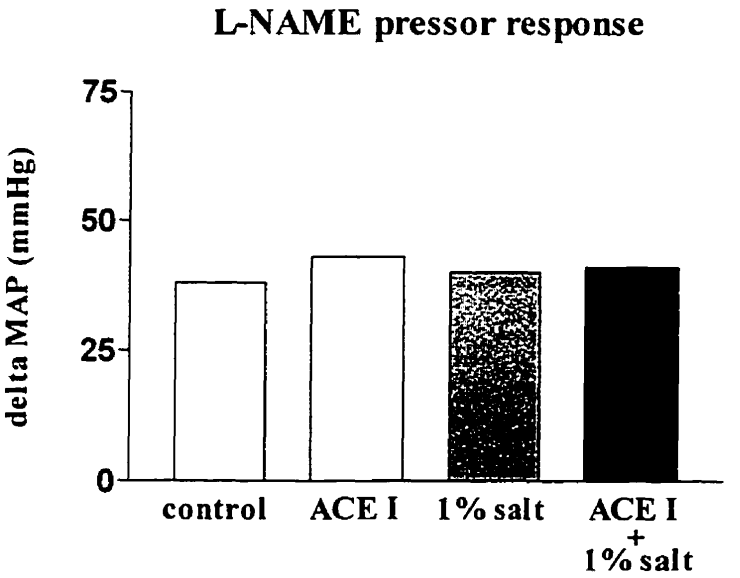

Figure 2-3: Illustrates the Δ mean arterial pressure (MAP) response to steady state NO synthase blockade with L-NAME following a 3 day pre-treatment with the angiotensin I converting enzyme inhibitor (ACE I) enalapril (30 mg/kg per day p.o.) and 1 % salt in drinking water alone or in combination. Values are expressed as mean change in MAP (± SD) above steady state baseline.

The studies presented expose the critical involvement of endothelin in the hypertension induced by NO synthase blockade. The results of Richard *et al* (1995), although done in anaesthetized and pithed rats, provide supportive evidence for the involvement of endothelin in L-NAME hypertension. In their study they demonstrated that pre-treatment with an ET_A/ET_B antagonist blunted the pressor response to NO synthase blockade. However, in that study they did not account for changes in the other circulatory control systems (e.g. α_1, AT_1 and V_1/V_2 receptors) related to the pithing or anaesthesia. In the present study, using conscious rats, we demonstrate in rats pre-treated with an endothelin receptor blocker that the L-NAME induced pressor response is inhibited by at least 70 % in the acute phase. The present results demonstrate that only endothelin receptor blockade was able to equalize mean arterial between the control and L-NAME treated groups.

Acute inhibition of the other vasoconstrictor systems did not change the L-NAME pressor response. Pre-treatment with the AT_1 receptor blocker did not modify the amplitude of the L-NAME pressor response. Sequential blockade of α_1, AT_1 and V_1/V_2 receptors also did not attenuate the increased pressure in the L-NAME-treated group. These findings appear, at first, to be in contrast with those of Pollock *et al*, in that they suggested primary involvement of the renin angiotensin system in hypertension following NO synthase blockade (Pollock *et al.* 1993). Their conclusions derived from demonstrating that concomitant treatment of an AT_1 receptor blocker with L-NAME

blocked the development of chronic hypertension (1-4 weeks). In the present study we first allowed a new steady-state pressure to be established before inducing further changes with L-NAME. Thus, we determined the amplitude of the L-NAME pressor response alone and not the physiological antagonism that can also occur when vasoconstrictor systems are removed or by vasodilators are added.

The results of Study 3 also are in contrast to the conclusions of Pollock *et al.* (1993). The results show that a 3 day pre-treatment period with the angiotensin I converting enzyme(ACE) inhibitor enalapril did not blunt the pressor response following NO synthase blockade. This also indicates a lack of involvement of the bradykinin, which has been shown to be enhanced with ACE inhibitor treatment (Hirata *et al.* 1994, Gilbert 1992). Study 3 also provides evidence that atrial natriuretic factor (ANF) is probably not involved in the L-NAME pressor response, as a short-term 1 % salt treatment has been demonstrated to enhance the plasma levels of ANF (Conte *et al.* 1992, Dietz *et al.* 1992).

This study focuses on the mechanism responsible for the acute pressor effects of L-NAME and not on the mechanisms contributing to the chronic phase of hypertension that follows NO synthase inhibition. The mechanisms involved in maintaining chronic L-NAME hypertension are more difficult to dissect because of the need to identify the adaptations of the different vasoactive systems as well as structural changes in the heart and blood vessels. Arnal *et al.* (1993) demonstrated that the adaptations to chronic NO

synthase inhibition were not consistent. Specifically, approximately 25% of the rats treated with L-NAME developed cardiac hypertrophy associated with a significant increase in plasma renin activity, whereas the remaining rats did not develop cardiac hypertrophy or have plasma renin activities different from saline control values. Therefore, the response to chronic NO synthase blockade has already been shown to have the potential for a renin-dependent response, at least in a sub-group of rats. A number of studies will be required to clearly elucidate the potential role of endothelin, neurohumoral systems, changes in blood volume as well as cardiovascular structural changes in the mechanisms of chronic L-NAME hypertension.

It has been previously speculated that NO may have a role in regulating the activity of the ET-1 vasoconstrictor system. Boulanger and Luscher (1991) demonstrated that NO released during thrombin stimulation in the porcine aorta, *in vitro*, has the capacity to inhibit ET-1 production. In addition, in human endothelial cells in culture, both ET-1 gene expression and ET-1 release were substantially increased by inhibition of endothelial-derived NO (Kourembanas *et al.* 1993). Conflicting results come from Tresham *et al.* (1994) who indicate that the plasma levels of immunoreactive ET-1 remain unchanged following NO synthase inhibition whereas Richard *et al.* (1995) report that plasma levels of ET-1 were modestly increased. It may be that plasma levels of ET-1 do not give an accurate representation of locally released ET-1 either as a result of rapid degradation (Luscher *et al.* 1993) or as a consequence of unidirectional release away from the circulation (Wagner *et al.* 1992). Despite the lack of change in the plasma levels of

ET-1 it remains likely that enhanced ET-1 actions can still occur at a local level.

It is widely acknowledged that inhibition of the NO system results in a ≈40 mmHg increase in MAP from the resting state. Assessment of the potential capacity of the endothelium to promote vascular relaxations *in vivo* (e.g. via acetylcholine or bradykinin) has shown a maximal decrease in MAP of approximately 20-25 mmHg (Gardiner *et al.* 1990). If the L-NAME induced increase in pressure (+ 40 mmHg) was mediated solely by the loss of NO-dependent vasodilation, this would indicate that the total vasodilator range of the NO system would be approximately 60 mmHg. This would imply that NO vasodilator activity is normally functioning at close to 70% of maximal capacity. This limitation is unlikely as this would leave very little reserve capacity, or 'gain', left to provoke vasodilation in physiological conditions that require rapid, local autoregulatory adjustments. The present data suggest that the basal vasodilator activity of NO accounts for about 5-10 mmHg. Further, the results suggest that inhibition of the local ET-1 vasoconstrictor system is a major role for the NO system. This mechanism would leave a much greater 'gain' in the NO system; i.e. it would have a reserve vasodilator capacity of up to ten fold. In conclusion, our studies provide evidence for an integrative control mechanism within the endothelium whereby NO-mediated mechanisms control both endothelin action and vasodilator effects where the latter actions are likely reserved for transient changes in vascular tone.

Abstract:

Objective: To determine the quantitative roles played by several endogenous vasoconstrictor systems to the pressor response in conscious rats before and after chronic nitric oxide synthase blockade with N^{ω}-nitro-L-arginine (L-NAME).

Methods: In conscious male Sprague-Dawley rats, previously instrumented with aortic and venous catheters, the contributions of the different vasoactive systems were assessed by maximal cumulative receptor blockade of α_1-adrenoceptors (1 mg/kg prazosin, intraperitoneally), AT_1 receptors (30 mg/kg losartan, intraperitoneally) and ET_A/ET_B receptors (10 mg/kg per min PD 145065, intravenously) following 12 days of either L-NAME (100 mg/kg, in drinking water). The antagonists were administered in a cumulative sequential manner to each rat in the study. The steady state following each antagonist administration served as the baseline level of mean arterial pressure for the following antagonist.

Results: Blockade of α_1-adrenoceptors resulted in a marked 'normalization' of the chronic L-NAME induced hypertension, relative to control. It was also demonstrated that endothelin receptor activation was playing an enhanced role, compared to control. Upon altering the order of antagonist administration, blocking ET_A/ET_B receptors first, resulted in a 3-fold larger decrease in mean arterial pressure compared to blocking ET_A/ET_B receptors as the third step in the cumulative sequential receptor blockade. The

contribution of AT_1 receptor activation was the same in the control and L-NAME treated rats.

Conclusions: These studies indicate that the enhanced vascular sensitivity to α_1-adrenoceptor activation plays an important role in the maintenance of the hypertension associated with chronic nitric oxide synthase blockade. We also demonstrated that the enhanced vascular sensitivity to α_1-adrenoceptor activation is mediated, at least in part, by enhanced levels of endothelin mediated vasoconstriction.

Introduction

Many experimental models of hypertension exist, where the factors involved in the maintenance of the hypertension are often distinct from the initiating cause(s)(Lever 1986). Elucidating the time course of change(s) in the neural, humoral and local vasoactive systems in these models is important if we are to understand the mechanisms involved in the development as well as the established phases of hypertension. Recent investigations into the etiology of these systems with respect to hypertension have focused on neuro-humoral systems and not on local systems. The nitric oxide synthase blockade induced model of hypertension presents a novel model to assess the contribution(s) local systems can make to the development and maintenance of hypertension.

Initial studies aimed at elucidating the circulatory control role(s) for nitric oxide, *in vivo*, revealed that this small molecule was functioning as a chronic vasodilator system (Moncada *et al* 1991). Further, it was proposed that nitric oxide mediates this tonic vasodilator influence on vascular smooth muscle cells via increased cGMP levels via activation of guanylyl cyclase (Arnal *et al* 1992). Thus, the hypertension that develops following nitric oxide synthase blockade was attributed to an increase in peripheral vascular resistance due a generalized increased sensitivity to the endogenous vasoconstrictor systems (Johnson and Freeman 1992, Tolins *et al* 1991, Bank *et al* 1994).

Many of the studies investigating the hypertension following NO synthase blockade have focused on neuro-humoral based mechanisms. For example, several studies have indicated a lack of involvement of the sympathetic nervous system in the NO synthase blockade induced pressor response (Kumagai *et al* 1993, Pucci *et al* 1992, Banting *et al* 1996). The contribution of the renin-angiotensin system (RAS) to the L-NAME induced hypertension has not been generally agreed upon, as Pollock *et al* (1993) indicate a significant role of the RAS to the hypertension following NO synthase blockade. Several others (Pucci *et al* 1992, Banting *et al* 1996, Arnal *et al* 1993) have demonstrated a complete lack of involvement of the RAS to the NO synthase blockade induced hypertension. Taken together, much of the investigations into the specific events involved in the development of hypertension following NO synthase blockade have focused on neuro-humoral, as opposed to local, systems.

Recent studies have revealed that the initiating mechanisms of nitric oxide synthase blockade induced hypertension was almost entirely due to enhanced local endothelin mediated vasoconstriction. (Banting *et al* 1996, Clozel *et al* 1995). This presents a potential conflict with respect to the hypothesis that nitric oxide functions as a chronic generalized vasodilator system. As such, the present study tested the hypothesis that the imbalance between nitric oxide and endothelin that mediates the acute pressor response to nitric oxide synthase blockade, may also play a role in the maintenance of the pressor response to chronic NO synthase blockade. The specific objective of the present study was to elucidate the contribution of several major vasoactive systems to the

hypertension associated with chronic nitric oxide synthase blockade. The vasoconstrictor mechanism(s) involved in maintenance of hypertension with chronic nitric oxide synthase blockade will be compared to the primary initiator of endothelin in the acute phase.

Methods:

Animals

Male Sprague-Dawley rats (325-400 g) obtained from Charles River Laboratories (Montreal, Canada) were housed individually under conditions of 12-hour light/dark cycle, with room temperature at 22-24°C, and were provided with Purina rodent chow and tap water ad libitum for at least 2 days before any experiments were started.

Measurement of MAP and Short Acting Drug Administration

The surgical method was based on the technique of Thompson *et al* (1992) In brief, rats were anaesthetized with ketamine/xylazine (70/5 mg/kg i.p.), and the descending aorta distal to the kidneys was catheterized with small bore Teflon® tubing (0.012-in. i.d., 30 gauge, Cole-Palmer, Laval, Canada) inserted into vinyl tubing (0.02-in. i.d., 0.060-in., 23 gauge). The inferior vena cava was also catheterized distal to the kidneys with small bore Teflon tubing (0.012-in. i.d., 30 gauge, Cole-Palmer) The catheters were filled with heparinized saline (10 IU/ml) and held in place by a small amount of cyanoacrylate glue at the puncture site. The catheters were tunneled subcutaneously and exteriorized at the back of the neck and sutured in place. Two days after surgery, MAP could be recorded (MacLab DAS, ADInstruments, Milford, MA). After connection, an equilibration period of approximately 30 minutes allowed for the determination of the steady state level of MAP before any recording began. Baseline

MAP was determined from readings averaged over 5 minutes, taken from each rat at 15-minute intervals for at least 1 hour prior to the start of any experiment.

Chronic NO synthase blockade.

The Chronic blockade of NO synthase was induced by including N^{ω}-nitro-L-arginine methyl ester (100 mg/kg per day) in drinking water. Drinking volumes were monitored over 24 hour periods to ensure proper dosages were administered.

Study 1: *Cumulative vasoconstrictor blockade following chronic (12 days) of NO synthase inhibition.*

After obtaining a baseline MAP, the effects of sequential pharmacological blockade of vasoconstrictor systems in conscious rats was investigated. The present approach has been validated previously (Banting *et al* 1996). In preliminary experiments we determined the doses of antagonists that were required to produce a maximal decrease in MAP (i.e. further increases in dose did not result in further lowering of MAP). MAP was determined at each new level of cumulative blockade as the average MAP over 2-3 minutes following the establishment of a new steady state.

In these experiments we assessed the contribution of vasoconstrictor systems at the neural (α_1-adrenoceptor), humoral (AT_1) and local (ET_A/ET_B receptors) levels in untreated rats (n=6) and after chronic L-NAME treatment (n=6) by determining the depressor response with each level of blockade. A sequential and cumulative blockade approach was used in order to eventually account for the difference between the L-NAME

and control groups, i.e. the objective was to step-wise add the different pharmacological antagonists until MAP's in the two groups were equalized.

Each rat received the antagonist drugs in the following order: (i) α_1-adrenoceptors were maximally blocked with prazosin (Sigma Chemical Company, St. Louis, MO, U.S.A.) at a dose of 1 mg/kg (5% ethanol [vol/vol] in 0.9% saline, total volume 1 ml/kg) which maximally decreased pressure for this drug, (ii) the non-peptide AT_1 receptor antagonist losartan (Dupont-Merck, 30 mg/kg i.p.) was used to maximally block the effects of Ang II, (iii) the effect of the endothelin-1 vasoconstrictor system, via both ET_A and ET_B receptors, was blocked with a continuous infusion of PD 145065 (10 mg/kg, i.v., Parke-Davis Pharmaceuticals).

Study 2: *Altered order of antagonist administration with chronic (12 days) nitric oxide synthase blockade.*

To elucidate any compensatory responses that may occur as a result of removing any other vasoactive system, the order of receptor antagonist administration was altered. As such, each of the rats in Study 2 received the antagonist drugs as follows: (i) the effect of the endothelin-1 vasoconstrictor system, via both ET_A and ET_B receptors, was blocked with an infusion of PD 145065 (10 mg/kg, i.v., Parke-Davis Pharmaceuticals). (ii) α_1-adrenoceptors were maximally blocked with prazosin (Sigma Chemical Company, St. Louis, MO, U.S.A.) at a dose of 1 mg/kg (5% ethanol [vol/vol] in 0.9% saline, total volume 1 ml/kg) which maximally decreased pressure for this drug, (iii) the non-peptide

AT_1 receptor antagonist losartan (Dupont-Merck, 30 mg/kg i.p.) was used to maximally block the effects of Ang II.

Results:

Study 1

As described in the Methods, we verified that the dose of each antagonist was maximal, on the basis of finding that there was no further lowering of MAP with repeated administrations (data not shown).

Figure 3-1 illustrates the stepwise MAP lowering after the sequential pharmacological blockade of α_1, AT_1 and ET_A/ET_B receptors. The chronic L-NAME treatment resulted in a 35 ± 8 mmHg (↑ 32 ± 8 %) increase in MAP, compared to controls. The data revealed that the absolute decrease in MAP (Δ MAP) with maximal AT_1 receptor blockade was similar to the responses in controls (Figure 3-2). In contrast, the effect of both α_1-adrenoceptor and ET_A/ET_B receptor blockade, in chronic L-NAME treated rats, were demonstrated to have an enhanced involvement compared to controls. α_1- Blockade induced a 35 ± 6 % larger decrease in MAP, in L-NAME treated rats, in comparison to control values. ET_A/ET_B receptor blockade resulted in a 65 ± 8 % larger decrease in MAP in L-NAME treated rats as compared to control. Consistent with previous studies (Banting *et al.* 1996), ET_A/ET_B receptor blockade did not induce any decrease in MAP in control rats.

In terms of the contribution these systems made to the level of MAP, based on the percentage of range analysis, only the ET_A/ET_B receptors demonstrated an enhanced

involvement of 3-fold above control values (Table 3-1). The contribution of α_1 and AT_1 receptors was similar to control levels.

Study 2

Figure 3-3 illustrates the Δ MAP following the sequential cumulative receptor blockade of ET_A/ET_B, α_1 and AT_1 with chronic L-NAME treatment. ET_A/ET_B blockade in comparison control to Study 1 and Study 2 demonstrated a marked increased involvement to the L-NAME pressor of 22 ± 6 % and 93 ± 5 %, respectively. α_1-adrenoceptor activation did not make an enhanced contribution to the level of mean arterial pressure in the chronic L-NAME treatment group. AT_1 contribution to the level of mean arterial pressure remained the same, regardless of the order of administration.

Analysis of Study 1 and Study 2 combined

Figure 3-4 represents a comparison of the absolute lowering of MAP with α_1, ET_A/ET_B receptor blockade, with alternate order of administration. The absolute contribution these systems contribute to the regulation of MAP in chronic L-NAME treated rats was similar to contributions in the control conditions. The overall impact of α_1, AT_1 and ET_A/ET_B receptor activation to the hypertension with chronic NO synthase blockade is presented in Figure 3-5. Banting *et al* (1997) and Li and Schiffrin (1994) demonstrated that vascular hypertrophy may increase peripheral vascular resistance properties in rats treated with L-NAME which may account for up to 8 % of the chronic L-NAME induced pressor response. The activation of α_1-adrenoceptors accounts for 25

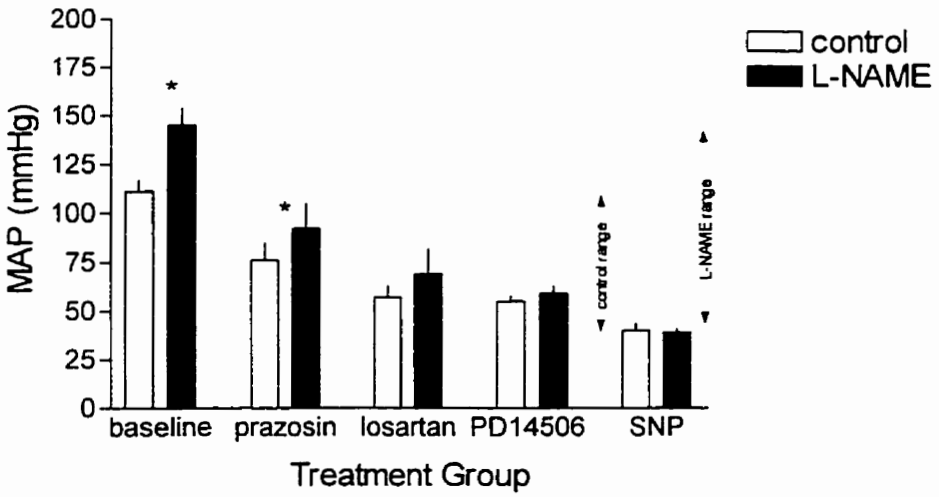

Figure 3-1: The MAP response from baseline (base) with N^{ω}-nitro-L-arginine methyl ester (L-NAME) or saline treatment with the sequential cumulative addition of prazosin, losartan and PD 145065 and sodium nitroprusside (SNP). Numbers of rats at each level of pharmacological blockade were 6, 6, 6, 6 and 6 for both control and 12 day L-NAME treated rats. Values are expressed as means ± SD. *P<0.05, versus control.

Table 3-1: Contribution of α_1, AT_1, ET_A/ET_B receptors with and without chronic NO synthase blockade via N^{ω}-nitro-L-arginine methyl ester treatment for 12 days. The percentage of range was calculated by dividing the mean arterial pressure lowering with each antagonist administration by the entire mean arterial pressure range (i.e. range = MAP_{max}-MAP_{min})

Receptor Antagonist	Percent of Range		
	Saline	L-NAME	Change
prazosin	48 ± 8 %	50 ± 12 %	↔
losartan	26 ± 8 %	22 ± 15 %	↔
PD 145065	2 ± 2 %	7 ± 2* %	↑

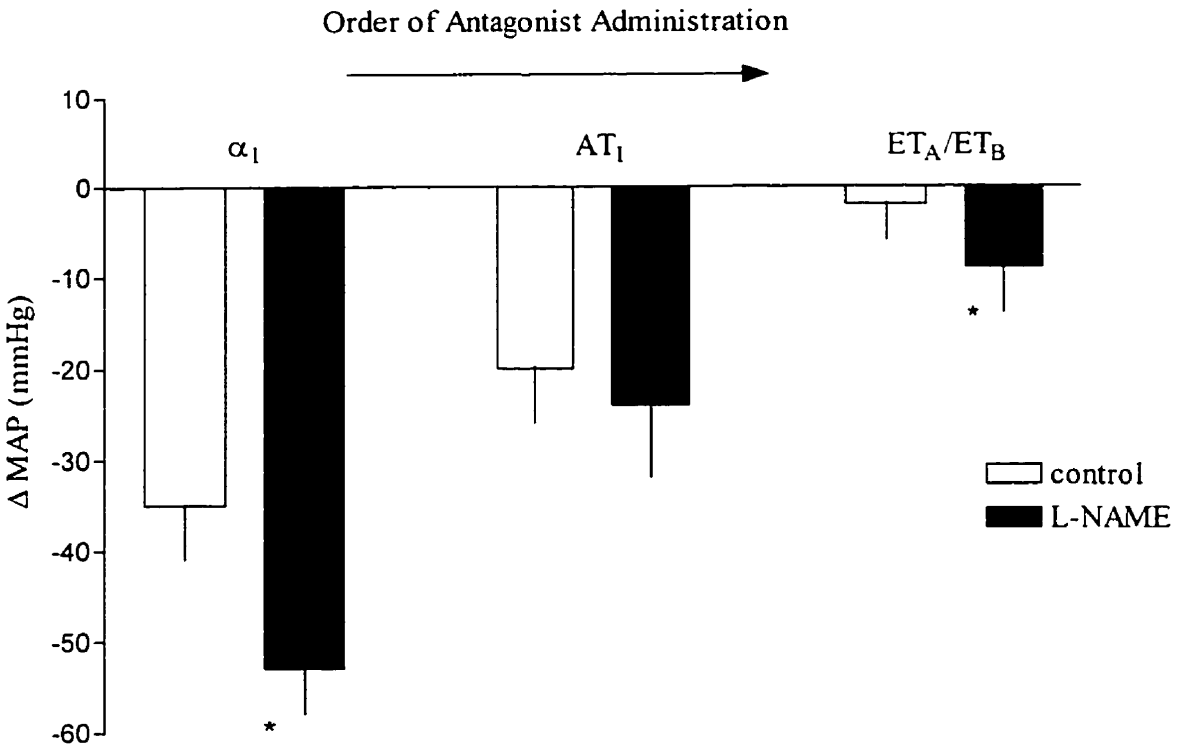

Figure 3-2: Δ MAP with α_1 (prazosin), AT_1 (losartan) and ET_A/ET_B (PD145065) in control and 12 day N^{ω}-nitro-L-arginine methyl ester (L-NAME) treated rats. Values are expressed as means ± SD. *P<0.05, versus control.

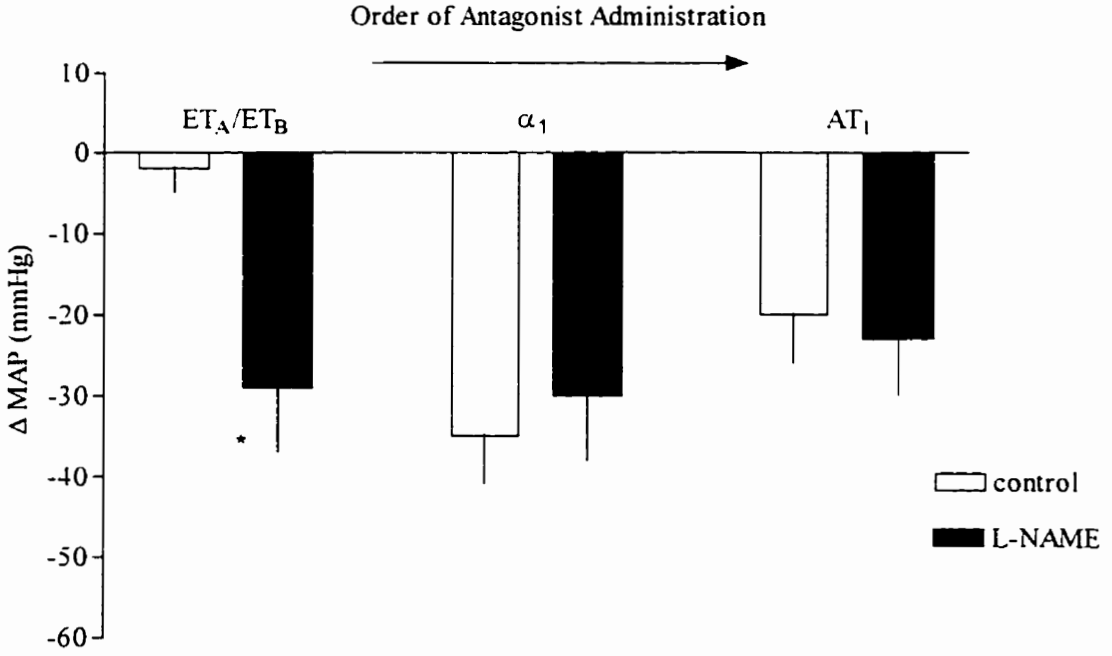

Figure 3-3: Δ MAP with ET_A/ET_B (PD145065), α_1 (prazosin) and AT_1 (losartan) in control and 12 day N^{ω}-nitro-L-arginine methyl ester (L-NAME) treated rats. Values are expressed as means ± SD. *P<0.05, versus control.

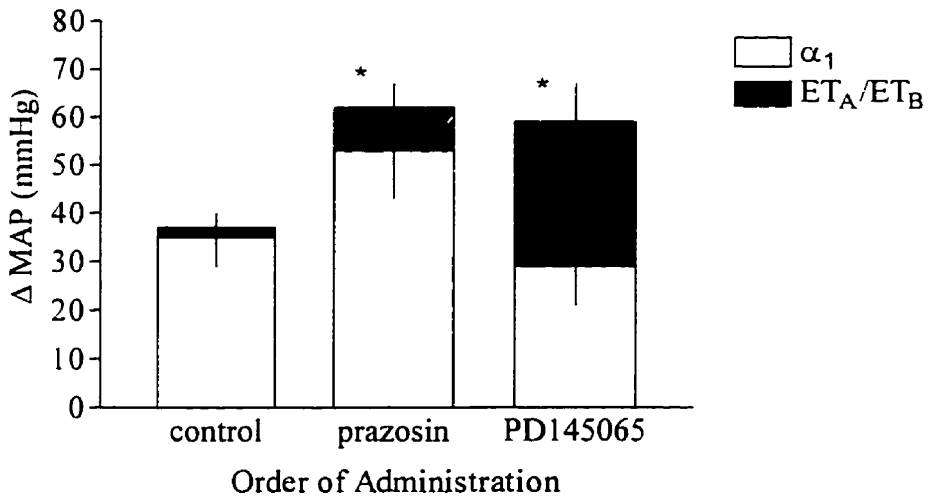

Figure 3-4: Comparison of the effect or absolute lowering in MAP when changing the order of administration of the antagonists. α_1-block, followed by endothelin block (left 2 sets of bars) to the lowering with endothelin block, followed by α_1 block (right set of bars). Values are expressed as means ± SD. *P<0.05, versus control.

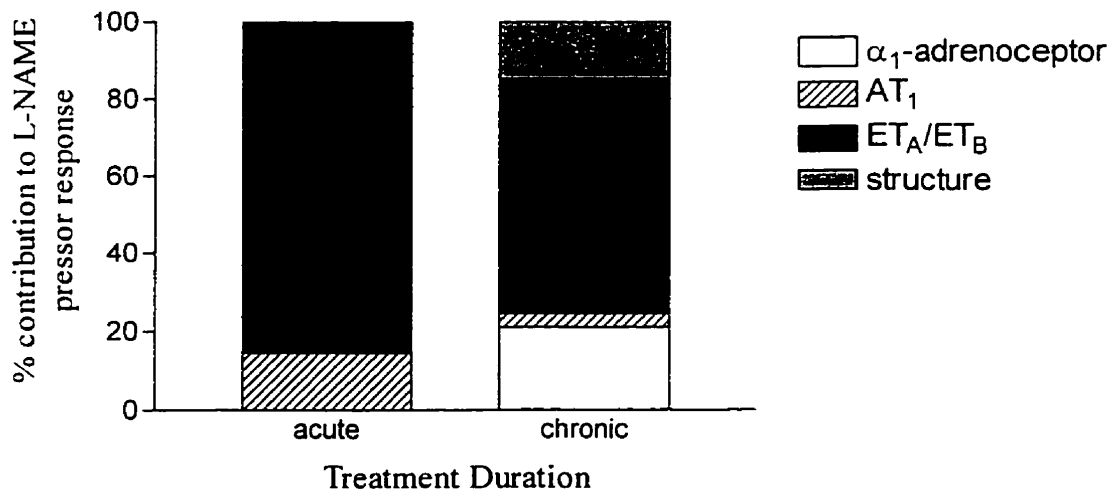

Figure 3-5: Presentation of the contribution of the different pressor mechanisms mediating the hypertension in the acute (adapted from Banting *et al*, 1996) and chronic phases of hypertension induced by NO synthase blockade.

± 5 % of the chronic L-NAME pressor response. Endothelin mediated vasoconstriction, via direct vasoconstriction and mediated the enhanced α_1 -adrenoceptor reactivity, accounts for 60 ± 9 % of the chronic NO synthase blockade induced hypertension. The contribution of AT_1 receptor activation remains as a 8 ± 4 % role in the chronic L-NAME pressor response.

Discussion:

A major finding of the present study was that augmented endothelin mediated vasoconstriction played an important role in the maintenance of the chronic hypertension associated with prolonged NO synthase blockade (12 days). This enhanced vasoconstrictor mechanism was demonstrated to be a consequence of: (i) direct actions of endothelin as a vasoconstrictor and (ii) via indirect actions, involving a sensitization or 'priming' action with respect to α_1-adrenoceptor mediated constrictor effects. Specifically, the data demonstrate that endothelin-mediated effects, both via direct and indirect actions, account for at least 60 % of the hypertension induced by chronic NO synthase blockade. In contrast to other studies, the present findings revealed a lack of involvement of enhanced AT_1 receptor activation in the hypertension. These data, combined with previous findings, provide evidence that endothelin plays a major role in both the initiation (Banting *et al*, 1996) and maintenance of the hypertension associated with NO synthase blockade.

The component of the L-NAME pressor response related to the marked increased sensitivity to α_1-adrenoceptor activation is consistent with the findings of other *in vitro* and *in situ* studies. Li and Schiffrin (1994) demonstrated a significantly increased sensitivity to norepinephrine in blood vessels from rats treated for two-weeks with L-NAME as compared to controls. Their study similarly demonstrated that acute treatment of the control vessels with L-NAME resulted in a leftward shift in the agonist-contractile response curve to norepinephrine (Li and Schiffrin 1994). Further, *in situ*, experiments have shown that the blockade of NO synthase with N^G-nitro-L-arginine (L-NOLA) markedly increased the vascular reactivity to both sympathetic nerve stimulation and norepinephrine in the isolated perfused rat kidney (Reid and Rand 1992). Thus, the enhanced pressor response (51% above control levels) that occurred in the chronic L-NAME treated rats of the present study is consistent with the increased α_1-adrenoceptor sensitivity observed in other studies both *in vitro* and *in situ*.

Despite the potential for hyperactivity of α_1-adrenoceptors activation during L-NAME treatment, previous evidence showed that the contribution of the sympathetic nervous system was actually diminished in the acute phase of L-NAME hypertension (Banting *et al* 1996). That is, the activation of α_1-adrenoceptors could not account for any of the L-NAME induced pressor response. This result is consistent with other investigations that have demonstrated a marked decrease in sympathetic nervous system activity, shortly after NO synthase blockade (Kumagai 1993, Pucci *et al* 1992), likely resulting from a baroreceptor reflex-induced suppression of sympathetic drive (Banting

et al 1996, Banting *et al* 1996a). In contrast, in the present longer term study, the contribution of increased α_1-adrenoceptor mediated constriction (prior to AT_1 and ET_A/ET_B blockade) accounted for at least 50 percent of the hypertension in the chronic stage of NO synthase blockade. This indicates that the role of α_1-adrenoceptor activation has progressed from a decreased involvement during the acute phase of the hypertension to an 'apparently' enhanced role in the chronic phase. That is, the resetting of sympathetic drive towards normal levels from the acute phase to the chronic phase produced a progressive increase in the effect of even normal levels of sympathetic tone. This hyper-reactivity has been 'accounted' for by a marked upregulation of endothelin mediated effects, as upon blocking ET_A/ET_B receptors the involvement of α_1-adrenoceptor activation was almost completely normalized.

In contrast, no differences in AT_1 receptor activation were observed following chronic L-NAME treatment. This lack of involvement in the chronic pressor response is similar to the contribution of AT_1 receptor activation in the acute phase of L-NAME hypertension (Banting *et al* 1996). The overall lack of an enhanced involvement of AT_1 receptor activation in both acute and chronic phases indicates that the renin-angiotensin system is not a major contributor to the development and/or maintenance of L-NAME induced hypertension. This concept is consistent with the findings of Arnal *et al* (1993) where plasma renin activity was normal in 90% of the L-NAME treated rats. In their study, only in a small portion of the rats was there abnormal increase in plasma renin activity associated with cardiac hypertrophy. As such, a role for the renin-angiotensin

system in the L-NAME model of hypertension may reflect a transition from a more benign towards a malignant phase (Ono *et al* 1996). Taken together, the results of the present and previous studies (Banting *et al* 1996) indicate an overall lack of involvement of increased AT_1 receptor activation to the development and maintenance of L-NAME induced hypertension.

Our findings, in contrast to others, propose that endothelin plays a pre-eminent role both in the initiation and in the maintenance of chronic L-NAME induced hypertension. Moreau *et al* (1997), using the orally active ET_A/ET_B antagonist bosentan, did not demonstrate normalization of mean arterial pressure in rats treated with L-NAME for 6 weeks. The 6-week duration of this study verges on the more malignant phase of hypertension making any direct comparisons to the present findings difficult. Further, it should be noted that there is substantial variability in the efficacy of different endothelin antagonists. For instance, discussions by Minamino *et al* (1997) highlight the fact that the ET_A selective antagonist BQ-123 was ineffective in attenuating the neointimal proliferation that occurs following balloon injury whereas the ET_A selective antagonist BMS-182874 did attenuate the proliferative response (Minamino *et al* 1997). A second example of the discrepancies in the efficacies of endothelin antagonists is revealed by the comparison of results of Banting *et al* (1996) and Richard *et al* (1995). Richard *et al* (1995) demonstrated using the ET_A/ET_B receptor blocker, bosentan, that only a portion of the hypertension following acute NO synthase blockade could be inhibited. In contrast, Banting *et al* (1996) demonstrated that ET_A/ET_B receptor blockade, using PD 145065,

both prevented and reversed almost all of the acute L-NAME induced hypertension,. While direct comparison of separate studies remains difficult, some evidence has accumulated regarding the differential efficacy of the endothelin receptor antagonists.

In a study by Sventek *et al* (1997) it was demonstrated that a three week treatment of L-NAME in combination with the non-peptide ET_A antagonist A-127722 failed to normalize mean arterial pressure. Their results, combined with the present study, suggest that ET_B receptor activation may be an important mediator of chronic L-NAME induced hypertension. However, the relative contribution of ET_A and/or ET_B receptor activation in a pressor response remains equivocal, as such it is difficult to discuss the specific physiological roles played by these receptors.

Consistent with our hypotheses regarding the endothelin mediated priming effects on α_1-adrenoceptor activation, Reid *et al* (1991) demonstrated that infusion of 0.3 nM of endothelin-1, a concentration which did not alter the basal perfusion pressure in the rat tail artery, resulted in a markedly increased sensitivity to norepinephrine mediated vasoconstriction. Similarly, Adams *et al* (1996) demonstrated in the isolated pudendal vasculature that sub-pressor concentrations of endothelin-1 had the capacity to convert a sub-pressor dose of the α_1-adrenoceptor agonist, methoxamine, to a pressor effect. Interestingly, Adams *et al* (1996) also demonstrated in this isolated perfused vasculature that sub-pressor doses of methoxamine in combination with L-NAME treatment resulted in a markedly enhanced pressor response that was fully reversed following ET_A/ET_B

receptor blockade. Overall these studies provide strong supportive evidence that endothelin serves to increase vascular sensitivity to α_1-adrenoceptor activation.

Our findings demonstrate that upregulation of endothelin mediated vascular effects represents not the only predominant mechanism involved in the initiation of NO synthase blockade hypertension, but appears to be the primary mediator of the increased reactivity to α_1-adrenoceptor activation in the chronic phase as well. The capacity for endothelin to modulate the vascular sensitivity or 'gain' of the α_1-adrenoceptor concentration-response relationship may represent a mechanism by which vascular tone can be regulated in specific regions without the necessity for alterations in the activity of more global circulatory control systems. Overall, the present study, in combination with previous work has demonstrated a causal involvement of a local vasoconstrictor system, endothelin, in the development and maintenance of hypertension following NO synthase blockade for 12 days.

Chapter 4: Hypertension without cardiac hypertrophy does not induce a cardiac baroreflex deficit.

Abstract:

Objective: To investigate the effects of prolonged hypertension, in the absence of cardiac hypertrophy, on the blood pressure-heart rate reflex during acute and chronic NO synthase blockade.

Methods: Male Wistar rats were treated acutely (N^{w}-nitro-L-arginine methyl ester; L-NAME, 50 mg/kg i.p.)or chronically (L-NAME, 2.5-3 weeks, 50 mg/kg per day p.o.). The cardiac baroreceptor reflex was assessed, in previously instrumented conscious, rats using a "steady-state" method which involved alternating vasoactive drug-induced step-wise increases and decreases in MAP with methoxamine and sodium nitroprusside. Following baroreflex assessment, the rats were sacrificed by anaesthetic overdose, the hearts were removed and the LV + septum were separated from the heart and weighed.

Results: The BP_{50} was shifted to higher arterial pressures consistent with the increase in the operating point of MAP following NO synthase blockade. No change in any of the baroreflex parameters could be detected despite prolonged L-NAME induced hypertension. Based on the study criteria, the data from one rat were not included in the group analysis because of the presence of cardiac hypertrophy.

Conclusions: The results of the present study indicate that increased blood pressure alone, both acutely and chronically, is not a sufficient stimulus to induce a baroreflex deficit.

Introduction:

Baroreceptor re-setting occurs in response to sustained hypertension both in human beings (Zanchetti and Mancia 1991) and in animals (Segar *et al.* 1994, Moreira *et al.* 1989, Moreira *et al* 1990). This resetting is usually associated with an overall reduced sensitivity of the baroreceptor reflex control of heart rate (Zanchetti and Mancia 1991, Sleight *et al.* 1977, Struyker-Boudier *et al.* 1982, Korner *et al.* 1972). The mechanisms which have been proposed to explain this deficit have not been clearly elucidated, although a number of causative factors have been postulated, including the development of left ventricular hypertrophy (Head and Minami *et al* 1992), alterations in the central nervous system (Gonzales *et al.* 1983) and vascular hypertrophy (Andresen and Yang 1989).

The development of left ventricular hypertrophy is a primary feature of chronic hypertension in both human beings (Zanchetti and Mancia 1991) and spontaneously hypertensive rats (SHR). In developing SHR, Head and Adams (1992) and others (Umemura *et al.* 1992, Moyses *et al.* 1992) indicated that the magnitude of cardiac hypertrophy and the decreased baroreflex sensitivity were associated. A strong correlation has been found between the baroreflex-heart rate range and cardiac hypertrophy in comparison to factors such as blood pressure or the presence of vascular hypertrophy (Head and Minami 1992, Head and McCarty 1987). For example, in studies in which cardiac hypertrophy in SHR (Head and Adams 1992) and in humans (Grassi *et al.* 1988) is progressively reversed normalisation of the baroreflex gain has been found

concomitantly. However, since the changes in cardiac hypertrophy and hypertension occur in parallel their causal relationship to the baroreflex deficit remains obscured.

A new model of hypertension involves the inhibition of the enzyme NO synthase. In this model administration of enzyme antagonists produces a rapid and sustained hypertension characterised by increased total peripheral resistance (Gardiner *et al.* 1990). A surprising finding with this model has been that despite prolonged hypertension cardiac hypertrophy is not a prominent result. Arnal *et al.* (1993) showed that only a small portion of the NO synthase blocked rats (25%) developed left ventricular hypertrophy, illustrating that there is a heterogeneous cardiac growth stimulus. The lack of a cardiotrophic response may result from the fact that there is minimal, if any, up-regulation of neuro-humoral systems which are known to have potent trophic capacity (i.e. renin angiotensin system (RAS), sympathetic nervous system). In fact, their data demonstrated that cardiac hypertrophy only occurred in a sub-group of rats, in which there was also an enhanced RAS activity (Arnal *et al.* 1993). The study of Morton *et al.* (1993) further supports the role of RAS activation in the cardiac growth response. They showed that after four weeks of L-NAME hypertension, but not before, the development of cardiac hypertrophy was strongly associated with increased RAS activity.

Some studies have assessed changes in baroreflex sensitivity in the NO synthase blockade model of hypertension but none of these have appropriately addressed the confounding issue of the presence of cardiac hypertrophy within the treatment groups

(Lantelme *et al.* 1994, Scrogin *et al.* 1994, Vasquez *et al.* 1994, Cerutti *et al.* 1995). In a study by Lantelme *et al.* (1994) they suggested that the development of the baroreflex deficit in the hypertensive rats did not correlate with the development of cardiac hypertrophy. The problem with their conclusions is that there was, in fact, a small significant increase in left ventricular to body weight ratio. Based on the findings of Arnal *et al.* (1993), the 'small' increase in cardiac mass could reflect the presence of cardiac hypertrophy in a sub-group of rats. The overall impact of cardiac hypertrophy in only a few rats would have been 'diluted' by a lack of hypertrophy in a majority of rats. In the present study our objective was to clarify this issue. Our experiments were designed to assess changes in baroreceptor reflex function resulting from acute and chronic NO synthase blockade-induced hypertension in rats that did not develop cardiac hypertrophy. Importantly, in our experiments, the baroreflex data from rats which developed cardiac hypertrophy, determined as being two standard deviations above the mean, were separated from the group in order to assess the impact of increased blood pressure alone on baroreflex sensitivity.

Methods:

Animals

Male Wistar rats (270-370g), obtained from Charles River Laboratories (Montreal, Quebec, Canada) were housed individually under conditions of 12-hour light/12-hour dark cycle (temperature of 22 to 24C), and received free access to rodent

chow and tap water for at least four days before any experiment.

Treatments

Rats were randomly assigned to two treatment groups: (i) NO synthase blockade with N^{w}-nitro-L-arginine methyl ester (L-NAME, 50 mg/kg per day in drinking water, n=12) for 2.5-3 weeks and (ii) saline control (n=12). In a separate group of rats control baroreflex assessments were performed followed by acute administration of L-NAME (50 mg/kg i.p., n=6) and the effect on baroreflexes assessed 30-60 minutes later.

Surgery

Two days before the end of treatment period, rats were instrumented with indwelling abdominal aortic catheters. The surgical method was based on the technique of Thompson *et al.* (1993). In brief, rats were anaesthetised with sodium pentobarbital (60 mg/kg i.p.), and the descending aorta distal to the kidneys was catheterized with small bore Teflon tubing (0.012-in i.d., 30 gauge, Cole-Parmer, Laval Quebec, Canada) inserted into vinyl tubing (0.02-in, 23 gauge). The inferior vena cava was cannulated and two catheters were inserted distal to the kidneys using small bore Teflon tubing (0.012-in. i.d., 30 gauge, Cole-Parmer). The catheters were filled with heparinized saline (10 IU/ml) and held in place by a small amount of cyanoacrylate glue at the puncture site. The catheters were tunnelled subcutaneously and exteriorized at the back of the neck.

Experiment Protocol

At least two days were allowed for recovery from surgery. On the experimental day, following connection of the catheters and a 30 minute acclimitization period, baseline MAP and HR were obtained for 60 minutes prior to the assessment of the blood pressure-heart rate reflex in both control (n=12) and in acute and chronic L-NAME treated (n=6, 12, respectively) rats. The heart rate and mean arterial pressure data was sampled at 150 Hz and stored on computer for later analysis (MacLab DAS, ADInstruments, Milford, MA). The baroreceptor-HR reflex was assessed using the "steady-state" method which involved alternating vasoactive drug-induced, step-wise increases and decreases in MAP. Alternating intravenous injections of 1-200 ul of the α_1-adrenoceptor agonist methoxamine (MXA, 200 ug/ml) and sodium nitroprusside (SNP, 100 ug/ml) were used to produce a series of short term increases and decreases in MAP (± 5-60 mmHg) in each rat.

The steady-state changes in MAP and HR were fitted to a sigmoidal logistic equation using a computer program (Sigmoid 5, Baker Medical Institute, Melbourne, Australia) that employed the algorithm of Marquart (1963). The computer program fitted the values to a sigmoidal curve using the following equation:

$$HR = P_1 + \frac{P_2}{1 + e^{P_3(MAP - P_4)}}$$

Where P_1 = the lower plateau, P_2 = HR range, P_3 = normalized gain (curve parameter) and $P_4 = BP_{50}$ as described by Head and McCarty (1987). The gain or sensitivity of the reflex was determined mathematically as the slope at the MAP point midway between the two plateaus of the curve (Head and Adams, 1991). The equation used to calculate maximum gain was:

$$MaxGain = \frac{(HRrange)x(Curvature)}{4}$$

Assessment of Cardiac Hypertrophy

Immediately following the baroreceptor-reflex HR assessments, each rat was euthanized by anaesthetic overdose (dilute sodium pentobarbital 60 mg/kg, i.v). The heart was excised and extraneous tissues were removed. The left ventricle plus septum (LV) was carefully separated from the right ventricle free wall (RV) and the tissues were weighed. The presence of LV hypertrophy was defined by a LV index (LV:BW ratio) greater than 2 standard deviations above the mean of the control group.

Statistical Analysis

Statistical analyses of the baroreflex parameters were performed using a one-way ANOVA followed by post-hoc comparisons with the Bonferroni correction method.

Values are plotted as means ± S.D. Values were considered significantly different when $p<0.05$, as compared with controls.

Results:

LV:BW ratios were similar in both treatment groups (Table 4-1) with one exception. A single rat in the chronic L-NAME treatment group was not included because it had a LV:BW ratio of 2.48 (> 2 SD from mean in control group). Assessment of conscious baseline MAP and HR in acute and chronic L-NAME treatment demonstrated that there was an increase in MAP in both groups of 33 ± 11 % and 42 ± 9%, respectively. A decrease in heart rate occurred in the acute L-NAME treatment group only.

Despite the presence of pronounced hypertension, the assessments of the blood pressure-heart rate reflex demonstrated that there were few differences (Table 4-2). The only significant difference found between the groups was a rightward shift in the position of the baroreflex curve in both the acute and chronic treated rats towards higher pressure. The magnitude of the shift was of a similar order as the increase in MAP (Figure 4-1). Specifically, there were no differences found in the maximum gain, heart rate range, or in the upper and lower plateaus between groups.

Interestingly, in the one L-NAME-treated rat with cardiac hypertrophy, the MAP was 165 mmHg, the HR was 290 bpm and there was an apparent decrease in the HR

range compared to all other treatment groups (185 bpm vs. 253 ± 72, 234 ± 40 and 236 ± 49 bpm from Table 4-2).

Table 4-1: MAP, HR and cardiac index for the control and treatment groups.

	MAP (mmHg)	HR (bpm)	LV:BW (mg/g)
control	109 ± 10	356 ± 37	1.97 ± 0.13
L-NAME$_a$	145 ± 15*	298 ± 40*	-
L-NAME$_c$	155 ± 13*	350 ± 49	1.89 ± 0.16

All values are expressed as means ± SD. *$p<0.05$, compared to control. MAP, mean arterial blood pressure; HR, heart rate; LV:BW, left ventricle to body weight ratio; L-NAME$_{a,c}$, acute and chronic N^w-nitro-L-arginine methyl ester, respectively. n=12,6,12

Table 4-2: Baroreflex parameter values for the control and treated groups.

	BP50 (mmHg)	Gain (bpm/mmHg)	Range (bpm)	Upper (bpm)	Lower (bpm)
control	107 ± 10	-2.4 ± 0.43	253 ± 72	479 ± 56	226 ± 39
L-NAME$_a$	128 ± 14 *	-2.1 ± 0.51	234 ± 40	458 ± 11	224 ± 36
L-NAME$_c$	138 ± 15*	-2.1 ± 0.91	236 ± 49	464 ± 69	227 ± 39

All values are expressed as means ± SD. *$p<0.05$, compared to control. BP_{50}, arterial pressure at half the heart rate range; L-NAME$_{a,c}$, acute and chronic N^{w}-nitro-L-arginine methyl ester, respectively.n=12,6,12

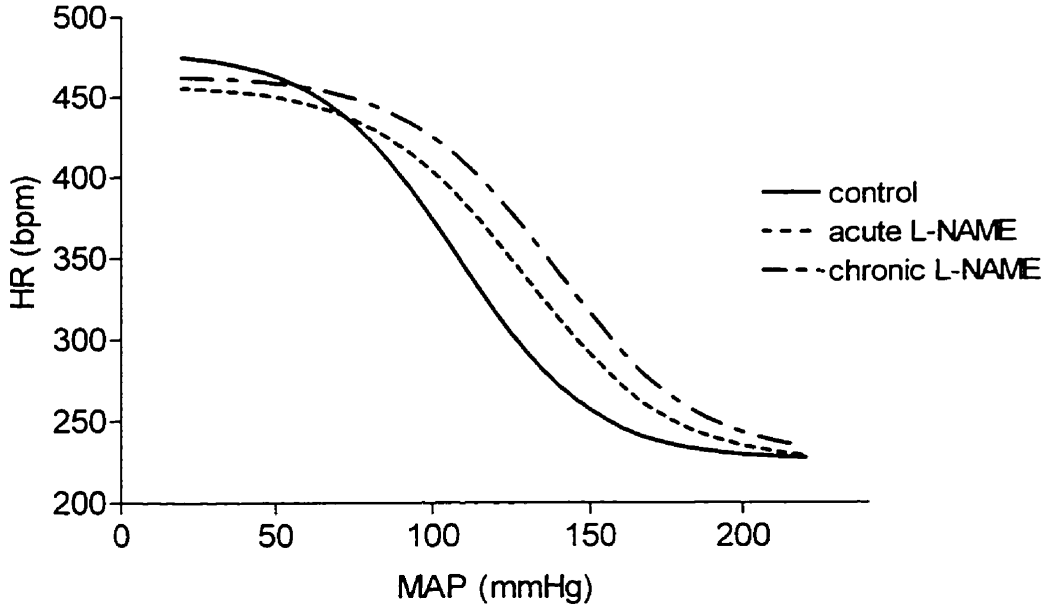

Figure 4-1: Averaged curve parameter values for blood pressure-heart rate baroreflex relationship with acute and chronic L-NAME treated rats compared to control (n=6,12,12, respectively).

Discussion:

The major finding of this study was that despite marked hypertension induced by acute and chronic NO synthase blockade, no deficit in the baroreceptor-heart rate reflex range or gain was induced. Taken together with previous findings (Head and Adams 1987, Umemura *et al.* 1992, Moyses *et al.* 1994, Head and McCarty 1987, Grassi *et al.* 1988), the results of the present study demonstrate that prolonged hypertension alone, in the absence of cardiac hypertrophy, is not a sufficient stimulus to induce a baroreflex deficit. In addition, the relationship between the operating point (MAP) and the mid-point of the baroreflex (BP_{50}) revealed that the set-point of the baroreflex had shifted to the same extent as the increase in arterial pressure. The lack of change in heart rate with the chronic L-NAME treatments is consistent with the resetting of baroreceptors, a phenomenon which has been demonstrated in other hypertensive models such as those induced by angiotensin II or phenylephrine infusion (Segar *et al.* 1994), subdiaphragmatic aortic constriction (Moreira *et al.* 1989), and renal clipping (Moreira *et al.* 1990). It is clear, however, based on the significant bradycardia the resetting of the heart rate has not yet occurred 30 minutes following acute NO synthase blockade in the present study.

The lack of change in the baroreflex sensitivity, in the present study, also indicates that the baroreflex does not play an important role as a mediator of L-NAME induced hypertension. This is consistent with the concept that L-NAME hypertension results from an imbalance of local factors in the vascular endothelium rather than due to neuro-humoral activation. The L-NAME pressor response has recently been shown to result

from a marked enhancement of endothelin mediated vasoconstriction (Banting *et al.* 1996, Richard *et al.* 1995, Sventek *et al.* 1996) and not from changes in sympathetic nervous system (Pucci *et al.* 1992) and renin-angiotensin system activity (Bank *et al.* 1994). The lack of neuro-humoral activation may account for the absence of a change in baroreflex sensitivity. A positive example of this association comes from the work of Hartikainen *et al.* (1995) in which decreased baroreflex sensitivity after myocardial infarct was associated with enhanced sympathetic activation. They also demonstrated that the decreased baroreflex deficit was not associated with changes in plasma atrial natriuretic factor, endothelin-1 or plasma renin activity (Hartikainen *et al.* 1995). Ferrari *et al.* (1991) have also revealed that enhanced sympathetic nerve activity exerts an antagonistic effect on baroreflex sensitivity both in Wistar rats and SHR and that sympathectomy potentiated the baroreflex.

Several other experimental models of hypertension, characterized by eccentric (Head and Minami 1992) and concentric (Moyses *et al.* 1994) cardiac hypertrophy have demonstrated a baroreflex deficit that was strongly correlated to the magnitude of the cardiac hypertrophy. In addition, Umemura *et al.* (1992) demonstrated, using a model of aortic valve insufficiency, that the development of cardiac hypertrophy in the absence of hypertension still produced a significant baroreflex deficit. Overall, it appears that the development of cardiac hypertrophy under these wide variety of conditions may represent a final common pathway in the development of a baroreflex deficit.

Head and Adams (1992) found that the baroreflex deficit in SHR occurred only after hypertension had been established, suggesting that it is a consequence, rather than a cause, of the increased blood pressure (Head and Minami 1992). In support of this concept, Struyker-Boudier *et al.* (1992), demonstrated that the baroreflex deficit that occurs in SHR was due to the abnormal development of the baroreflex paralleling the marked increase in MAP in developing SHR in comparison to that normal development of the baroreflex in WKY rats. In SHR, the baroreflex deficit develops from approximately six weeks of age a time interval during which cardiac hypertrophy develops (Head and Adams 1988, Head and Adams 1992]. The strong temporal correlation between these two events lead to the hypothesis that cardiac hypertrophy, rather than an increase in MAP, may be a factor in the deficit (Head and Adams 1992). Minami and Head (1993) further demonstrated that the deficit in the baroreflex showed a strong correlation with the level of cardiac hypertrophy and not with the level of vascular hypertrophy. This conclusion was based on the demonstration that the normalization of the baroreflex in stroke-prone spontaneously hypertensive rats (SpSHR) progressed concomitantly with the regression of cardiac hypertrophy during anti-hypertensive treatment with the ACE inhibitor perinodpril. Grassi *et al.* (1988) have similarly demonstrated, in human studies, the association between increased cardiopulmonary reflex activity and regression of left ventricular hypertrophy during anti-hypertensive treatment.

Overall, these studies suggest that there is a direct link between the degree of

cardiac hypertrophy and the level of deficit in the baroreflex. The L-NAME model of hypertension is characterized by a lack of the development of cardiac hypertrophy (Arnal *et al.* 1993). This experimental manipulation presented the opportunity to distinguish between the confounding factors of cardiac structural changes and increased blood pressure in the mechanism of the baroreflex deficit. Taken together, the results of the present study provide useful evidence as a 'negative control' in support of the association between cardiac hypertrophy and the baroreflex deficit, i.e. prolonged hypertension, in the absence of cardiac hypertrophy, does not induce a baroreflex deficit.

There have been a few of studies which have assessed baroreflex function during acute and chronic L-NAME hypertension, and these have been conflicting. Similar to the present study, both Castellano *et al*, (1995) in humans and Matsuda *et al.* (1995) in rabbits demonstrated that there was no impairment of the baroreflex sensitivity with acute L-NAME. Results with chronic treatments have been more inconsistent. For example, Scrogin *et al.* (1994) found, in Sprague-Dawley rats, a decreased heart rate range and gain following 5-weeks of L-NAME treatment. Similarly, Lantelme *et al.* (1994), in Wistar rats, also demonstrated a decreased baroreflex gain, although with no change in heart rate range, following 4-weeks of L-NAME treatment. In contrast, Cerutti *et al.* (1995) reported no changes in the reflex mechanism during a 4-week L-NAME treatment using a statistical method of spontaneous baroreflex assessment. On the other hand, the Vasquez *et al.* (1994) is the only one of any duration, that found an enhanced baroreflex range following 6 days of L-NAME treatment in Wistar rats. Several points of concern make

direct comparisons between the Vasquez study and all others difficult. First, the baroreflex assessments were performed only six hours after surgery. The lack of recovery time would contribute markedly to the variability of the baroreflex curves. Secondly, baseline HR for the L-NAME treated group was relatively high (418 ± 16 bpm), indicating increased baseline sympathetic activation , whereas in other studies HR was either unchanged or was lower following L-NAME treatment compared to control. Thirdly, the HR range of the control Wistar rats was only 185 bpm whereas in other studies the control Wistar HR range is always above 250 bpm (Ferrari *et al.* 1991, Head and Adams 1988).

The discrepancies in the conclusions of the different chronic L-NAME studies can be explained, at least in part, by (i) inadequate recovery time from surgery (Lantelme *et al.* 1994, Scrogin *et al.* 1994, Vasquez *et al.* 1994), (ii) increased stress at the time of assessment as indicated by elevated heart rates (Vasquez *et al.* 1994) and (iii) the development of cardiac hypertrophy in a sub-group of L-NAME treated rats (Lantelme *et al.* 1994, Scrogin *et al.* 1994). In particular, if present, cardiac hypertrophy in a small number of rats could skewed the baroreflex analysis of the entire group. For example, Lentelme *et al.* (1994) reported a significant (8%) increase in LV:BW ratio. In other studies cardiac structural measurements were not done (surgery (Lantelme *et al.* 1994, Scrogin *et al.* 1994, Vasquez *et al.* 1994). Based on the results of Arnal *et al.* (1993) the expectation is that approximately 25 % of the L-NAME treated rats could develop cardiac hypertrophy, the other rats remaining completely normal. In the study by Lentelme *et al.*

(1994), however, the results of the baroreflex assessments of the individual rats have been pooled regardless of the level of cardiac hypertrophy. Hence, the decrease in the baroreflex gain of the entire L-NAME group may be confounded by a baroreflex deficit in a sub-group of the treated rats with marked cardiac hypertrophy.

In summary, an objective of the present study was to clarify the issue of involvement of chronically increased MAP alone with respect to changes baroreflex sensitivity. Our findings demonstrate that the cardiac baroreflex sensitivity does not change following acute and chronic L-NAME hypertension in Wistar rats, specifically in those animals that do not develop cardiac hypertrophy. In conclusion, the results of the present study indicate that increased blood pressure alone is not a sufficient stimulus to induce a baroreflex deficit.

Chapter 5: Blunted Cardiovascular Growth Induction During Prolonged Nitric Oxide Synthase Blockade.

Abstract:

The goal of the present study was to characterize the activation profile of the growth related enzyme, ornithine decarboxylase, in cardiovascular tissue during hypertension induced by chronic NO synthase blockade in relation to the development of structural based changes in the heart and blood vessels. In previously instrumented conscious rats, mean arterial pressure (MAP) and ornithine decarboxylase (ODC) activation were measured in cardiovascular tissue of rats treated with 4 hours, 1, 6 and 12 days of N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 mg/kg per day p.o.). After 12 days of L-NAME treatment alone or in combination with 3% L-ornithine, structurally based hindlimb resistance properties were assessed. A marginal activation of ODC in the left ventricle and aorta was seen at 4 hours but returned to control levels at 1, 6 and 12 days of L-NAME treatment. A slightly prolonged, yet transient, activation of ODC occurred in the mesenteric vascular bed. Structurally-based hindlimb vascular resistance was enhanced by 15 % at maximum vasoconstrictor tone and no change in cardiac mass occurred with L-NAME treatment. L-NAME + 3 % L-ornithine treatment resulted in a similar level of structural up-regulation as compared to L-NAME treatment alone. In summary, 12 days of L-NAME treatment resulted in only a modest change in vascular resistance, and only at maximum constriction, and no cardiac hypertrophy despite the presence of marked hypertension. The results of the present study indicate either that (i)

pressure-alone is not a sufficient stimulus to induce cardiovascular growth processes or (ii) L-NAME may be "non-specifically" inhibiting cardiovascular growth processes.

Introduction:

It is widely acknowledged that structural changes in the heart and blood vessels occur in almost all forms of hypertension. A less defined association is the cause-effect relationship between the level of cardiovascular hypertrophy and the magnitude of the hypertension. The development of cardiovascular structural changes is a feature commonly associated with experimental models of hypertension such as 2-kidney-1-clip(2K1C), SHR or angiotensin II (Ang II) infusion (Folkow 1990, Griffin *et al* 1991, Dostal and Baker 1992). In contrast, in studies in which hypertension was induced by nitric oxide (NO) synthase blockade this relationship does not hold (Arnal 1992 & 1993, Delacretaz *et al* 1994, Jover *et al* 1993, Fernadez-Rivas *et al* 1995).

Studies by Arnal *et al* (1992, 1993) have demonstrated that increases in cardiac mass are normally lacking with hypertension induced by NO synthase blockade using the antagonist N^{ω}-nitro-L-arginine methyl ester (L-NAME). A small subgroup of these rats (25%), however, developed cardiac hypertrophy that was strongly correlated with increased plasma renin activity (PRA). On the other hand, in the rats that did not develop cardiac hypertrophy the PRA was not elevated (Arnal *et al* 1992 & 1993). The mechanism of the differential activation of the renin angiotensin system (RAS) has not been elucidated. Taken together the findings indicate that, in the majority of rats, during the first 2-3

weeks of NO synthase blockade, the lack of development of cardiac hypertrophy is consistent with the absence of neuro-humoral activation (Arnal 1992 & 1993, Delacretaz *et al* 1994, Jover *et al* 1993, Fernadez-Rivas *et al* 1995).

Consistent with these findings with respect to the heart, Schiffrin *et al* (1995) as well as Dunn and Gardiner (1995) have both demonstrated that short term L-NAME-induced hypertension leads to minimal (Sventek *et al* 1996) or no changes (Dunn and Gardiner 1995) in vascular structure. Regardless of whether changes in structure occur, the magnitude of these changes was found to be inconsistent with the degree of hypertrophy found in other models of experimental hypertension (Schiffrin 1995). The reason for the lack of cardiovascular structural changes has not been elucidated. Schiffrin has proposed that L-NAME, despite inducing hypertension, may have growth inhibitory properties independent of the effects on NO generation (Schiffrin 1995, Li *et al* 1996). Alternatively, Dunn and Gardiner (1995) have proposed that the lack of growth response is because increased pressure alone is not a potent enough trophic stimulus, which is consistent with the lack of activation of trophic neuro-humoral systems.

Recently, studies by Banting *et al* (1996) and another group (Richard *et al* 1995) have revealed that the primary initiation mechanism of L-NAME hypertension results from the rapid up-regulation of a powerful local vasoactive

system, endothelin. Endothelin has also been shown to be a potent inducer of cardiovascular growth both *in vitro* (Weber *et al* 1994) and *in vivo* where Schiffrin *et al* (1995) has demonstrated its involvement in the development of vascular growth in the SHR DOCA-salt model of hypertension. Given that endothelin has been shown to have this growth-promoting capacity, the lack of evidence for trophic changes during L-NAME hypertension is in conflict with the putative role of endothelin as a trophic factor.

To address the some of the conflicts presented by these findings we have determined the time course of activation of an obligatory growth-related enzyme, ornithine decarboxylase (ODC) (Thyberg and Fredholm 1987, Davis 1990, Soltis *et al* 1991). ODC is the first and rate-limiting step in the biosynthesis of polyamines, which are essential for protein synthesis, cellular proliferation and tissue repair processes. Thus, activation of ODC is an essential step in the induction of cellular growth in all cells (Thyberg and Fredholm 1987, Davis 1990, Soltis *et al* 1991). To characterize the pattern of growth induction, or lack thereof, with NO synthase blockade we assessed ODC activity levels throughout a 12 day treatment with L-NAME. In addition, we have determined cardiovascular structural changes by assessing cardiac mass and structurally-based vascular resistance properties in the hindlimb circulation after L-NAME treatment.

Methods:

Animals

Male Sprague-Dawley rats (290-350g), obtained from Charles River Laboratories (Montreal, Quebec, Canada), were housed individually under conditions of a 12 hour light / 12 hour dark cycle, room temperature 22-24°C, and were provided with Purina rodent chow and tap water *ad libitum* for at least two days before starting any procedures.

Measurement of ODC activity in blood vessels and heart:

ODC activity in thoracic aortic (aortic arch to diaphragm), mesenteric vasculature (including vessels considered to be resistance vessels as well as the elastic and muscular segments of the superior mesenteric artery) and left ventricle (LV) supernatant fractions was determined by the method of Russell and Synder (1968) and later modified (Thompson 1994) in which $^{14}CO_2$ released from DL-[l-^{14}C] ornithine HCl was measured. Mesenteric and aortic tissues were homogenized in 10 volumes and single LV in 5 volumes of 10 mM Tris buffer (pH 7.2), 0.5 mM dithiothreitol and 0.4 mM pyridoxal-5'-phosphate. The homogenates were centrifuged at 13, 000 x g at 4°C for 15 minutes. The reaction mixture contained 400 ul of supernatant, 2.0 μCi (96 μM - aorta and mesentery) or 0.5 μCi (24 μM - LV) of DL-[l-^{14}C] ornithine HCl (specific activity: 42.5 mCi/mmol, New England Nuclear, Mississauga, Ontario, Canada),

10 mM Tris buffer (pH 7.2), 0.5 mM dithiothreitol, 0.4 mM pyridoxal-5'-phosphate in a final volume of 0.5 ml. After incubating (60 minutes, 37°C) in a shaking water bath, HCl (100 µl, 3.0 N) was injected and the mixture was shaken for another period (60 minutes, room temperature). Radioactivity in the CO_2 trapping agent (Solvable, New England Nuclear) was counted. Blank values obtained from identical sample containing 5 mM difluoromethyl-ornithine (DFMO) (Merrell Dow Pharmaceuticals Inc., Cincinnati, Ohio, USA) were subtracted from determinations of each tissue supernatant. Protein concentrations of the supernatant was determined by the method of Lowry *et al* (1951). The results of each ODC activity determination in treated rat tissues were compared to the control sample in that particular experiment. The data are expressed, therefore, as a fold difference relative to the control values.

Hindlimb vascular resistance properties with NO synthase blockade

This procedure, comparing the perfusion of the isolated right hindlimb vasculature of rats with L-NAME for 12 days, alone or in combination with 3% L-ornithine, treated rats compared to control treated rats, is based on a technique established by Folkow *et al* (1970). Control rats were treated acutely (≈30 min.) with L-NAME (100 mg/kg i.p.) to control for the leftward shift of the cumulative methoxamine (MXA) dose-response with NO synthase blockade [24]. A heated box maintained both the temperature of the rats and the perfusion apparatus at 37-38°C. The perfusion system consisted of a heated reservoir, an injection port

and a bubble/mixing chamber connected to a single peristaltic pump (Gilson, Minipuls 3). The perfusate was a Tyrode-dextran solution (1.5%, average mol wt: 71,200, Sigma Inc.) composed (in mg / 100 ml fluid) of KCl 20, $CaCl_2 \times H_2O$ 32.3, $MgCl_2 \times 6H_2O$ 5.1, $NaH_2PO_2 \times H_2O$ 6.2, $NaHCO_3$ 100, glucose 100 and NaCl 800. The solution was maintained at pH 7.4, 37-39°C and oxygenated with 95% O_2 and 5 %CO_2. The rats were anaesthetized (60 mg/kg sodium pentobarbital) and heparinized (1000 I.U./kg i.v.). After a mid-line abdominal incision, the right iliac artery was cannulated proximal to the iliac bifurcation with a 21 gauge needle and connected to the perfusion apparatus. After sectioning the vena cava and spinal cord, to eliminate neural influences and to remove venous resistance, the exsanguinated rat was perfused at a constant flow rate (1 ml/min per 100g BW). The perfusion pressure (PP) was continuously recorded on a data acquisition system (MacLab, ADInstruments, Houston, TX). After allowing time for the blood vessels to flush free of blood, sodium nitroprusside (SNP, 20 ug/ml) was infused to produce maximum vasodilation based on a comparison to the maximal lowering of PP induced by papaverine. To ensure a common baseline condition, only preparations which had a stable PP at minimum vascular resistance at the end of a wash-out period were used. A flow rate-PP relationship was characterized by measuring the PP at minimum vascular resistance (PP_{min}) at flow rates of 0.5, 1, 2 and 4 ml/min per 100g BW. A cumulative concentration-response curve to methoxamine (MXA, 0.5- 64 ug/ml, 3 min per level) was generated until PP at maximum constriction with

MXA was achieved. Subsequently, an infusion of supramaximal concentrations of constrictors (vasopressin,10 IU/ml; angiotensin II 200 ng/ml; MXA 64 ug/ml) was given to ensure that a maximum constrictor response (PP_{maxcon}) was achieved that was not dependent on the activation of a single receptor type. After completing the concentration response assessments, flow was stopped to ensure that the pressure returned to zero.

The flow rate - PP_{min} relationships were plotted and the slope was calculated by linear regression analysis. The slope (PP/flow rate, mmHg/(ml/min per 100g BW)) was used as an index of changes in lumen diameter integrated with vascular distensibility (Li and Schiffrin 1994). The PP responses to MXA were computer-fitted to a sigmoidal logistic curve (Sigmoid Version 5, Baker Medical Research Institute, Melbourne, Australia) to fit data points by the algorithm of least squares estimates of non-linear parameters. Values obtained directly from the collected data were PP_{min} and PP_{maxcon} (after bolus constrictor cocktail). The only values that were obtained from the fitted curves were EC_{50} and the maximum slope. The determination and comparison of these parameters were used as the best method to assess differences between treatment groups. At maximum dilation, the changes in PP reflect the hemodynamic consequences of changes in the average cross-sectional area of the vessel lumens (Folkow *et al* 1970). Any structural change in the vessel wall would alter the PP relationships. According to Poiseuille's law, any change in

the lumen radius averaged throughout the entire vascular bed would produce an inversely proportional change in resistance amplified to the fourth power. The PP_{maxcon} is used as a direct index of the contractile mass in the vascular bed. This characteristic has been shown to correlate directly with structural changes of increased wall thickness and media:lumen ratio (Folkow 1970). The EC_{50}, the [MXA] that produced 50% of the maximal PP response, was used as an indication of the smooth muscle sensitivity to α_1-adrenergic stimulation. Further, an enhancement of the wall/lumen ratio of the vessels will increase the reactivity to constrictor agents whether it be the result of lumen and/or wall changes (Folkow 1970).

Measurement of conscious MAP:

The surgical method for the implantation of catheters was based on the technique described by Head and Adams (1988). Rats were anaesthetized with ketamine/xylazine (70/5 mg/kg i.p.), and the descending aorta distal and inferior vena cava to the kidneys were catheterized with small bore Teflon tubing (0.012-in. i.d., o.d. 30 gauge, Cole-Parmer. i.d., 0.060-in. o.d., 23 gauge). The catheter was filled with heparinized saline (10 IU/ml) and held in place by a small drop of cyanoacrylate tissue glue at the puncture site. The catheters were tunneled subcutaneously and exteriorized at the back of the neck and sutured in place. Two days after surgery, MAP were recorded (Narco Physiograph or MacLab DAS, ADInstruments). After connection, an equilibration period of

approximately 30 minutes average MAP reading over 5 minutes was taken from each rat at 15-minute intervals for at least 1 hour before (control baseline period) before treatment period.

Time-course NO synthase blockade:

Acute NO synthase blockade (4 hours) was produced by a single injection of L-NAME (100 mg/kg, i.p.). Chronic NO synthase blockade, longer than 4 hours, was produced by an initial injection of L-NAME (100 mg/kg i.p.) followed by 100 mg/kg per day in drinking water. The ODC activity following NO synthase blockade was assessed at 4 hours and 1, 6 and 12 days (n=8,8,5,7). LV/BW ratios were calculated at all time points as an index of cardiac structural alterations. In a separate group of rats, hindlimb vascular resistance properties were assessed in controls (n=6) and after 12 days of NO synthase blockade alone or with 3 % L-ornithine (n=6,6).

Data Analysis:

All values are expressed as groups means ± S.D. or S.E.M., as indicated. The Student's unpaired t-test with the Bonferroni correction method was used for statistical comparisons between groups. $P<0.05$ was considered significant.

Results:

Mean arterial pressure with NO synthase blockade

There was a 35 mmHg increase in MAP (Figure 5-1) observed within 5 minutes after L-NAME administration, an elevation which persisted throughout the 12 days of treatment. 3 % L-ornithine treatment in the tap water did not alter the MAP profile in a separate group of L-NAME treated rats (data not shown).

Time course of ODC activation with chronic NO synthase blockade:

The time course of activation of ODC in the LV (Figure 5-2, upper panel) throughout the L-NAME treatment revealed only a brief, short-lived increase. After 4 hours of NO synthase blockade, ODC activity increased to 492 ± 75 pmol of $^{14}CO_2$/mg protein per hr above control (116 ± 22 pmol of $^{14}CO_2$/mg protein per hr) returning to control levels at 1, 6 and 12 days.

The aortic ODC activation profile paralleled the LV ODC activation after NO synthase blockade (Figure 5-2, middle panel). After 4 hours of NO synthase blockade, aortic ODC activation was 1063 ± 255 compared to control (571 ± 53.8 pmol of $^{14}CO_2$/mg protein per hr) returning to control levels at 1, 6 and 12 days.

The mesenteric ODC activation profile (Figure 5-2, lower panel) with chronic L-NAME treatment differed from the profile in the LV and aorta. ODC activation in the mesentery did not return to control levels on day 1 remaining significantly increased (893 ± 322 versus a control value of 364 ± 77 pmol of $^{14}CO_2$/mg protein per hr) but returning to control levels at 6 and 12 days.

Cardiac Mass:

Chronic NO synthase blockade did not induce an increase in cardiac mass. LV:BW determinations made at 4 hours (1.91 ± 0.159 g/Kg), 1day (2.1 ± 0.110 g/Kg), 6 days (2.15 ± 0.091 g/kg) and 12 days (1.89 ± 0.095 g/kg) were not elevated above saline. control values (1.97 ± 0.27 g/kg).

Hindlimb vascular resistance properties after 12 day L-NAME treatment:

Analysis of the hindlimb vasculature revealed only modest changes in resistance properties, only at maximum constriction, were induced by chronic NO synthase blockade. Chronic L-NAME treatment did not alter the PP_{min} at flow rates (in ml/min per 100g B.W.) of 0.5 (14 ± 2 vs. 15 ± 1 mmHg), 1 (21 ± 2 vs. 21 ± 2 mmHg), 2 (30 ± 3 vs. 29 ±3 mmHg) and 4 (44 ± 5 mmHg vs. 45 ± 4

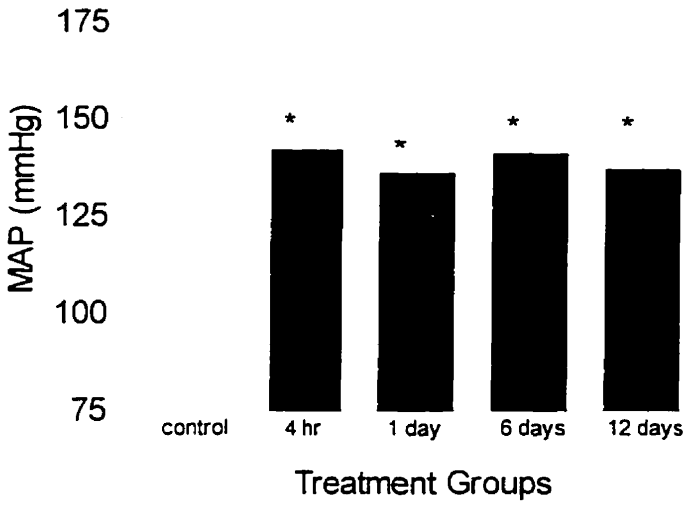

Figure 5-1: The steady state MAP with 4 hr, 1, 6 and 12 days of NO synthase blockade (L-NAME 100 mg/kg per day p.o.) compared to saline. n=8,8,8,5,7 respectively. Values are expressed as mean ± S.D. *denotes significant difference, $p<0.05$.

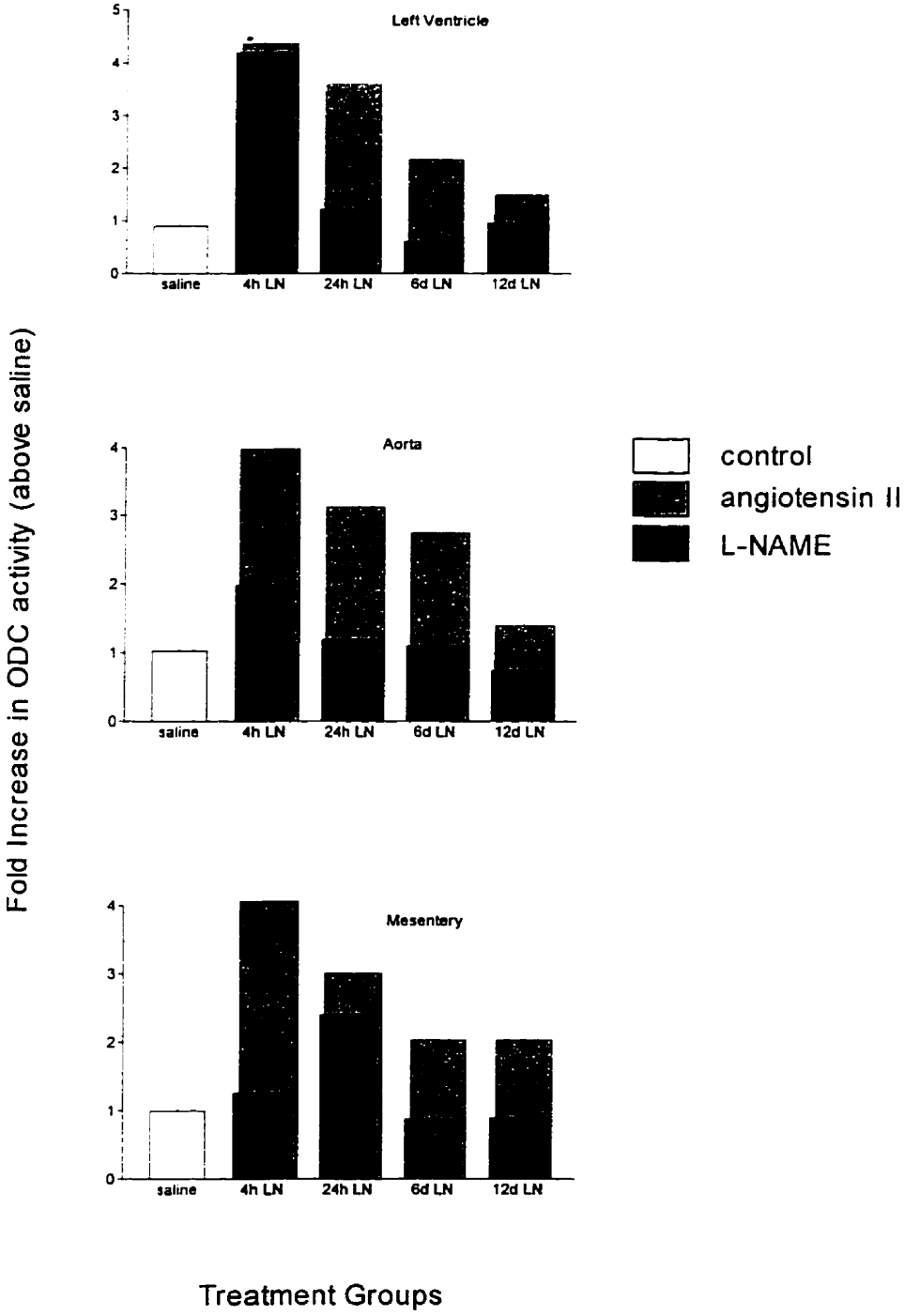

Figure 5-2: Time course of induction of ODC activity with 4 hr, 1, 6 and 12 day L-NAME (100 mg/kg per day p.o.) and control treatment in the left ventricle (top panel), aorta (middle panel) and mesenteric vasculature (lower panel). The ODC activation with 1, 6 and 12 days of angiotensin II treatment (unpublished observations). Values given as mean ± S.E.M. above saline control, n=8,8,8,5,7. * denotes significant difference compared to control, $p<0.05$. L-NAME - (LN).

mmHg) as compared to control, respectively (Figure 5-3). A logistic function analysis of the cumulative log [methoxamine]-PP response curves demonstrated that there was also no change in the sensitivity of the hindlimb vasculature after the treatment as indicated by similar EC_{50} and maximum gain values compared to saline control.

On the other hand, the PP_{maxcon} obtained after bolus administration of methoxamine, vasopressin and angiotensin II showed that there were significant increases ($p<0.05$) in maximum resistance in the L-NAME treated group (Figure 5-4, lower panel). Specifically, the PP_{maxcon} was increased by 15 % in the L-NAME and L-NAME + 3 % L-ornithine treated group compared to saline control (310 ± 20, 308 ± 15 vs. 270 ± 19 mmHg). The increase in maximum vascular resistance after a cocktail of vasoconstrictor agents has been previously demonstrated to be associated with an increase in 'bulk' of the medial smooth muscle layer.

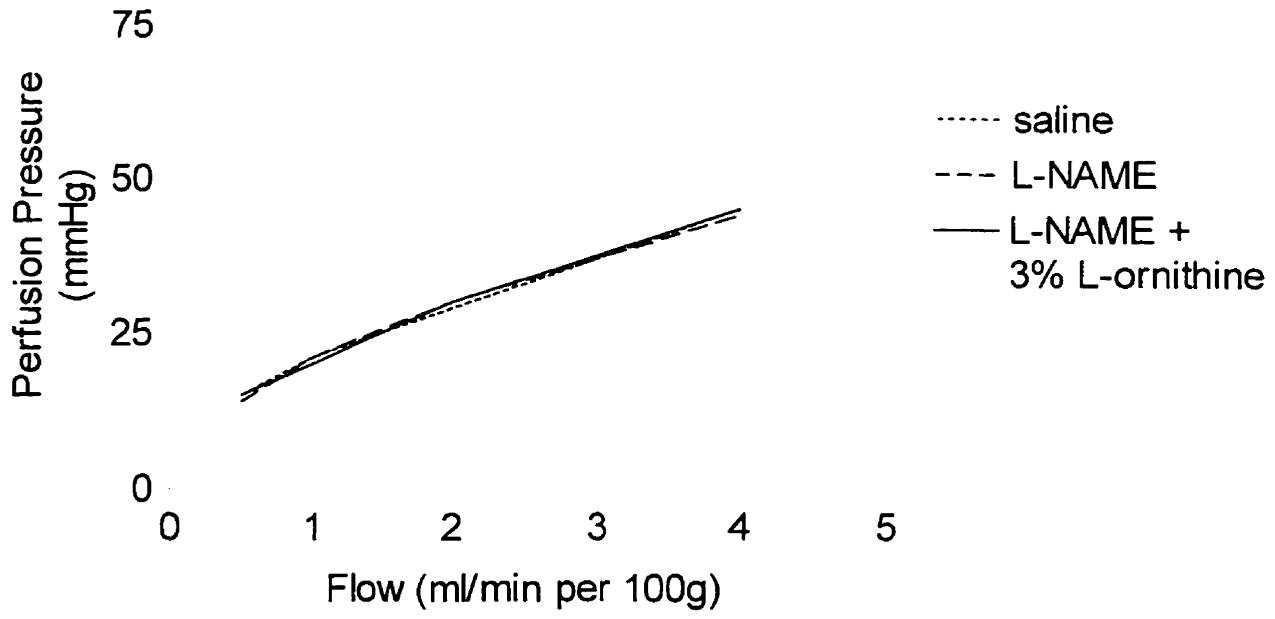

Figure 5-3: Effects of 12 day L-NAME (100 mg/kg per day p.o.) alone or in combination with 3 % L-ornithine treatment and control (treated acutely with L-NAME 100 mg/kg i.p.) treatment on the hemodynamic hindlimb vascular resistance properties. The perfusion pressure at maximum vasodilation (at 1 ml/min per 100g BW, upper panel) and maximum constriction after bolus administration of constrictor agents (lower panel) during constant flow rate (1 ml/min per 100g BW). The values represent n=6,6 respectively. Values as mean ± S.D., * denotes significant difference compared to control, $p<0.05$.

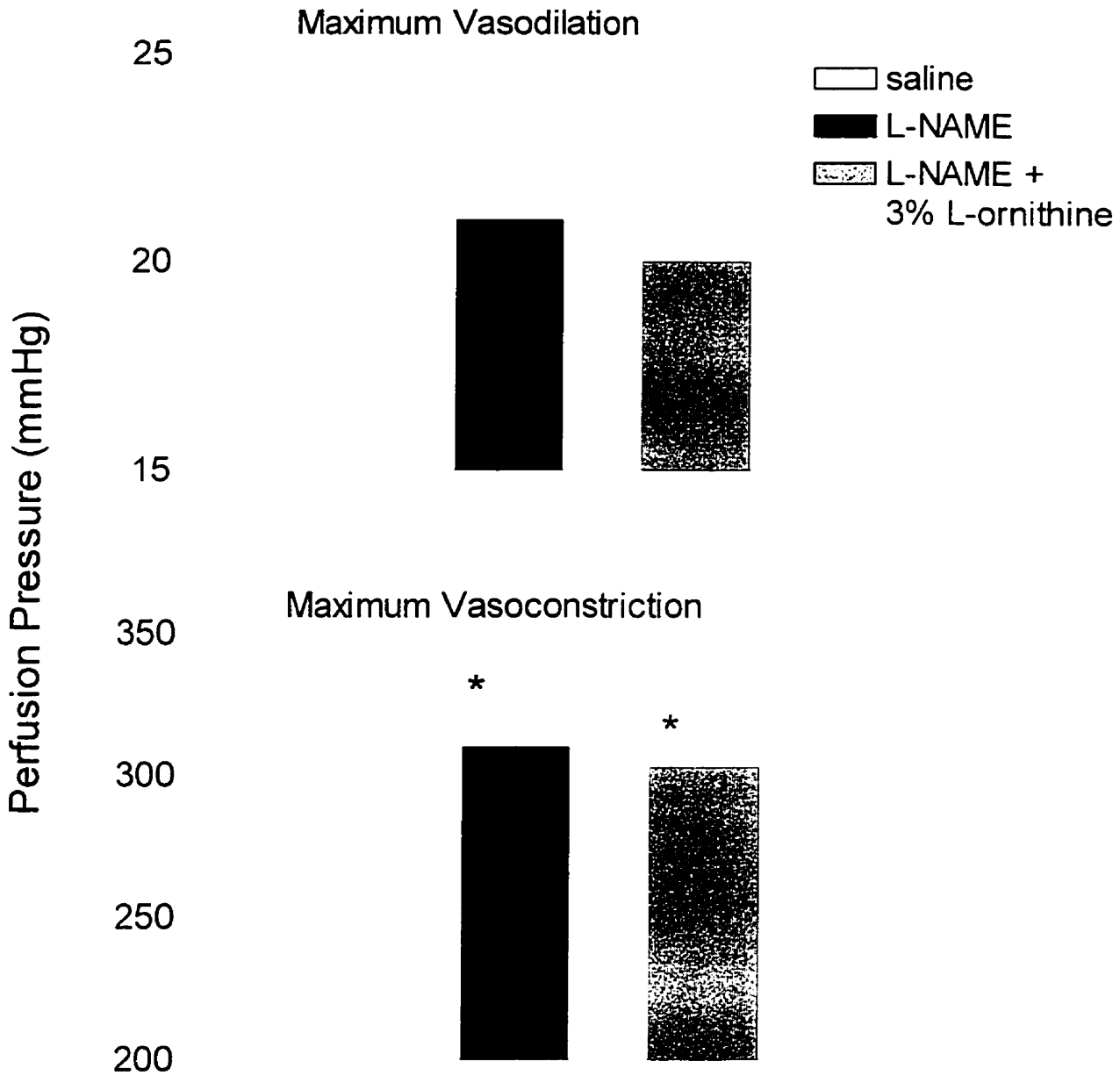

Figure 5-4: The flow-perfusion pressure relationship at maximum dilation in the isolated right hindlimb of rats treated for 12 days with L-NAME (100 mg/kg p.o.) alone or in combination with 3 % L-ornithine compared to control (treated acutely with L-NAME 100 mg/kg i.p.) n=6,6, respectively at the multiple flow rates. Values as mean ± S.D, * denotes significant difference compared to control, $p<0.05$.

Discussion:

The major findings of the present study include: (i) that despite the prolonged hypertension induced by L-NAME treatment there were no changes in left ventricular mass and only minimal changes in vascular resistance properties, which were consistent with (ii) the finding that there was only transient activation of the obligatory growth-related enzyme, ornithine decarboxylase (ODC) in the LV, the aorta, and in the mesenteric vasculature and (iii) the supplementation with the ODC substrate, L-ornithine (3 %) did not alter the effect of L-NAME hypertension on cardiovascular structural changes.

The finding of both minimal cardiac and vascular structurally-based changes during prolonged hypertension with L-NAME, although consistent with the previous data (Arnal 1992 & 1993, Sventek *et al* 1996, Dunn and Gardiner 1995) is in contrast to the findings in almost every other model of hypertension. Thus, the 'expected' result based on the level of hypertension induced by L-NAME would have been approximately a 35% up-regulation of structurally-based vascular resistance. This result was not achieved. Using the hindlimb perfusion technique, we determined that there was an 'uncoupling' between the consequences to pressure and changes in vascular resistance properties (i.e. there was no change in resistance at maximum dilatation). This perfusion technique, particularly under conditions of maximum dilatation, takes advantage of Poiseuille's Law and involves the assessment of structurally-based resistance

properties in all orders of vessels. This method provides a sensitive index of the hemodynamic impact of even very small changes in vascular dimensions according to the fourth power relation with resistance. Our results confirm and extend previous findings regarding the lack of vascular structural changes during L-NAME treatment. Our novel findings, in the intact vascular bed, demonstrate that the minimal vascular changes are likely similar throughout the entire vascular bed.

It has been widely acknowledged previously, that in different forms of experimental hypertension 'slow pressor mechanisms', often distinct from the 'initiating' cause, can account for long term blood pressure elevation (Lever 1986). Thus, in established hypertension, in almost all cases the 'slow pressor mechanisms' have involved a critical role of vascular structural changes. In the last few years it has become apparent that the quantitative contribution that these structural alterations make is greatest in the chronic, steady-state phase of hypertension, at a time when the contribution of the basic initiating cause or stimulus (Lever 1986) has returned towards normal levels. This was previously explained, by Folkow and co-workers (1970 & 1990) as being part of a positive feedback loop in which the response to hyperactivity of a pressor mechanism is amplified by the development of pressure-dependent vascular hypertrophy, slowly leading to a substantial level of hypertension. Alternatively, others have proposed mechanisms that involve direct effects of trophic factors (e.g. growth factors, Ang

II, catecholamines) on the induction of cardiovascular growth processes. Regardless of the underlying mechanism, the consequence of these processes is increased structurally-based vascular resistance and elevated blood pressure. In the present study, the results clearly do not support the concept that elevated blood pressure alone will necessarily result in proportional changes in cardiovascular structure. Previous results have demonstrated that there is, in general, a lack of activation of neurohumoral systems (Banting *et al* 1996, Bank *et al* 1994) with L-NAME-induced hypertension. Hence, in the present study the incomplete structural adaptation to the chronic hypertension may be causally related to the lack of prolonged activation of trophic systems.

Recently, Banting *et al* (1996) demonstrated a role of endothelin-1 (ET-1)-mediated vasoconstriction as the initiating mechanism of acute L-NAME hypertension. Thus, the abrupt removal of the inhibitory actions of NO (by NOS inhibition) resulted in a rapid up-regulation of ET vasoconstrictor actions. In the context of the present study, the specific role played by endothelin in the chronic model of L-NAME hypertension has not been elucidated. However, based on both previous data which demonstrates that endothelin has the capacity to be a cardiovascular trophic factor it may be that this mechanism plays a role in the minimal vascular structural changes found in the present study. Recent findings, in particular from Schiffrin's group (1995), in DOCA-salt hypertension have supported the concept of a trophic role for endogenous endothelin in the

development of vascular structural changes. They found that there is both increased ET-1 gene expression and immunoreactivity in blood vessels, but not in the plasma, of DOCA-salt hypertensive rats. They further showed that the development of vascular hypertrophy in the DOCA-salt model was markedly attenuated by treatment with an ET_A/ET_B receptor antagonist. Schiffrin's group has also demonstrated that L-NAME + DOCA-salt resulted in a blunted of the structural changes compared to DOCA-salt treatment alone (1996). The vascular trophic capacity of ET-1 has also been supported by findings in cultured vascular smooth muscle cells showing that addition of endothelin produces a mitogenic response (Weber *et al* 1994). ET-1 is approximately 100 times more potent as a vasoconstrictor than Ang II or catecholamines. However, in the culture studies the maximal growth response to ET-1 was less than half that for Ang II. We are not aware of any studies that have assessed the *in vivo* cardiovascular growth responses to chronic endothelin infusion.

In the present study, the profile of the ODC activation was markedly blunted when compared to a similar time course of hypertension using equipressor levels of angiotensin II (Ang II, unpublished observations). We have determined that Ang II infusion unequivocally induces a proportional hypertension and up-regulation of cardiovascular structure (unpublished observations). The activation of ODC occurs prior to increases in mass in any tissue and the duration of the increased ODC activity represents the time course

of the growth phase (Thyberg and Freholm 1987, Davis 1990, Soltis 1991). ODC is highly regulated in mammalian cells which provides polyamine levels correlating with the cellular growth rate (Auvinen *et al* 1992). Not surprisingly, pharmacological blockade of ODC inhibits cardiovascular growth responses in various experimental conditions (Thyberg and Freholm 1987, Davis 1990, Pegg *et al* 1994, Pedersen 1993). Thus, activation of ODC indicates a state of elevated cellular growth processes whereas a return to basal ODC activity denotes a return to a 'new' quiescent steady-state. The lack of any growth signal at six days in any tissue suggests that the growth response follows an on/off trophic stimulus rather than a slow progressive time course.

Overall, the present study clearly indicates that rapid and sustained hypertension induced by NO synthase blockade is not a sufficient stimulus to induce a persistent activation of the growth related enzyme, ODC. These results indicate that either (i) pressure-alone is not a potent trophic stimulus or that (ii) the NO synthase inhibitor L-NAME may be "non-specifically" blocking cardiovascular growth processes as has been proposed by Schiffrin *et al* (1995, 1997). Our data demonstrate that 3 % L-ornithine supplementation neither augmented nor inhibited the L-NAME induced vascular structural up-regulation suggesting that, at least, alterations in the ODC-polyamine pathway are not a likely mechanism for the lack of growth induction. Despite this latter finding further work is required to elucidate the specific mechanisms involved in the

lack of marked structural adaptation with chronic NO synthase blockade. We further speculate that an important component of the blunted development of cardiovascular structural changes is that there has not been a prolonged activation of neuro-humoral systems acting as trophic factors.

Chapter 6: Evidence that the nitric oxide synthase inhibitor L-NAME inhibits cardiovascular growth processes

Abstract:

We tested the hypothesis that the lack of cardiovascular structural up-regulation during treatment with the NO synthase antagonist N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 mg/kg, i.p.) is a consequence of non-specific inhibitory effects on growth. *In vivo*, we determined the level of activation of the obligatory growth-related enzyme, ornithine decarboxylase (ODC), with L-NAME treatment in comparison to an equipressor stimulation using angiotensin II infusion (200 ng/kg/min). *In vitro*, we assessed the effects of L-NAME and L-arginine alone and in combination on aortic vascular smooth muscle cell proliferation at various levels of growth stimulation (2.5, 5 and 10% fetal calf serum). *In vivo*, equipressor levels of hypertension induced by L-NAME activated ODC, in general, to a lesser extent as compared to activation with angiotensin II. The *in vitro* results demonstrated that the L-NAME treatment had an inhibitory effect on growth and was dependent on the magnitude of the growth stimulus. L-NAME blunted cell proliferation at 2.5% fetal calf serum by approximately 20%, but not at higher levels (5 and 10% fetal calf serum). L-arginine supplementation alone also blunted VSMC growth to a similar degree. Together, L-NAME + L-arginine further blunted the mitogenic response. These results indicate that L-NAME treatment has the capacity to induce cardiovascular growth mechanisms *in vivo*. However, based on the inhibition of aortic vascular smooth muscle cells at a low level of growth stimulation with 2.5 % fetal calf serum, a potential growth inhibitory effect of the L-NAME may also be present. Although it remains to be investigated, the results of the present study clearly outline the potential

for L-NAME as an inhibitor of cardiovascular growth processes. This issue must be resolved before the specific mechanisms leading to the lack of cardiovascular growth response with chronic L-NAME treatment can be properly elucidated.

Introduction:

The discovery of endothelium-derived relaxing factor as nitric oxide (NO), or a closely related compound, led to a change in the concept that the endothelium operated simply as an inert vascular barrier system (Palmer *et al.* 1987, Palmer *et al.* 1988). In particular, several lines of investigation have proposed that the role of NO is as a chronic vasodilator. The development of this hypothesis has been based almost completely on the results obtained in studies using analogues of L-arginine to inhibit NO synthase. Thus, investigators by demonstrating that there is a rapid increase in peripheral resistance (Sigmon and Beierwaltes 1994) and hypertension within minutes (Tresham *et al* 1994) following administration of compounds such as N^{ω}-nitro-L-arginine methyl ester (L-NAME) concluded that it was a result of a lack of the chronic NO vasodilator tone. However, recent findings of Banting *et al.* (1996) have demonstrated that it is not the lack of NO that induces the hypertension but rather an up-regulation of endothelin mediated vasoconstriction, at least initially. Thus, although compounds such as L-NAME have been widely used in both acute and chronic models of NO deficient hypertension the mechanism of action remains equivocal.

Studies investigating the cardiovascular structural alterations associated with NO synthase blockade-induced hypertension (2-3 weeks) have demonstrated a surprising lack of a change (Sventek *et al.* 1996, Dunn and Gardiner 1995) as compared to the levels of structural up-regulation that occurs in other equipressor models, such as 2-kidney 1-clip

or angiotensin II infusions (Delacratz *et al.* 1995, Griffin *et al.* 1991). A lack of a cardiovascular structural up-regulation with NO synthase blockade presents a conflicting issue with respect to the proposed physiological role(s) of NO in regulating cardiovascular growth processes. *In vitro* evidence has demonstrated the capacity for NO to inhibit vascular smooth muscle cell proliferation (Kolpakov *et al.* 1995, Garg and Hassid 1989) and, *in vivo*, administration of the NO pre-cursor L-arginine also markedly attenuated proliferation of smooth muscle cells following balloon injury in rats (Taguchi *et al.* 1993).

One concept proposed by Schiffrin (1995) to explain this conflict, suggests that the L-arginine analogues, particularly L-NAME, which are used to block NO synthase activity may, in fact, 'non-specifically' inhibit cardiovascular growth processes. If this were true the evidence either of an anti-trophic role of NO or the enhanced trophic actions mediated by the up-regulation of ET-1 would be obscured in the experimental findings. Thus, the explanation for a lack of cardiovascular growth responses with NO synthase blockade following L-NAME administration has several possibilities that clearly need to be resolved. Schiffrin's group (Jin-S *et al.* 1996) has also provided *in vivo* evidence that that cardiovascular hypertrophy that normally occurs when rats are treated with DOCA-salt becomes markedly blunted with concomitant L-NAME treatment.

To address this concern, the present study was designed to investigate the specific issue of whether the widely used NO synthase inhibitor, L-NAME, has the capacity to

inhibit the induction of growth processes either *in vivo* or *in vitro*. In the first study, *in vivo*, we compared the potency of angiotensin II infusion and L-NAME treatment, at equipressor levels, on the activation of an obligatory growth-related enzyme, ornithine decarboxylase (Thompson and Adams 1994, Banting *et al* 1997). The activation of this enzyme can be used as an index of the capacity for growth induction. In the second study, *in vitro*, we tested the capacity of L-NAME alone or in combination with L-arginine to alter growth responses in cultured aortic vascular smooth muscle cells.

Methods:

Study 1:

Animals

Male Sprague-Dawley rats (290-350g), obtained from Charles River Laboratories (Montreal, Quebec, Canada), were housed individually under conditions of a 12 hour light / 12 hour dark cycle, room temperature 22 ± 2°C, and were provided with Purina rodent chow and tap water *ad libitum* for at least two days before starting any procedures.

L-NAME treatment protocol and Mean Arterial Pressure measurements:

NO synthase blockade was obtained by a single injection of L-NAME (100 mg/kg i.p., Sigma Chemicals Inc., St. Louis, MO). Infusion of angiotensin II was used as the positive control (200 ng/kg per min i.v.).

Rats were instrumented with catheters in the abdominal aorta and inferior vena cava, exteriorized to the back of the neck under ketamine/xyzaline anesthesia (70/5 mg/kg i.p.). On the day of the experiments, rats were connected to a MacLab DAS (ADInstruments, Milford, MA) and allowed a 1 hour acclimatization period before any recordings began. Baseline mean arterial pressures were recorded for 45-60 minutes prior to L-NAME or Ang II treatment. The L-NAME or Ang II treatment was monitored for a period of 4 hours.

Measurement of ODC activity in blood vessels and heart:

ODC activity in thoracic aortic (aortic arch to diaphragm), mesenteric vasculature (including vessels considered to be resistance vessels as well as the elastic and muscular segments of the superior mesenteric artery) and left ventricle (LV) supernatant fractions were determined by the method modified as previously reported by Thompson and Adams (1994) in which $^{14}CO_2$ released from DL-[l-^{14}C] ornithine HCl was measured. Aortic tissues were homogenized in 10 volumes and single LV in 5 volumes of 10 mM Tris buffer (pH 7.2), 0.5 mM dithiothreitol and 0.4 mM pyridoxal-5'-phosphate. After incubating (60 minutes, 37°C) in a shaking water bath, HCl (100 ul, 3.0 N) was injected and the mixture was shaken for another period (60 minutes, room temperature). The data are expressed as a fold difference relative to the control values.

Study 2:

Vascular Smooth Muscle Cell Culture:

Aortic vascular smooth muscle cells (VSMC) from a previously established and characterized cell line originating from Wistar rats (Pang and Venance 1991) were cultured in Dulbecco's Modified Eagle medium (DME, Gibco) supplemented with 10% calf serum (CS), 5 % fetal calf serum (FCS), 8 mM HEPES, 100 U/ml pencillin and 100 ug/ml streptomycin. Cells were kept at in a humidified incubator at 37 ° C maintaining an atmosphere of 95% air: 5%CO_2. Medium was changed every other day until cells reached confluence. One day after reaching confluence, cells were trypsinized, counted using a haemocytometer and re-plated at a density of 10^5 cells/ 3 ml in 60 mm petri dishes. Cell used were between passages 4 to 8.

VSMC experiments: Effects of L-NAME on VSMC proliferation:

To determine the effects of various concentrations of FCS on VSMC proliferation, cells were cultured at three different levels of FCS (2.5%, 5 % or 10 %) in DME. Cells were collected by trypsinization after 3, 4 and 5 days in culture, fixed in 4% paraformaldehyde and stored at 4 ° C unitl counting. Cell counts were assessed using a haemocytometer.

The effects of L-NAME were determined by assessing the VSMC proliferative response starting two days after cells were plated in DME containing the three concentrations of FCS. At this time cells were firmly attached to the substratum. Two concentrations of L-NAME were used in initial experiments (10^{-4} M or 10^{-6} M). Cells were collected and counted after 3, 4 and 5 days of treatment.

In a separate series of experiments we assessed whether L-arginine would reverse the inhibitory effect of L-NAME on VSMC proliferation. In these experiments cells were cultured in DME supplemented with 2.5% FCS for 3, 5 and 7 days. The different treatments were: (i) L-NAME 10^{-4} M (ii) L-arginine $3x10^{-4}$ M (iii) L-NAME 10^{-4} M + L-arginine 3×10^{-4} M (iv) control. In preliminary experiments, Trypan blue exclusion tests revealed that L-NAME did not induce cell death.

Data Analysis

All values are represented as group means ± SEM. Statistical comparisons were made using Student's unpaired t-test with the Bonferroni correction method.

Results:

Mean arterial pressure with NO synthase blockade

There was a 37 mmHg increase in MAP (138 ± 9 mmHg) observed within 5 minutes after L-NAME administration. Similarly, the 4 hour angiotensin II infusion resulted in an increase in MAP (139 ± 7 mmHg) of 34 mmHg as compared to saline control values (105 ± 7 mmHg). Hence, we were able to use an "equipressor" effect of angiotensin II infusion as a positive control for growth induction to compare with the trophic effect of NO synthase blockade.

Time course ODC activation with chronic NO synthase blockade:

In the LV with L-NAME treatment, ODC activity (Figure 6-1, upper panel) increased to 378 ± 57 pmol of $^{14}CO_2$/mg protein per hr above control (154 ± 63 pmol of $^{14}CO_2$/mg protein per hr). Equipressor angiotensin II infusion increased ODC activity significantly to 386 ± 80 pmol of $^{14}CO_2$/mg protein per hr.

The aortic ODC activation profile paralleled the LV ODC activation after NO synthase blockade (Figure 6-1, lower panel). After 4 hours of NO synthase blockade, aortic ODC activation was 1063 ± 90 compared to control (571 ± 19 pmol of $^{14}CO_2$/mg protein per hr). Angiotensin II infusion resulted in an increase in aortic ODC activity to 1807 ± 126 pmol of $^{14}CO_2$/mg protein per hr.

L-NAME and VSMC proliferation:

Cultured aortic VSMC with DME media +2.5%, 5% and 10 % fetal calf serum induced a rank order increase in cell numbers from 3 to 7 days (Figure 6-2). The slope of the growth response with 2.5%, 5% and 10% FCS treatment groups were 2193 ± 54. 8168 ± 58 and 31880 ± 4700 cells per day, respectively. The fractional slope (the relative increase in cell number per day) for the 2.5%, 5% and 10% FCS treatment groups were 8%, 25% and 40 %, respectively.

L-NAME treatment with 5% and 10% did not alter the rate of proliferation as compared to the control values (Figure 6-3) . At a low level stimulus with 2.5% FCS,

however, L-NAME at 10^{-4} M and 10^{-6} M resulted in a significant decrease in cell number by day 5 (Figure 6-3).

In the experiment that utilized 2.5% FCS alone, L-NAME treatment resulted in a decreased rate of cell proliferation, as compared to control (Figure 6-4). L-arginine treatment alone resulted in a decrease in cell proliferation by day 5. The combination of L-NAME and L-arginine produced a decrease in cell proliferation at 3 and 5 days. Consistent with the previous result, L-NAME alone decreased cell proliferation by day 5. By day 7 the cell lines were reaching confluence, hence little proliferation was occurring and no changes were detected as compared to the control values. These experiments were repeated three-times with the same trend occurring each trial.

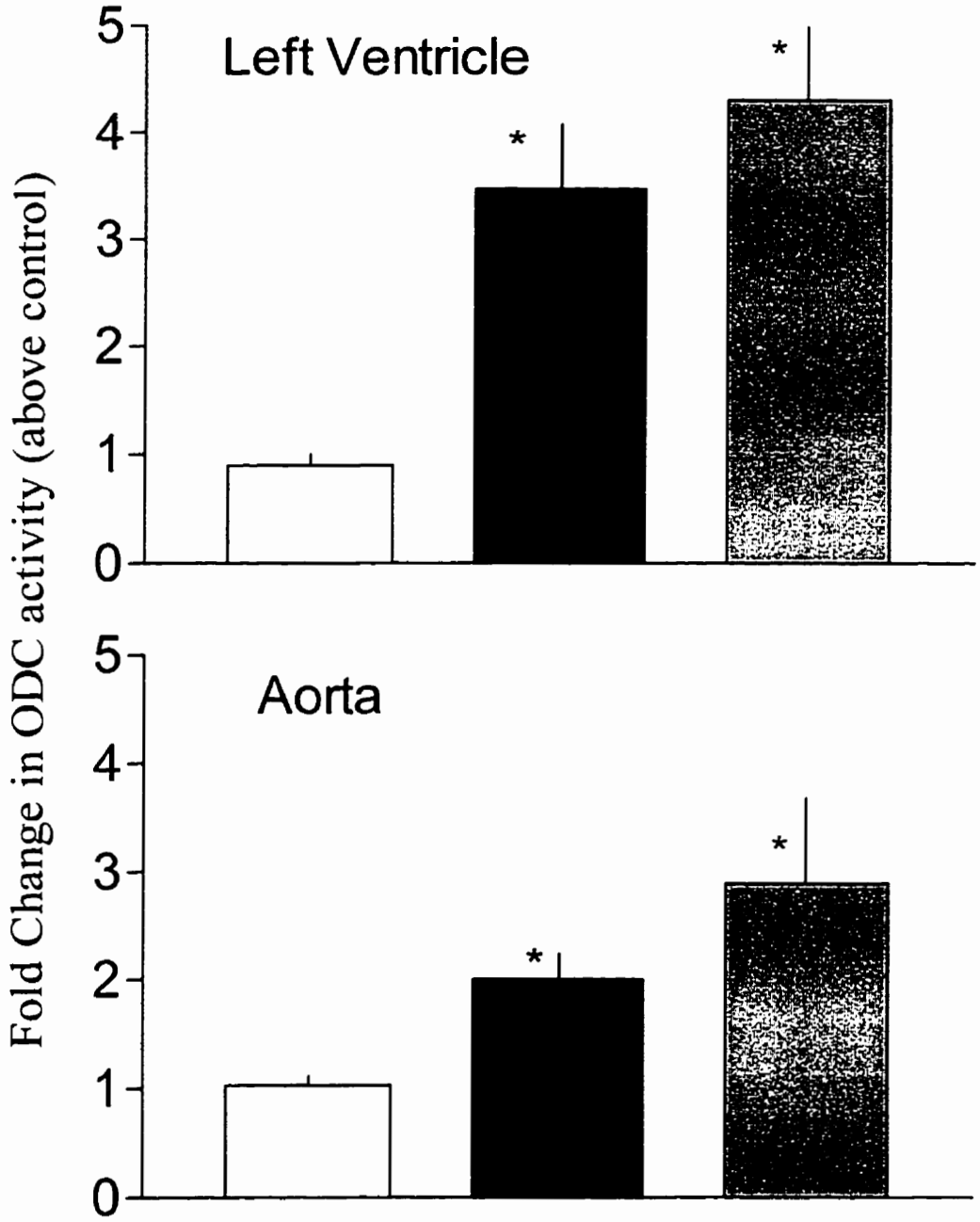

Figure 6-1: Illustrates the fold increase in ornithine decarboxylase activity (ODC) in the left ventricle (upper panel), aorta (lower panel) following either N^{ω}-nitro-L-arginine methyl ester (L-NAME, black bar) treatment (100 mg/kg , intraperitoneally) or angiotensin II infusion (200 ng/kg per min, intravenously, grey bar). Values expressed as group means ± SEM (n=6,8 for each panel).

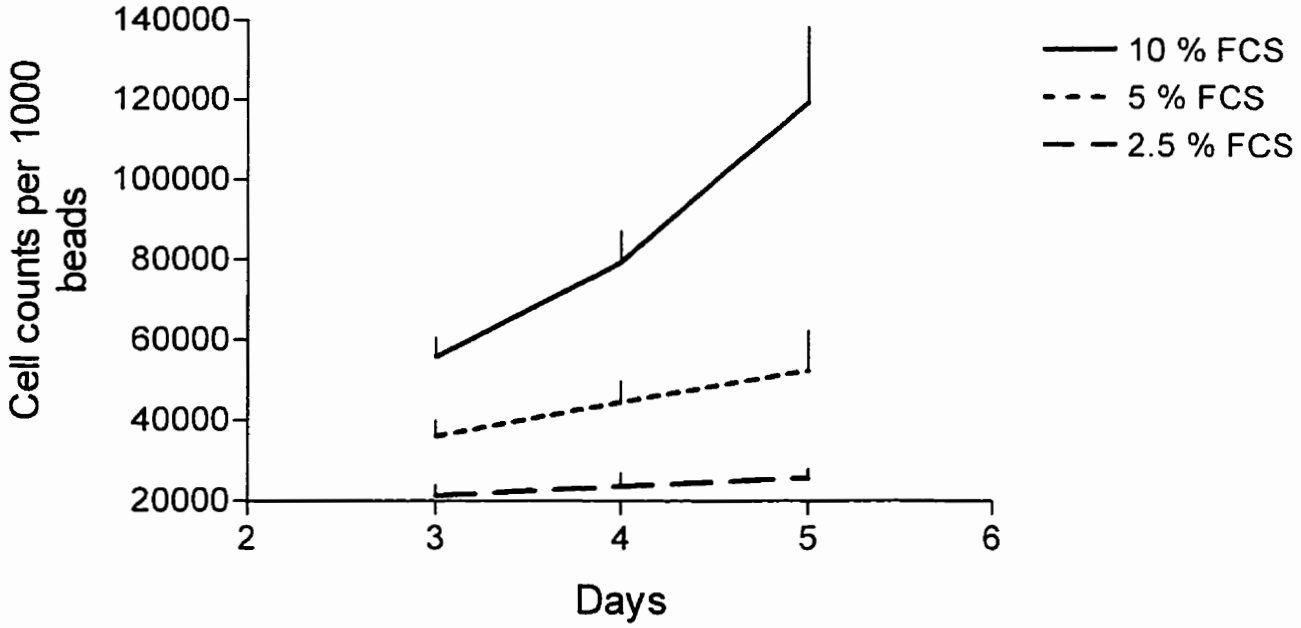

Figure 6-2: The growth rate of aortic vascular smooth muscle cells (VSMC) with 2.5 %, 5 % and 10 %fetal calf serum (FCS) in Dulbecco's modified Eagle's medium (DME) for 1, 3 and 5 days. Values expressed as group means ± SD.

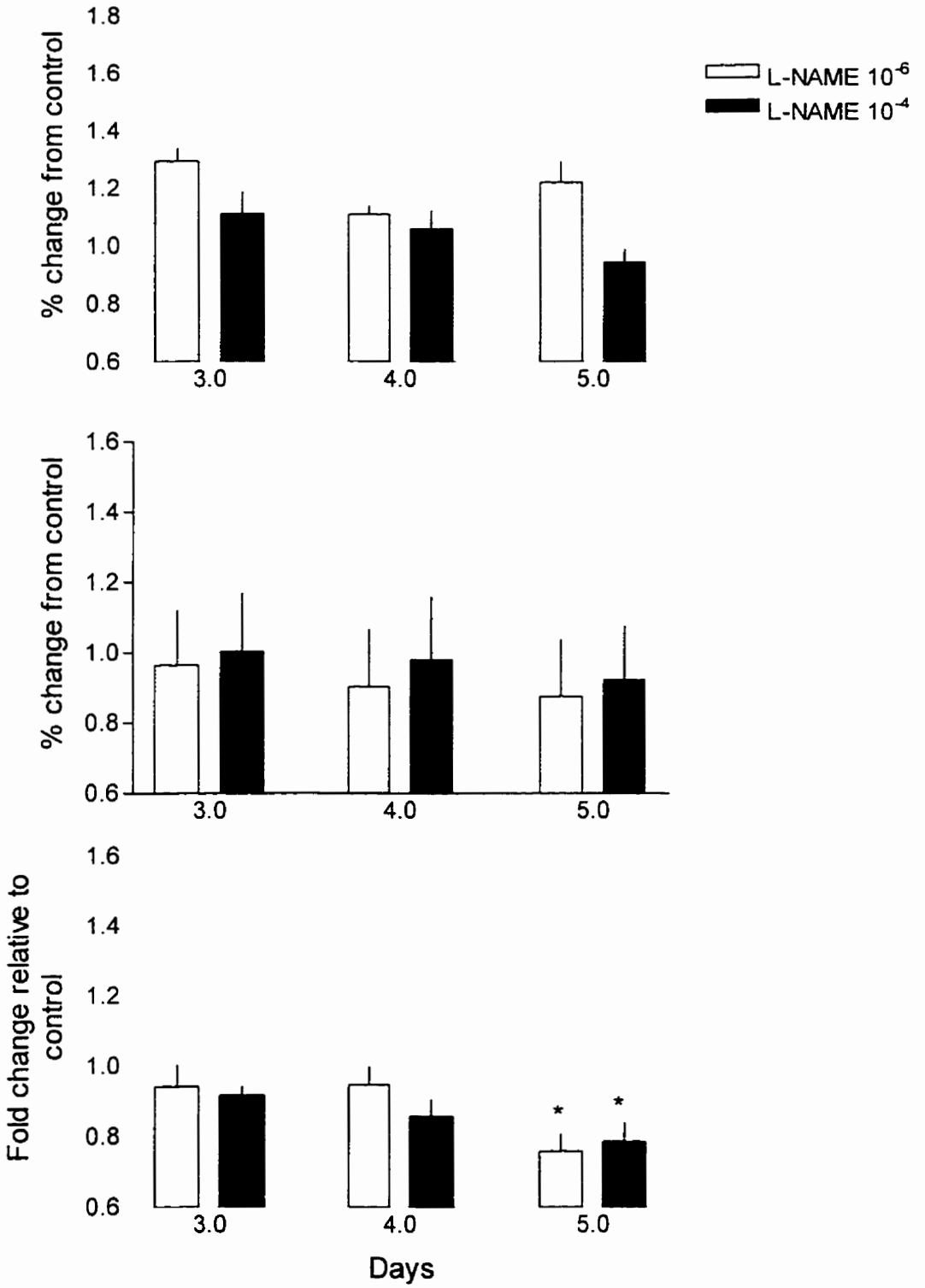

Figure 6-3: The growth inhibitory effects of N^{ω}-nitro-L-arginine methyl ester (L-NAME, 10^{-4} and 10^{-6} M) on aortic VSMC proliferation with 10 % (upper panel), 5 % (middle panel) and 2.5 % (lower panel, n=6,6,6, respectively). Data is presented as mean ± SEM.

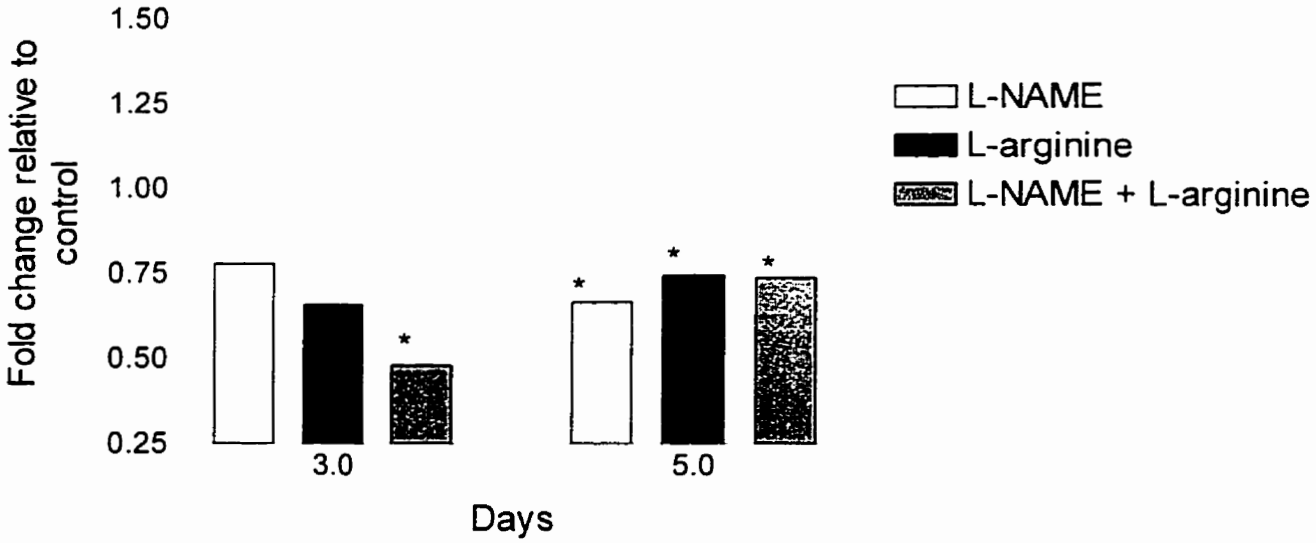

Figure 6-4: The effect of N^{ω}-nitro-L-arginine methyl ester (L-NAME, 10^{-4}M) on the proliferation of aortic vascular smooth muscle cells with 2.5% FCS. Data is presented as fold change from control (n=6,6,6, respectively).

Discussion:

A major finding of the present study was that L-NAME was demonstrated to have the capacity to inhibit proliferation of vascular smooth muscle cells at a relatively low level of growth stimulation with 2.5 % fetal calf serum. In addition, our results *in vivo* demonstrated that L-NAME had the capacity to induce activation of the growth-related enzyme ornithine decarboxylase (ODC). However, the L-NAME induced activation was, in general, to a lesser extent then the activation induced via equipressor levels of angiotensin II infusion. Further, in the *in vitro* experiments we found that L-arginine did not reverse the inhibitory effects of the L-NAME treatment on VSMC proliferation. In fact, we found an additional inhibitory effect of L-arginine on VSMC growth responses.

Activity levels of ODC increase in all tissues, prior to changes in mass, consistent with a role as an obligatory enzyme in all growth processes (Auvinen *et al.* 1992) . The duration of the increased ODC activity patterns the time course of the active growth phase. ODC is highly regulated in mammalian cells producing polyamine levels which correlate with the rate of cell growth (Pegg *et al.* 1994). Not surprisingly, pharmacological blockade of ODC inhibits cardiovascular growth responses in various experimental conditions (Moreau *et al.* 1996). Thus, activation of ODC indicates a state of elevated cellular growth processes whereas a return to basal ODC activity denotes either a lack of activation or a return to a quiescent steady-state. In the present study, L-NAME-induced ODC activation in the heart and aorta indicates that L-NAME did not

prevent, non-specifically, the early induction of growth processes in these tissues. The possibility remains, however, that the overall blunted growth induction previously found with chronic L-NAME hypertension may also be related to the fact that although pressure-alone can induce acute trophic responses it may not provide a potent enough stimulus to induce a chronic growth signal.

Increases in the proliferation of VSMC's by growth factors *in vitro* occur in a concentration-dependent manner. Our results in culture using passaged VSMC's, demonstrate that although L-NAME can inhibit the growth responses during low level growth stimulation it does not inhibit cell proliferation when the magnitude of the growth stimulus is very large. Specifically, the 2.5% FCS stimulus induces an 8% fractional growth rate per day. While small for *in vitro* studies this growth response, *in vivo*, would represent a dramatic growth stimulus (i.e. an 8% growth rate of VSMC in the aorta would result in a 60% increase in medial mass over 7 days). Thus, the demonstrated inhibitory effect of L-NAME at 2.5% FCS is more representative of the effect on growth stimulation *in vivo* than is the 5 and 10% stimulus. However, the finding that 5 and 10% FCS-growth stimulus is not altered suggests that the L-NAME mediated inhibition is surmountable.

The finding that L-arginine caused a decreased rate of cell proliferation in the culture experiments is consistent with the proposed anti-trophic role of NO (Taguchi *et al.* 1993, Garg and Hassid 1989, Kolpakov *et al.* 1995). The further decrease in cell

proliferation produced by the combination of L-NAME and L-arginine suggests that the inhibitory mechanisms of these two substances are separate, at least in part. If the inhibitory mechanism for L-NAME was related directly to the arginine-NO pathway the combination treatment should have restored the balance. In this case the inhibition of NO production would result in the removal of the tonic NO anti-trophic signal leading to an enhanced rate of cellular proliferation. Our results indicate that the inhibition by L-NAME was enhanced with L-arginine treatment suggests that other non-arginine-NO pathways are involved and remain to be elucidated.

Results from *in vivo* studies involving chronic treatment with L-NAME are consistent with L-NAME having a growth inhibitory effect. For example, the cardiovascular structural changes associated with deoxycorticosterone acetate (DOCA)-salt-treated SHR (Schiffrin *et al.* 1995) treatment are much more severe than with the equivalent level of malignant hypertension induced in SHR by chronic administration of L-NAME, despite high renin-angiotensin system activity. In addition, a recent study by Luscher's group provides evidence that L-NAME will blunt the vascular growth induced by Ang II infusion despite a further enhancement of the hypertension (Moreau *et al.* 1996). Overall these studies clearly outline the "uncoupling" that occurs between the level of mean arterial pressure and the level of cardiovascular structural adaptations with hypertension induced by NO synthase blockade.

The results of the present study raise an important issue with respect to the NO synthase blockade induced model of hypertension. These results of the present study indicated that L-NAME may have an inhibitory property with respect to cardiovascular growth processes. However, since we have demonstrated that L-NAME has the capacity to induce a marker of cardiovascular growth induction *in vivo*, the inhibitory mechanism is not related to the blockade of the cardiovascular system's ability to start the cascade of events leading to a growth response. Regardless, it remains to be determined whether the inhibitory effects of L-NAME that occurred in aortic VSMC in the present study reflects an inhibitory process on the *in vivo* growth processes. Currently, it is attractive to speculate that the marked increase in mean arterial pressure that occurs with NO synthase blockade is not a sufficient stimulus to induce cardiovascular growth processes, without concomitant induction of other trophic systems. The results of the present study demonstrate that the widely used NO synthase inhibiting compound, L-NAME, has the capacity to inhibit VSMC proliferation *in vitro* that may account for the overall lack of cardiovascular structural alterations with chronic NO synthase blockade. This issue of L-NAME as an inhibitor of growth processes must be resolved before cardiovascular structural changes, or lack thereof, associated with the chronic phase of this model of hypertension can be fully elucidated.

Chapter 7: Upregulation of endothelin tone associated with depressed nitric oxide function provokes marked penile vascular hyper-reactivity.

Abstract

Vascular tone is influenced by a balance between multiple and overlapping vasoactive systems. In this study our objective was to investigate whether decreased levels of nitric oxide (NO) results in enhanced endothelin (ET)-mediated vasoconstriction in the rat perfused pudendal vasculature. Changes in perfusion pressure (PP), at a constant flow rate, were assessed with methoxamine (MXA), the selective α_1-adrenocepter agonist, at sub-pressor concentrations, $MXA_{subpress}$ (0.5-1μg/ml) and at submaximal concentration, MXA_{submax} (6-9μg/ml). A nitric oxide synthase inhibitor, L-NAME (100μg/ml) was infused alone or in combination with MXA. Then, an ET_A/ET_B antagonist, PD145065 (30 μg/ml)was given when the PP was elevated by $MXA_{subpress}$ and L-NAME. In separate rats, cumulative concentration-response curves to ET-1 and MXA with $ET_{subpress}$ were obtained. $MXA_{subpress}$, $ET_{subpress}$ and L-NAME alone did not increase basal PP. L-NAME markedly potentiated the vasoconstrictor responses to both levels of MXA which was almost completely restored by PD145065. $ET_{subpress}$ also potentiated (4-fold) the vasoconstrictor responses to MXA. Our findings demonstrate that 'priming' effect on α_1-adrenoceptor mediated vasoconstriction due to decreased NO levels is mediated by endothelin. In the pudendal vasculature, this hypersensitivity to α_1–adrenergic stimulation induced by decreased NO leading to increased ET activity may play a critical role in erectile dysfunction.

Introduction

An important element underlying human erectile dysfunction frequently relates to disturbances in "vascular" mechanisms. Normally, a coordinated pattern of peripheral vascular events are involved in the generation of a penile erection. This outcome involves a temporal integration of a number of activating and inactivating processes (local, neural, humoral) which can act in concert or in opposition (Lerner *et al* 1993, Anderson 1993, Krane *et al* 1989, Saenz de Tejada *et al* 1991). This multiplicity of control systems should be anticipated for any process that is fundamental for reproductive function and the survival of the species (Adams *et al* 1996). In addition, since the dynamic regulation of any system is most effective if "the system" can respond dynamically in both directions it becomes very important to have an understanding of "the players". Thus, the operating point of a functioning system should be at the midpoint of the stimulus-response relationship, thereby providing the maximum gain to sustain homeostasis. In the penis one objective of the control systems which regulate penile arterial flow is to permit a small neural signal to achieve tumescence despite changes in homeostatic conditions within certain limits.

A fully functional penile erection is recognized as involving at least three sets of peripheral nerves (thoracolumbar sympathetic, sacral parasympathetic, and pelvic somatic). At the level of penile tissue these operate as sympathetic and parasympathetic as well as non-adrenergic, non-cholinergic (NANC) nerve terminals utilizing a number of different neurotransmitter systems (norepinephrine, neuropeptide Y, acetylcholine,

calcitonin gene related peptide (CGRP), vasoactive intestinal polypeptide (VIP) and nitric oxide (Luscher *et al* 1990, Zhang *et al* 1994). A component of the erectile control systems that has received much less attention is the more subtle balance that occurs at the local level between vasoactive substances derived from the vascular endothelium and smooth muscle or from the circulation. This concept is important as we have not yet fully accounted for the range of local systems in particular that might be involved under normal and abnormal conditions of erectile function.

Regardless of the mechanism that a particular system uses, if an alteration in function diminishes the capacity for smooth muscle relaxation the consequence is high penile vascular resistance. As such enhanced vasoconstriction leads to insufficient arterial inflow and inadequate venous occlusion ultimately resulting in erectile dysfunction (Lerner *et al* 1993, Krane *et al* 1989, Anderson *et al* 1993, Saenz de Tejada 1991). Thus, the level of vascular tone *in vivo* results from a critical balance of countervailing vasoactive systems at the neural, humoral and local levels. A widely discussed concept in the present literature subscribes to the concept that an imbalance in countervailing systems, particularly nitric oxide, will result in a modified vascular sensitivity to the other systems (Zhang *et al* 1994, Ito *et al* 1991, Lerman *et al* 1992, Luscher and Noll 1995, Greenberg *et al* 1991, Vo *et al* 1992, Jones *et al* 1993, Shinozuka *et al* 1992). The physiological role for NO was elucidated based on this concept whereby the marked increase in mean arterial pressure that occurs following NO synthase blockade was proposed to be a 'tipping of the balance' towards the now 'unopposed'

vasoconstrictor systems. Specifically, a number investigators proposed that this dramatic vasoconstrictor response after NO levels were depressed strongly indicated that NO functioned as a chronic vasodilator system (Ribeiro *et al* 1992, Rees *et al* 1989, Navarro *et al* 1994).

Recent evidence, however, regarding the mechanism(s) involved in the increased vascular resistance that occurs following NO synthase blockade has revealed a divergence with respect to which vasoconstrictor systems exert their effects 'unopposed'. Several investigators have demonstrated an increased sensitivity to α_1-adrenoceptor activation (Deng *et al* 1993, Li and Schiffrin 1994), but not to the renin-angiotensin system end-point angiotensin II receptor activation in blood vessels for rats with chronic NO synthase blockade (Arnal *et al* 1993). These results indicate a substantially more complicated interaction may exist between the countervailing vasoactive systems that dictate the level of vascular tone, then previously proposed in the literature.

Banting *et al* (1996) have demonstrated that up to 90 % of the hypertension following acute NO synthase blockade could be accounted for by endothelin mediated vasoconstriction. Also, the contribution of the sympathetic nervous system mediated vasoconstriction was found to be less or equal to that which it makes under control conditions. In addition, Banting *et al* (1996) also demonstrated that since the role for endothelin in the normal circulation was minimal, indicating that the chronic role of nitric oxide, in blood vessels, was more subtle and likely reserved for transient local vasodilator

function as well as suppression of local endothelin mediated vasoconstriction. In the present study our objective was to determine whether a similar relationship between NO and vasoconstrictor systems could also be found in the specialized circulation of the penile vasculature. To elucidate this concept we analysed the role of local control systems in regulating the vascular resistance properties of the isolated perfused penile vasculature of the rat following inhibition of NO production.

Methods

Materials

Methoxamine (MXA, α-[1-aminoethyl]-2,5-dimethoxybenzyl alcohol) and N^{w}-nitro-L-arginine methyl ester (L-NAME) were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). Endothelin-1 (ET-1) was obtained from American Peptide Company (Sunnyvale, California, USA) and gas mixtures were obtained from BOC Gases (Mississauga, Ontario, Canada). PD145065 is kindly given from Parke-Davis Pharmaceuticals (Ann Arbor, Michigan, USA).

The stock solution of ET-1 of 60 μg/ml was made in 0.1% acetic acid in 0.9% sterile saline solution (Baxter Corp., Toronto, Canada), stored at -70°C and diluted with saline immediately prior to use. Other drugs were made in 0.9% sterile saline solution.

Animals

Male Wistar rats (350 to 450 g), obtained from Charles River Laboratories (Montreal, Quebec, Canada), were housed individually under conditions of a 12-hour light/12-hour dark cycle (temperature of 22 to 24°C), and received free access to Purina® rodent chow and tap water for at least 2 days before any experiment.

Surgical Preparation of the Perfused Penile Vasculature

This protocol was approved by the Animal Care Committee at Queen's

University, Kingston, Ontario. The model used in the present study was the isolated perfused penile vasculature in male rats, previously established by Banting *et al* (1995). As described in detail before, the isolation of pudendal vasculature was achieved by ligatation all the arteries except pudendal arteries and cauterize to ensure that only penile vessels are perfused. After finishing surgical procedure, a flow of perfusate (1.0 ml/min/kg body weight) through the abdominal cannula was started. The perfusion pressure was recorded either by a physiograph (E & M Instrument Co., Inc., Houston, Texas, USA) or a computer system, MacLab (AD Instruments, Houston, Texas, USA) via a pressure transducer that was connected to the catheter in the aorta. The perfusate was infused for at least 20 minutes to flush the penile vasculature of blood and obtain a stable resting perfusion pressure before the beginning of any experiment.

The perfusate consisted of dextran (1.5%, average molecular weight:71400, Sigma Chemical Co., St. Louis, Missouri, USA) in Tyrode's solution, which was aerated with 95% O_2 and 5% CO_2, was adjusted its pH around 7.4. The composition of the Tyrode's solution was KCl 20, $CaCl_2\,2H_2O$ 32.3, $MgCl_6\,H_2O$ 5.1, $NaH_2PO_4\,2H_2O$ 6.2, $NaHCO_3$ 155, glucose 100 and NaCl 800 mg/100ml fluid. The perfusate was held in a reservoir, and passed through a bubble trapping/mixing chamber and heating bath. An injection port was located distal to the bubble trap for the introduction of pharmacological agents. An in-line peristaltic pump was used to establish flow at 1.0 ml/min/kg body weight (Minipuls-2, Gilson Medical Electronics, Inc., Middleton, Wisconsin, USA). The pharmacological agents were delivered by a syringe pump (Harvard Apparatus

Infusion/Withdrawal Pump, Millis, Massachusetts, USA). A servo-controlled heated chamber served to maintain the ambient temperature and the entire preparation at 37-38°C.

Cumulative concentration-response curve of methoxamine(MXA):

A cumulative concentration response curve to α_1-adrenoceptor agonist, MXA was obtained in 5 rats by step-wise increases of MXA from 0.5 to 16 μg/ml. MXA was infused for 2 minutes with the 2 minutes intervals between infusions. At each concentration, a perfusion pressure (PP) plateau was reached before the next concentration of MXA was given. The concentrations of MXA were calculated by the body weight of each rat to normalize run to run variations MXA was washed out at least 30 minutes before any next experiments were performed.

Cumulative concentration-response curve of endothelin-1.

After MXA cumulative concentration-response was done, a cumulative-concentration response curve to endothelin-1 (ET-1) was obtained in 5 rats by step wise increasing of ET-1 from 0.02 to 1.28 μg/ml. ET-1 was infused for 2 minutes as in MXA, but we found that a 3-minute interval was needed for the PP to reach the plateau before the next concentration was given. The effect of ET-1 was long lasting, such that a 1 hour wash out period was required to return the PP to basal levels before subsequent experiments were performed.

The effect of sub-threshold concentrations of ET-1 on α_1-adrenergic mediated vasoconstriction

After MXA and ET-1 cumulative concentration-responses were completed, MXA cumulative concentration-response curves were repeated in the same rats (n=5) in the presence of sub-threshold concentration of ET-1 (0.02-0.04 μg/ml). ET-1 was infused for the entire period that MXA cumulative concentration-response curves were performed.

The effect of decreased NO levels on α_1-adrenoceptor mediated vasoconstriction and the effect of PD145065

First, the MXA concentration-response was investigated as described above. In this protocol, the tissue was not subjected to the perfusion pressure greater than 150mmHg, in order to protect the vascular tissue from damage due to sustained high perfusion pressure.

The sub-threshold concentration (0.5-1.0μg/ml, n=4) and the sub-maximal concentration (6-8 μg/ml, n=4) of MXA were infused alone or in combination with L-NAME (100 μg/ml) to investigate whether decreased NO level can potentiate α_1-mediated vasoconstriction. The concentration-response curve to MXA with decreased NO levels was generated in multiple animals followed by ET_A/ET_B antagonist administration, PD145065 (30 μg/ml) was also infused for 2-3 minutes, when the PP was increased by L-NAME, to assess the involvement of endothelin in this response.

The effect of endothelin antagonist on MXA-mediated vasoconstriction

In this preparation, rats were likely exposed to some degree of hypoxia during the 2-3 hours of surgical preparation under sodium pentobarbital anaesthetic . It is conceivable that endothelin may have been already activated by prolonged hypoxia before experiments being performed in some preparations. Accordingly, the role of hypoxia, presumably via increasing ET-1, in MXA-meditating vasoconstriction was assessed. MXA (4-8μg/ml) was infused following PD145605 (30-100μg/ml) to examine if the response was blocked by an endothelin antagonist (n=5). PD145065 was also given after the plateau was established by MXA to determine if the response was reversed by an endothelin antagonist (n=4).

Data Analysis

A logistic function curve was found to be suitable to describe the relationship between MXA concentration and change in PP; a computer program (Sigmoid 5, Baker Medical Institute, Melbourne, Australia) that employed the algorithm of Marquardt (1963) was used to fit the data points. Each curve was characterized by the following parameters: (i) EC_{50}, the concentration of pharmacological agents to reach 50% of the pressure range of the curve between the minimum and maximum pressures with agonist, and (ii) the normalized slope of the concentration-response curve which represents the steepness of the curves. Since the maximum slope of the logistic curve depends in part on the range of the changes in PP, we calculated the normalized (range-independent) slope of the curve , where the range is regarded as 100% under all conditions, by the

following equation.

$$\text{normalized slope} = \frac{\text{maximal slope}}{\text{maximal PP/100}}$$

The multiple comparison of parameters between different treatments of the vasculature was performed by the Bonferroni correction. P value less than 0.05 was considered to be significant.

Results

Concentration response curve to MXA, ET-1 and MXA in the presence of sub-threshold ET-1 in the penile vascular bed

In a previous study, we demonstrated that the basal PP, which is the PP at the resting state, represents the maximal vasodilation of this preparation (Banting *et al* 1995). We performed the concentration-response to MXA, ET-1 and MXA combined with sub-threshold levels of ET-1 in the same rats (n=5). The actual representative traces and the average concentration-response curves for each of these different manipulations are shown in Figures 7-1 to 7-4 and the curve parameters of each are shown in Table 7-1. In the concentration-response curves, all points are expressed as a percentage of maximal perfusion pressure. The ED_{50} of ET-1 is 0.64±0.48 μg/ml which is significantly smaller than that of MXA, 6.16±1.50 μg/ml ($P<0.001$; Figure 7-4, Table 7-1). As such, consistent with literature, ET-1 is approximately 10 times more potent then MXA. The normalized slope is significantly smaller in ET-1 approximately half value than the MXA value($P<0.05$, Table 8-1), which may indicate that the response of penile vasculature to ET-1 is more blunt than to MXA.

A sub-threshold concentration of ET-1 (0.02-0.04μg/ml), that has no pressor effect by itself, when perfused in combination with MXA, resulted in a marked leftward shift in comparison to the MXA curve by approximately 4-fold ($P<0.001$; Figure 7-5, Table 7-1).

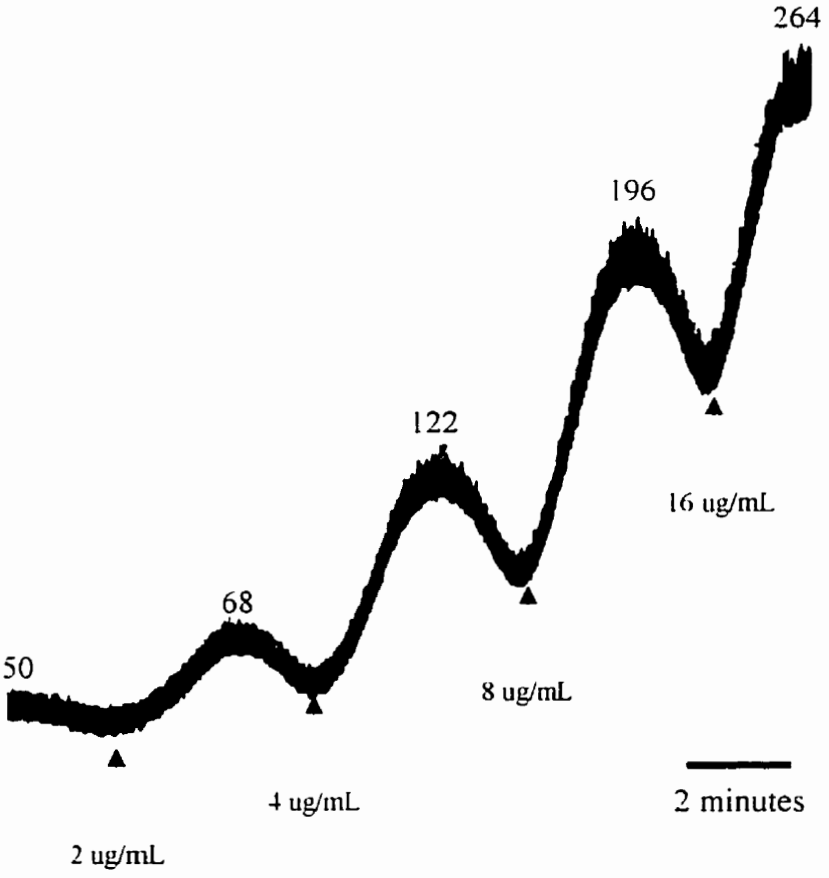

Figure 7-1: A representative trace of concentration-response of methoxamine (MXA) in the rat perfused pudendal vasculature. MXA was infused in a cumulative fashion for 2 minutes at each concentration with 2 minutes wash-out before the next concentration was infused. Values are expressed as raw perfusion pressure (mmHg).

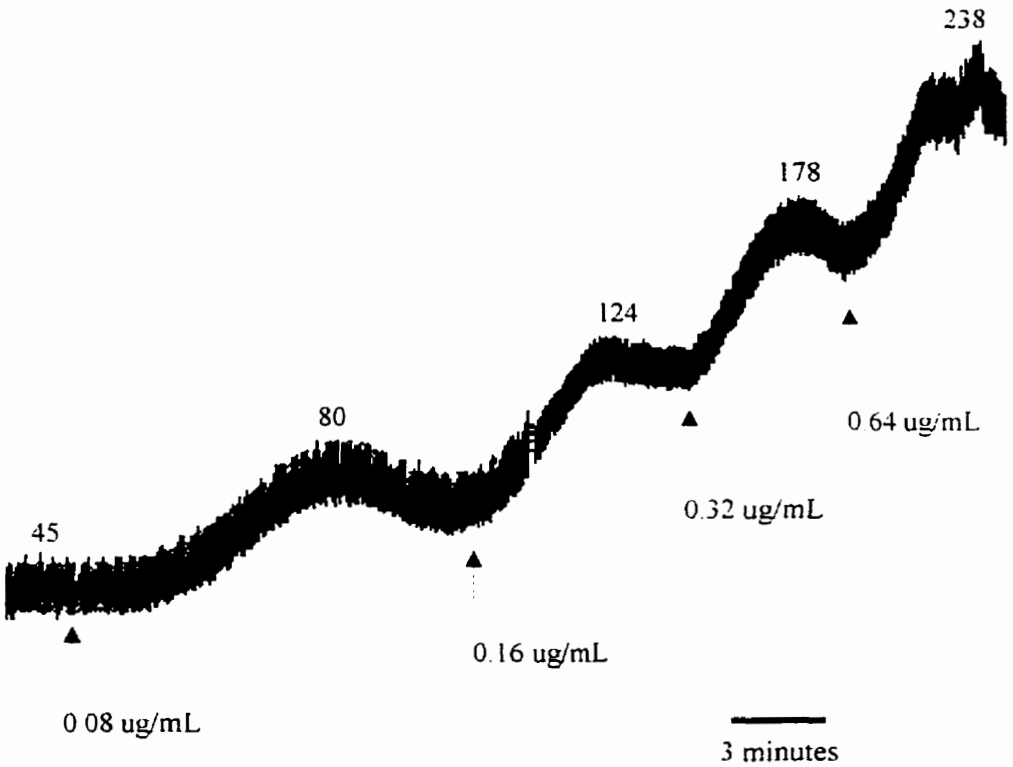

Figure 7-2: A representative trace of concentration-response of endothelin-1 (ET-1) in the rat perfused pudendal vasculature. ET-1 was infused in a cumulative fashion for 2 minutes at each concentration with 3 minutes wash-out before the next concentration was infused. Values are expressed as raw perfusion pressure (mmHg).

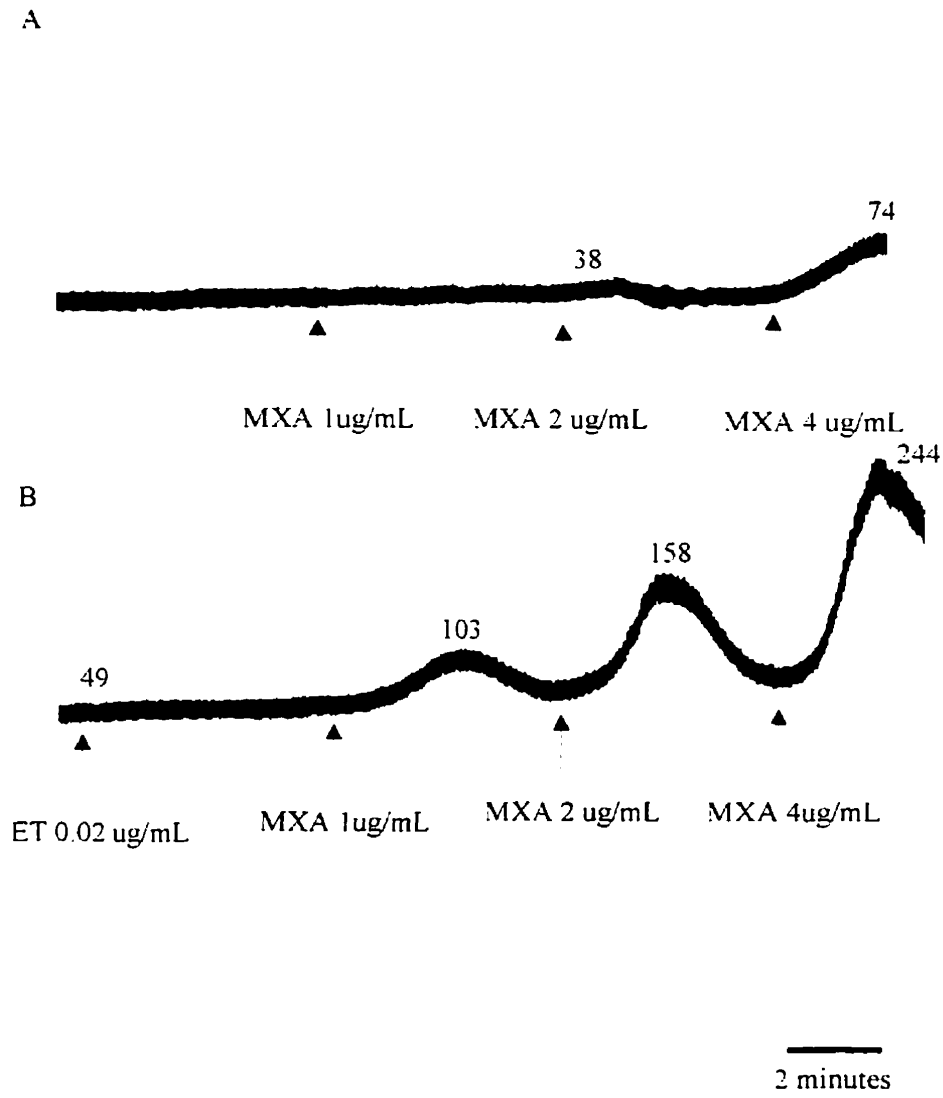

Figure 7-3: A representative trace showing the potentiated response to methoxamine (MXA) by sub-threshold endothelin-1 (ET-1). In the rat perfused pudendal vasculature, (A) MXA dose-responses were investigated at first, followed by dose-response to (B) methoxamine in combination with sub-threshold concentration of ET-1 (0.02 μg /ml) in the same animal. The responses of MXA were enhanced markedly in the presence of sub-threshold of ET-1.

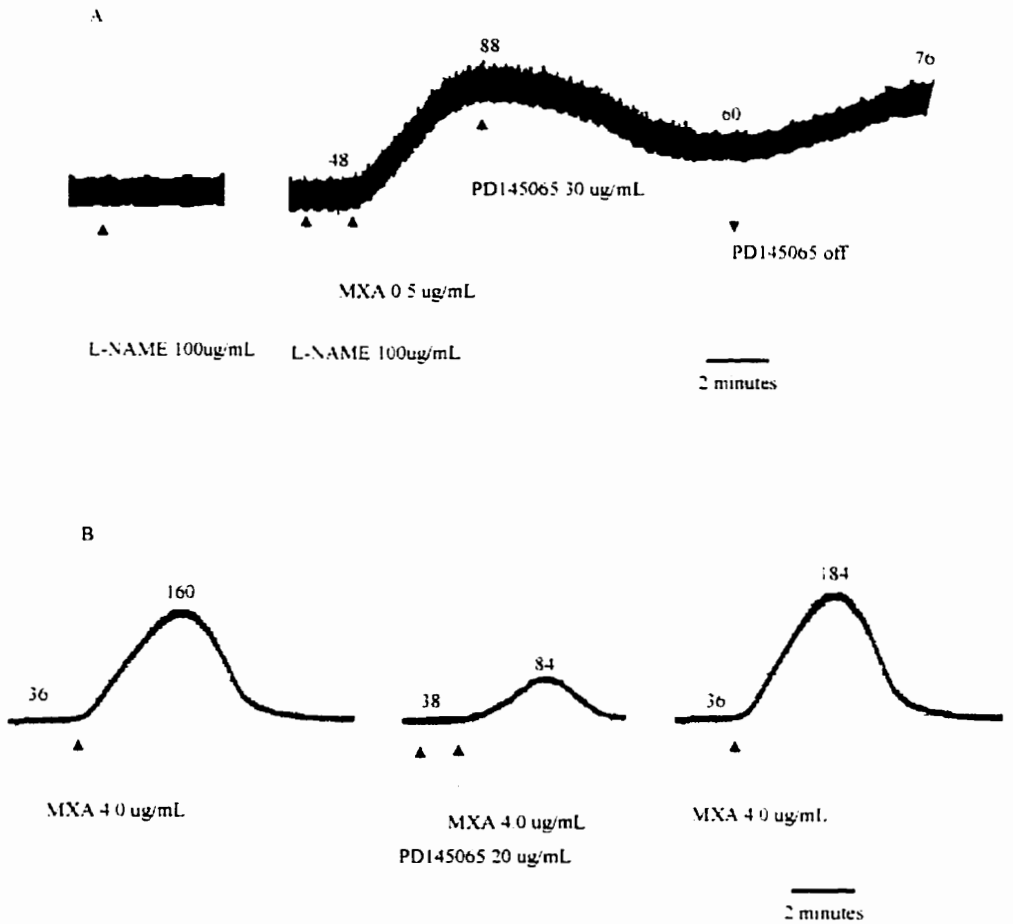

Figure 7-4: Potentiation of response to MXA and the effect of PD145065. (A) A representative trace of the potentiation of the pressor response of sub-pressor methoxamine (MXA, 0.5 μg/ml) by NO synthase inhibition via L-NAME (100 μg/ml) and the effect of ET_A/ET_B antagonist, PD145065 (30 μg/ml). Only L-NAME has no effect on perfusion pressure. In combination with MXA, L-NAME enhanced the pressor response of MXA, and it was restored by ET_A/ET_B antagonism. Note after PD 145065 was stopped, the perfusion pressure returned to control levels. (B) A representative trace showing inhibition of response to sub-maximal MXA by ET_A/ET_B antagonist, PD145065. Note that after wash-out PD145065, response to MXA was recovered.

Table 7-1. Mean curve parameters of concentration-response curve of methoxamine (MXA), endothelin-1 (ET-1) and MXA with sub-threshold of ET-1. Values are expressed by mean±SE. * expresses the significant difference ($P<0.05$, Bonferroni correction) compared with the values from those of MXA concentration-response curve.

	ED50 (μg/ml)	normalized slope
MXA (n=5)	6.16 ± 1.50	217±33
ET (n=5)	0.64± 0.48 *	116±26 *
MXA with $ET_{subpress.}$ (n=5)	1.43± 0.37*	204±52

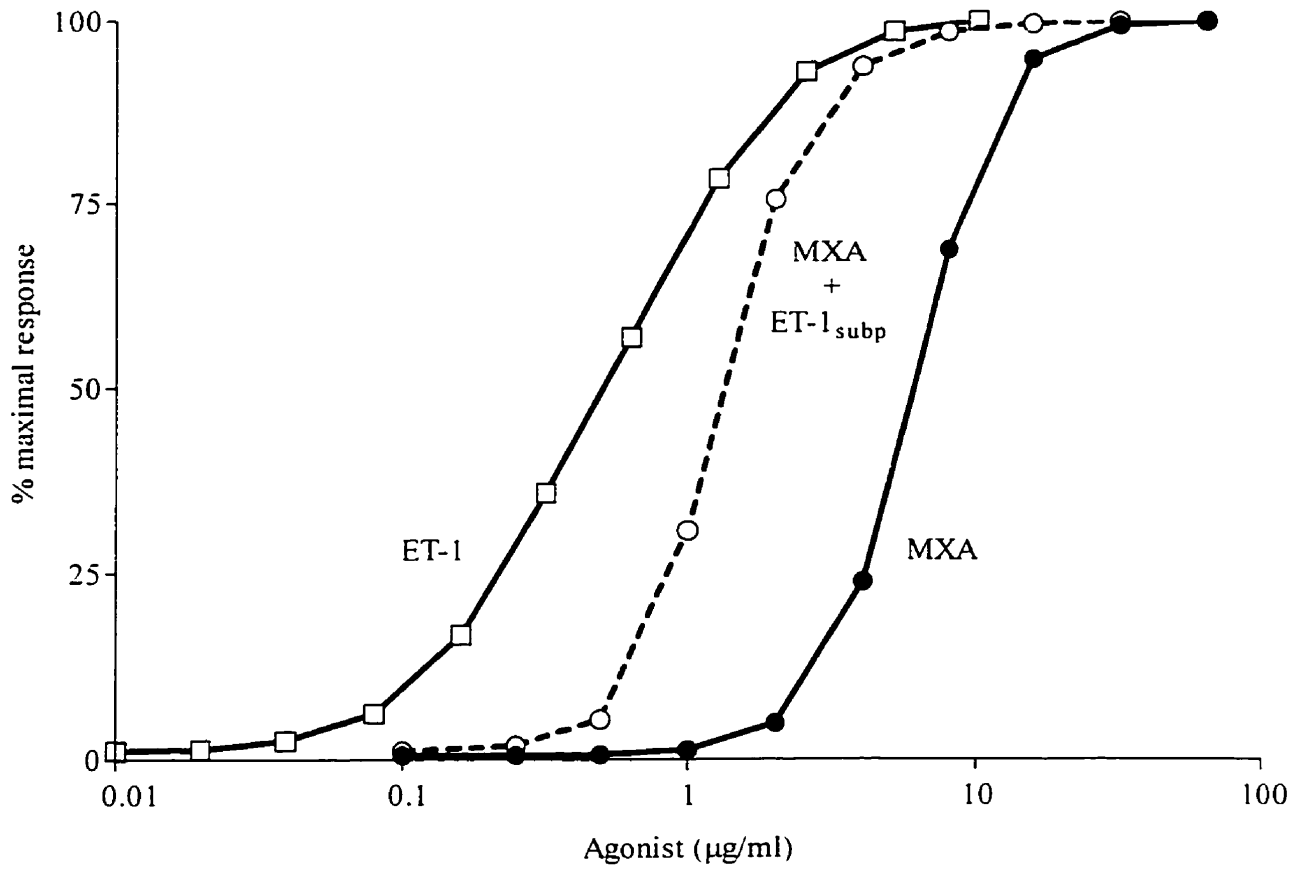

Figure 7-5: An averaged cumulative concentration-response curve generated by different agonists in the rat perfused pudendal vasculature. The responses to methoxamine (MXA, solid circle, n=5),endothelin-1 (ET-1, open square, n=5) and MXA in the presence of sub-threshold of ET-1 (open circle, n=5) were investigated. Points are expressed as percentage of maximal perfusion pressure

As a result, MXA at concentration 2 μg/ml could produce 75% of maximal response in combination with ET-1, whereas only less than 5% of maximal was obtained with MXA alone.

The effect of decreased NO level on α_1-adrenoceptor mediated vasoconstriction and the effect of PD145065:

The decreased NO levels by L-NAME (100 μg/ml) treatment did not increase the PP by itself, but potentiated the response to MXA (n=8). The sub-threshold concentration of MXA (0.5-1.0μg/ml) alone did not change PP. However, when infused in combination with L-NAME, even the sub-threshold concentration of MXA could elevate the PP by approximately 30mmHg (Figure 7-4), this response would correspond to the infusion of approximately 3.5 μg/ml of MXA alone. Similarly, the sub-maximal MXA (8 μg/ml) increased PP by approximately 150mmHg, and it was potentiated by L-NAME (100 μg/ml) to 214mmHg. In addition, 86±17% of the increased PP, above baseline, due to L-NAME + sub-pressor or sub-maximal MXA infusion was reversed by PD145065 (30 μg/ml), ET_A/ET_B antagonist (n=6, Figure 7-4), demonstrating that the potentiation of α_1-signals by decreased NO level was mainly mediated by enhanced endothelin-mediated vasoconstriction.

The concentration-response curve to MXA with the decreased NO levels demostrated a left-ward shift in the ED_{50} from 7.18μg/ml to 2.15 μg/ml. Representing an overall leftward shift of the curve 3.3 fold (Figure 7-6). Moreover, as shown in Figure 7-

7, infusions of sub-threshold concentrations of ET-1 with L-NAME have the almost same effect on the enhancement of α_1-signals, which further indicating that they share the same mechanism.

The effect of PD145065 on MXA

In several preparations, L-NAME treatment did not further enhance the MXA-induced vasoconstriction (n=6). In these preparations, we speculate that levels of ET-1 mediated vasoconstriction may already be enhanced, at least in part, due to the prolonged exposure to hypoxia during the surgical procedure, prior to starting the perfusion experiments. In fact, in such preparations, PD 145065 antagonist blocked the enhanced sensitivity to MXA by 57±11% (Figure 7-4, n=5) as well as reversed the enhanced sensitivity to MXA following NO synthase blockade (n=4) by 69±9%.

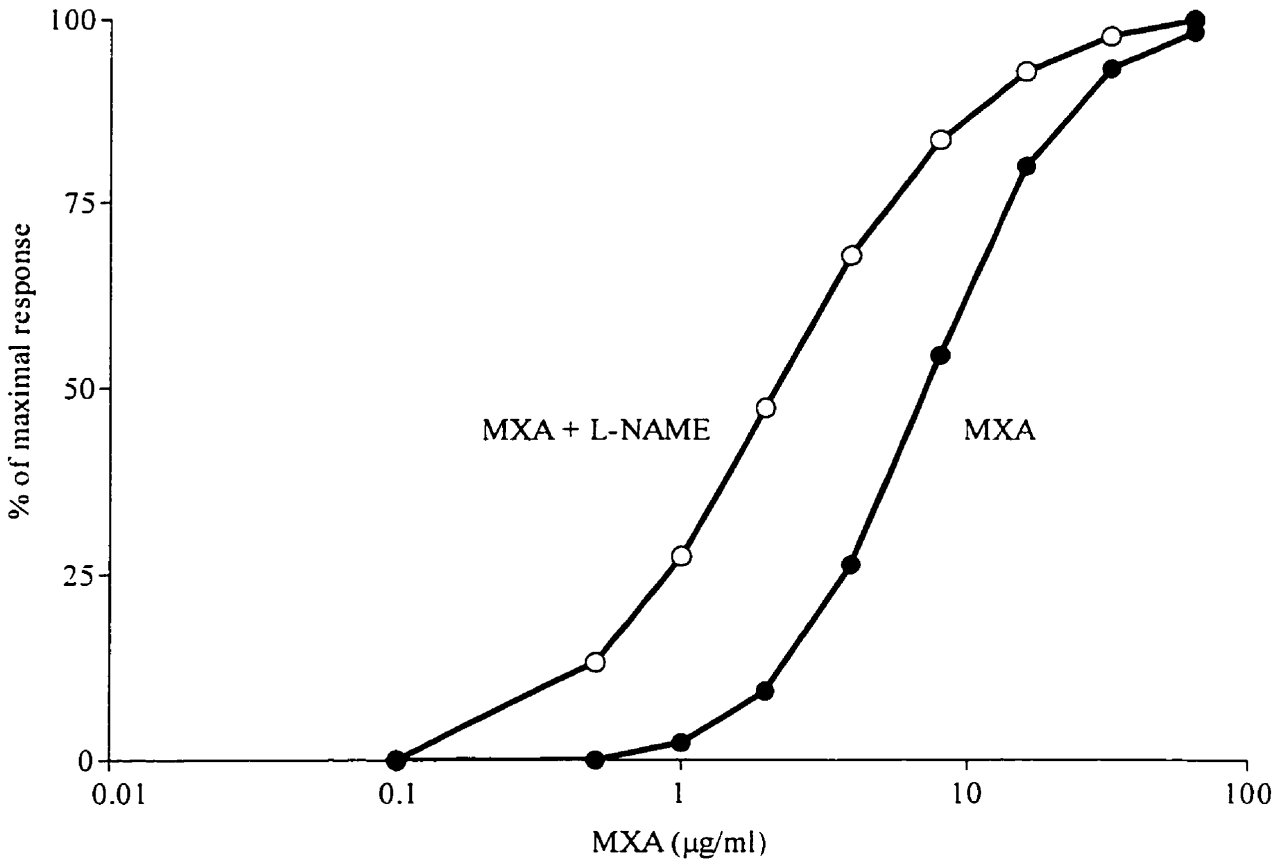

Figure 7-6: Concentration-response curves of methoxamine in the absence (solid circle, n=8) and presence (open circle,n=8) of L-NAME (100 μg/ml). Points are expressed as percentage of maximal perfusion pressure. ED_{50} shifted leftward from 7.18μg/ml to 2.15μg/ml by NOS inhibition. The concentration-response curve of MXA with decreased NO levels was generated in multiple animals using pooling responses.

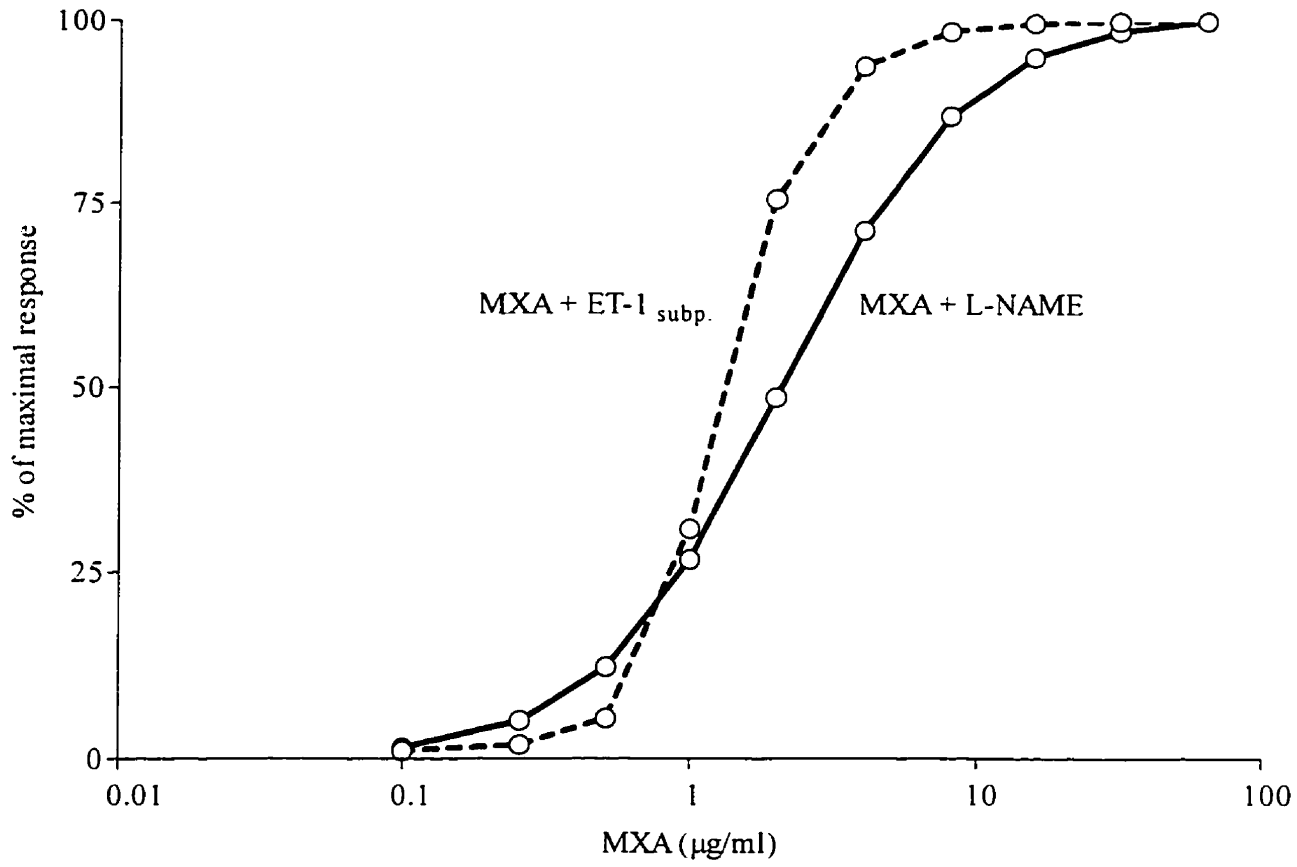

Figure 7-7: Comparison of enhanced concentration-response curves of methoxamine (MXA) by L-NAME (solid line, n=8) and sub-threshold ET-1 (dotted line, n=5). Points are expressed as percentage of maximal perfusion. NO inhibition and sub-threshold ET-1 have similar effect in respect to modulation of MXA concentration-response.

Discussion

The major findings of the present study are that (i) the hyperreactivity of the penile vasculature that occurs following inhibition of NO synthase is mediated by a marked upregulation of endothelin-mediated vasoconstriction, (ii) the enhanced role of endothelin also mediates a substantial increased sensitivity to α_1-adrenoceptor activation, and (iii) the levels of endothelin required to produce this level of hyperreactivity are, in fact, near-threshold i.e. the levels present induce very little vasoconstriction on their own.

In this study, we have demonstrated that decreasing levels of NO by L-NAME treatment, led to a more than 3-fold increase in sensitivity to α_1-adrenoceptor mediated vasoconstriction. Many studies have shown that inhibition of NO synthase results in the potentiation of the agonist-induced vasoconstriction (both directly and neurally initiated) in a number of species and in a number of vascular bed, including canine arteries (Greenberg *et al* 1991), rat caudal artery (Vo *et al* 1992), canine coronary artery (Jones *et al* 1993) and rabbit pulmonary artery (Shinozuka *et al* 1992). Clearly, NO modulation of α_1-adrenergic vasoconstrictor activity can play a significant role in controlling vascular tone in many vascular beds in a wide ranges of species. Therefore, it is crucial to elucidate the underlying mechanisms mediating the enhanced vascular sensitivity to α_1-activation, following NO synthase blockade to understand the regulatory systems of vascular tone.

Several mechanisms have been proposed to account for the increased sensitivity to

α_1-adrenoceptor activation following NO synthase blockade. It has been shown that NO can inhibit noradrenaline release from adrenergic nerve terminals, at least in part, via activation of the cyclic GMP signal transduction pathway (Greenberg *et al* 1991, Vo *et al* 1992, Jones *et al* 1993). Norepinephrine has also been demonstrated to be involved in a negative feedback loop regulating NO product through either a direct effect or by increasing shear stress during vasoconstriction. Taken together, pre and post synaptic mechanisms exist whereby NO inhibits of the release and or activity of noradrenaline has been widely demonstrated, at least, *in vitro*.

In vivo, however, the hypertension induced by inhibition of NO production has been shown to lack an enhanced role of α_1-adrenoceptor activation in the acute phase. In fact, the role of α_1-adrenoceptor activation was found to be diminished in NO synthase blocked rats, as compared to control values (Banting *et al* 1996). Therefore, in the acute phase of this model, enhancement of α_1-adrenoceptor activation could not account for the increased mean arterial pressure after NO synthase inhibition.

It has been widely accepted that the major function of NO, *in vivo*, is that of a general vasodilator leading to a generalized opposition of the endogenous vasoconstrictor systems (Ribeiro *et al* 1992, Rees *et al* 1989). This role has been derived from studies where the removal of NO has been thought to induce changes by unmasking the pressor effects of the remaining vasoactive systems. Clearly the concept of the pressor response following NO synthase blockade being mediated simply due to the unmasking of all other

existing endogensous systems is not consistent with the enhanced α_1-adrenoceptor activation, at least in the acute phase. Accordingly, there should be other mechanisms which are responsible for the early increase in vasoconstrictor sensitivity in a NO-deficient condition.

Evidence from Banting *et al.* (1996) and others (Kourembanas *et al* 1991, Richard *et al* 1995) revealed that NO inhibition, *in vivo*, unmasks ET mediated vasoconstriction. Banting *et al.*(1996) have investigated, in conscious rats, the quantitative contribution of each vasoconstrictor system to hypertension after acute nitric oxide synthase blockade with L-NAME. They demonstrated that ET receptor activation accounts for approximately 85% of the acute pressor response by inhibiting NO production, while α_1-adrenergic system remains the same or lessor contribution compared with control. From their results, they proposed that the major role of nitric oxide is to suppress vasoconstrictor activity of endothelin rather than as a vasodilator itself.

In this study, the enhanced pressor response to an α_1-adrenergic agonist that occurs following decreases in NO levels was almost completely reversed by an ET_A/ET_B antagonist (approximately 90%). We have also shown that sub-threshold concentration of ET-1, which elicit no contractile effect alone, can potentiate the contractile effect of MXA, a selective α_1-adrenergic agonist by approximately 4-fold. This result is consistent with previous findings in the human corpus cavernosum (Kim *et al* 1996) and rabbit aorta (Henrion and Laher 1993) preparations. Although the specific intracellular

pathways in this potentiation are beyond the scope of this experiment, it is likely that ET-1 and α_1-adrenergic agonists pathways converge at the same point (Henrion and Laher 1993, Zhao and Christ 1995).

It has been shown that both α_1- and ET-1 receptor activations lead to an increase of intracellular calcium concentration via several pathways. Specifically, it has been shown that both of these systems activate phospholipase C and in turn lead to the formation of inositol triphosphate and diacylglycerol. The former liberates calcium from intracellular storage sites, and the latter is suggested to increase the sensitivity to Ca^{2+} via the activation of protein kinase C. It is also known that both norepinephrine and ET-1 activate voltage-dependent Ca^{2+} channels which evokes the influx of extracellular Ca^{2+} (Zhao and Christ 1995, Luscher *et al* 1992). It has been suggested that ET-1-induced potentiation of α_1-adrenergic contraction may be related to an increased influx of Ca^{2+} into cells or the increased sensitivity of the cells to Ca^{2+} by protein kinase C activation. (Henrion and Laher 1993, Zhao and Christ 1995, Luscher 1992).

Our results demonstrate an almost complete reversal of the increased sensitivity to α_1-adrenoceptor activity following NO inhibition by blockade of ET_A/ET_B receptors. This is consistent with the results that demonstrate that there is a similar shift of MXA concentration-response curve either by sub-threshold levels of ET-1 or following L-NAME administration. These results further demonstrate that in response to decreased NO production an increase to near-threshold concentrations of ET-1 are induced thereby

potentiating the α_1-adrenergic response in the vasculature.

Interestingly, in some preparations, L-NAME treatment did not lead to further enhancement of the pressor response to MXA in several rats. This finding appeared to be associated with an 'already' enhanced effect since without L-NAME treatment the responses of MXA alone were markedly attenuated (by 60 %) following endothelin antagonism. This particular result may be explained in part by the variability in the time and difficulty of each preparation. That is, it is widely acknowledged that general anaesthesia depresses cardiac and respiratory function, which would result in lowering the oxygen tension in peripheral tissues. Vender *et al.* (1995) demonstrated that oxygen tension after general anaesthesia can drop to less than 60mmHg in rats. It has been shown that hypoxia is associated with both a stimulation of ET-1 production and decreased NO production (Kourembanas *et al* 1991, Levin 1995). Based on a 2-2.5 hours surgical preparation to isolate the pudendal vasculature, it is conceivable that ET-1 may already be enhanced as a result of depressed NO following to exposure to hypoxia. If this is the case, based on our hypothesis, it is expected that the lack of potentiating effect following L-NAME would be observed in the tissue where endothelin levels had already been activated (i.e. this preparation may represent a circumstance in which endothelin levels can be intrinsically upregulated.

In the flaccid state, it has been demonstrated that low oxygen tension in corpus cavernosum decreases NO production (Kim *et al* 1993). In turn, based on the results of

the present study, the activity of ET-1 may be up-regulated, leading to increased contractile tone of smooth muscle cells. Conversely, when erectile response is initiated, NO released either from non-adrenergic, non-cholinergic neurons or endothelial cells, will serve not only as a direct relaxant agent, but also to supress ET-1 activity. In pathophysiological conditions, locally elevated ET-1 activity due to insufficient NO function, may be a major cause of erectile dysfunction, as it will shift the balance in favour of vasoconstrictor mechanisms. This shift in balance, even with normal constrictor stimuli, can lead to a high level of smooth muscle tone in the penile vasculature which may prevent penile tumescence. In a pudendal circulation, which is dependent on active neural stimuli for dynamic control, a disturbance in the reciprocal relationship between NO and ET-1 may profoundly influence vascular tone, mediating erectile dysfunction.

Normally, it is considered that in human beings and in rats, endothelins appear to play a minimum role , if any, in the maintenance of mean arterial pressure in normal physiological conditions. That is, a minimal role is presumed based on the fact that administration of an endothelin antagonists has no effect on the resting levels of arterial pressure (Banting *et al*, Weber *et al* 1996). In some pathophysiological conditions, however, endothelial control of vascular tone is disturbed. This has normally been attributed to, at least in part, an impaired NO production although elevated ET-1 activity has also been suggested as a factor leading to enhanced vascular reactivity. (Luscher and Noll 1995, Levin 1995, Tanner *et al* 1993). In a severe disease state such as

atherosclerosis or ischemic heart disease, it has been demonstrated that ET-1, either in tissue or plasma levels, is elevated, and may play a role as a local mediator in the progression of these conditions. (Luscher and Noll, 1995, Levin 1995). Thus, it is noteworthy that local control systems have the capacity to induce a profound influence on functional changes of vascular homeostasis.

The penile vasculature represents a specialized circulation which is subjected to extremes of hemodynamic conditions (i.e. tumescence and detumescence). In the penis, there is little to no intrinsic autoregulation or metabolic regulation, as in the kidney, heart, brain, gut and skeletal muscle (Krane *et al* 1989). The dual action of neural systems in concert with local systems would appear to take almost of the control of the smooth muscle tone which dictates resistance and therefore arterial inflow in this specialized circulation. A change in the local balance between NO and ET-1, as presented in this study, will have a critical impact in dictating arterial inflow and thereby erections.

The major role of NO was widely believed to be that of a powerful, chronic vasodilator, and in the penis, an the obligatory mediator of erections (Rajfer *et al* 1992). The results of the present study revealed that NO actually serves as a local suppressor of a novel local endothelin. *In vivo* experiments provide further evidence in support of this concept. Additional support is found in experiments with apomorphine-induced erections in conscious rats are diminished or almost completely abolished following NO blockade in acute phase (Adams *et al* 1994), and yet administration of an endothelin antagonist can

completely normalize erectile function (Whittingham *et al* 1996).

In conclusion, in this study, we have characterized a novel countervailing regulating system occurring between NO and ET which is involved in the regulation of penile vascular tone, which in turn will very likely impact on erectile function, *in vivo*. Further, we have shown that in the penis, endothelial-derived nitric oxide serves as a powerful suppressor of the local activity of endothelin rather than as a potent vasodilator itself. Elevated levels of endothelin have been shown to markedly sensitize α_1-adrenergic stimuli potentially providing a major impediment to erectogenesis. The full impact of these findings in the complex etiologies of erectile function remain to be elucidated.

Chapter 8: Preliminary studies assessing the 'quantity' of NO-donor compounds required to maintain normal vascular function in NO synthase blocked rats.

Abstract

Objective: The present study examined the contribution of nitric oxide to the level of mean arterial pressure following acute and chronic NO synthase blockade, with respect to quantitating the exogenous levels of NO required to restore normal vascular tone in these phases of this model of hypertension.

Methods: Mean arterial pressure was recorded in conscious male Sprague-Dawley rats, previously instrumented with aortic and venous catheters. Rats were treated either acutely with L-NAME (100 mg/kg, intraperitoneally) or for 12 days with L-NAME (100 mg/kg per day) in drinking water or tap water alone. In the acute animals, a cumulative concentration-mean arterial pressure response assessment was performed with sodium nitroprusside (0.5-32 ug/ml per min, intravenously) and glyceryl trinitrate infusion (0.5-32 ug/kg per min, intravenously) prior to NO synthase blockade. Following acute NO synthase blockade this protocol was repeated. Following chronic NO synthase blockade, the cumulative concentration-mean arterial response assessment was performed with both sodium nitroprusside (0.5-32 ug/kg per min, intravenously) and glyceryl trinitrate (0.5-32 ug/kg per min, intravenously).

Results: Both sodium nitroprusside and glyceryl trinitrate demonstrated a marked increased sensitivity in both the acute and chronic phased of NO synthase blockade induced hypertension.

Conclusions: The results of the present study indicate that the quantities of exogenous NO required that are required to restore normal vascular tone are 5-10 fold less than the concentrations that induce a direct vasodilator response in healthy, normal tissue.

Introduction

The acute effects of exogenously administered nitric oxide (NO) donating compounds in normal animals has been of therapeutics interest. Further, the physiological role of nitric oxide has been described as that of a chronic vasodilator agent, based on the marked increase in vascular tone following NO synthase blockade (Johnson and Freeman 1992, Tolins 1991). As discussed by Banting *et al* (1996), based on this rationale the NO vasodilator system would normally have an overall activity level at close to 70% of maximal capacity, leaving this system little reserve or gain to protect against deviations in mean arterial pressure homeostasis.

Banting *et al* (1996) proposed that the chronic role of nitric oxide, *in vivo*, is not as that of a vasodilator but rather an inhibitor of the activity of local vasoconstrictor agents, such as endothelin. That is, the hypertension following blockade on NO production was completely reversed with the administration of an endothelin receptor antagonist. This finding combined with the understanding that endothelin appears to play almost no role in the physiological maintenance of resting mean arterial pressure indicates that the function of NO may be more subtle than previously proposed.

The objective of the present study was to characterize quantitatively the level of exogenous NO required, to restore 'normal' vascular function following the acute and chronic blockade of NO synthase.

Methods

Animals

Male Sprague-Dawley rats (325-400 g) obtained from Charles River Laboratories (Montreal, Canada) were housed individually under conditions of 12-hour light/dark cycle, with room temperature at 22-24°C, and were provided with Purina rodent chow and tap water ad libitum for at least 2 days before any experiments were started.

Measurement of MAP and Short Acting Drug Administration

The surgical method was based on the technique of Thompson *et al* (1992). In brief, rats were anaesthetized with ketamine/xylazine (70/5 mg/kg i.p.), and the descending aorta distal to the kidneys was catheterized with small bore Teflon tubing (0.012-in. i.d., 30 gauge, Cole-Palmer, Laval, Quebec, Canada) inserted into vinyl tubing (0.02-in. i.d., 0.060-in., 23 gauge). The inferior vena cava was also catheterized distal to the kidneys with small bore Teflon tubing (0.012-in. i.d., 30 gauge, Cole-Palmer) The catheters were filled with heparinized saline (10 IU/ml) and held in place by a small amount of cyanoacrylate glue at the puncture site. The catheters were tunneled subcutaneously and exteriorized at the back of the neck and sutured in place. Two days after surgery, MAP could be recorded (MacLab DAS, ADInstruments, Milford, MA). After connection, an equilibration period of approximately 30 minutes allowed for the determination of the steady state level of MAP before any recording began. Baseline

MAP was determined from readings averaged over 5 minutes, taken from each rat at 15-minute intervals for at least 1 hour prior to the start of any experiment.

Sodium nitroprusside and glyceryl trinitrate concentration - mean arterial pressure response curves following acute and chronic N^{ω}-nitro-L-arginine methyl ester.

Rats were randomly assigned treatment with N^{ω}-nitro-L-arginine methyl ester for 30 minutes (100mg/kg, intraperitoneally) or 12 days (100mg/kg, in drinking water) or tap water. Two days prior to the day of the experiment rats were instrumented with catheters, as described above. Following baseline measurements of MAP, rats were given a infusions of sodium nitroprusside (SNP, 0.5-32 ug/kg per minute dissolved in 0.9 % sterile saline) with a step-wise increase in concentration every two minutes. Rats were allowed 30 minutes to recover from the SNP administration. Rats were then infusions of glyceryl trinitrate (GTN, 0.5-32 ug/kg per minute dissolved in 0.9 % sterile saline) with a step-wise increase in concentration every two minutes.

Throughout all of these pharmacological manipulations, MAP and HR were recorded at a sampling rate of 100 Hz and the data was stored on a disk drive for later analysis.

Results

The concentration-response relationship with increasing levels of sodium nitroprusside (SNP) was markedly shifted for both the acute and chronic NO synthase blockade treated rats. The concentration-mean arterial pressure response curves were divided into two groups, concentrations that did not and those that did induce a lowering of mean arterial pressure in the controls (Figure 8-1). A similar increased sensitivity to low levels of SNP in the L-NAME treated rats was demonstrated both in the acute and chronic phases of NO synthase blockade treatment. This level of SNP administration (0.5-8 ug/kg per minute) did not significantly decrease mean arterial pressure in the control rats but did resulted in a marked decrease in mean arterial pressure. As an illustrative example of this finding, the depressor response to 2 ug/kg per minute in the NO synthase blockade phase was superimposed over the 2 ug/kg per minute response in the control period (Figure 8-2). Interestingly, the mean arterial pressure lowering induced by 12-32 ug/kg per minute induced a similar lowering in both the control and treated rats (Figure 8-1, lower panel), indicating a convergence with respect to the depressor response to SNP.

The cumulative glyceryl trinitrate concentration-mean arterial pressure response demonstrated results similar to SNP. The cumulative concentration-mean arterial pressure response curve was also shifted leftward in the NO synthase blockade treated rats (Figure 8-3). Again, the similar trend of concentrations that do not lower mean

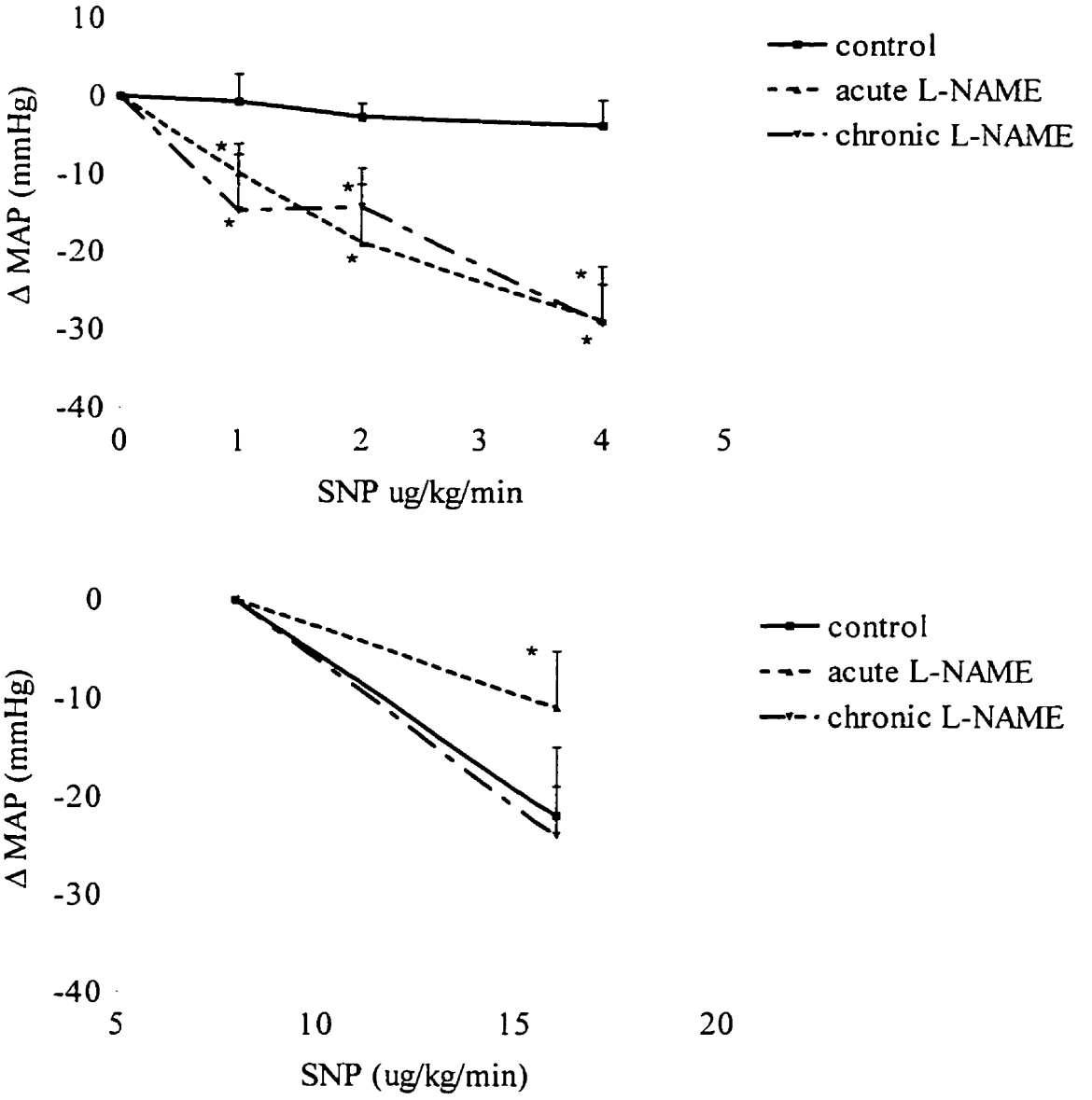

Figure 8-1: The differential impact on mean arterial pressure (mmHg) of infusions of sodium nitroprusside infusion in control and L-NAME treated rats. The concentration response curve to sodium nitroprusside was arbitrarily divided into two parts, doses that did (lower panel) and did not (upper panel) induce a lowering of mean arterial pressure.

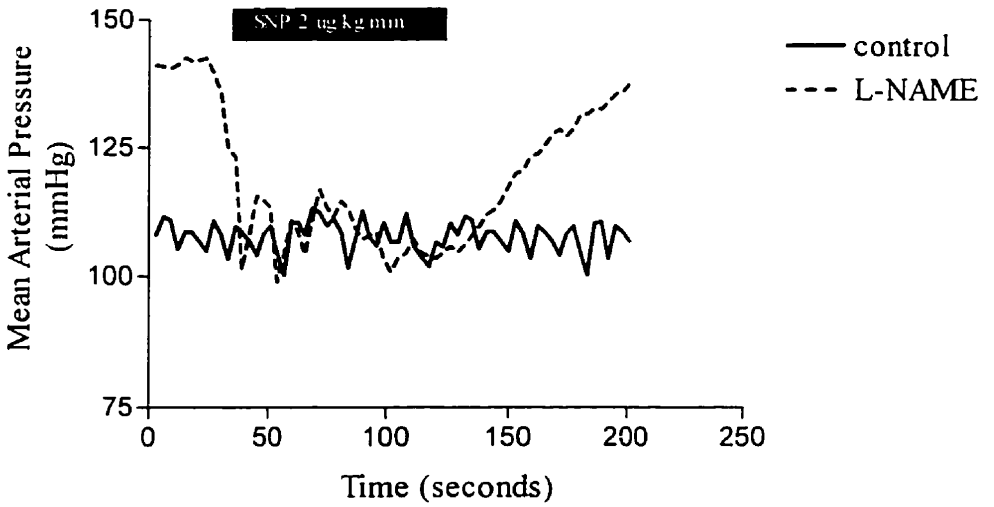

Figure 8-2: Representative tracing demonstrating the contrasting effect of low level infusions of sodium nitroprusside in control and acute L-NAME treated from a single rat (control and L-NAME traces has been superimposed for direct comparison). The 2 ug/kg per minute infusion of sodium nitroprusside resulted in a complete normalization of mean arterial pressure in the L-NAME phase, but did not lower mean arterial pressure under control conditions. The black bar indicate the duration of the sodium nitroprusside infusion.

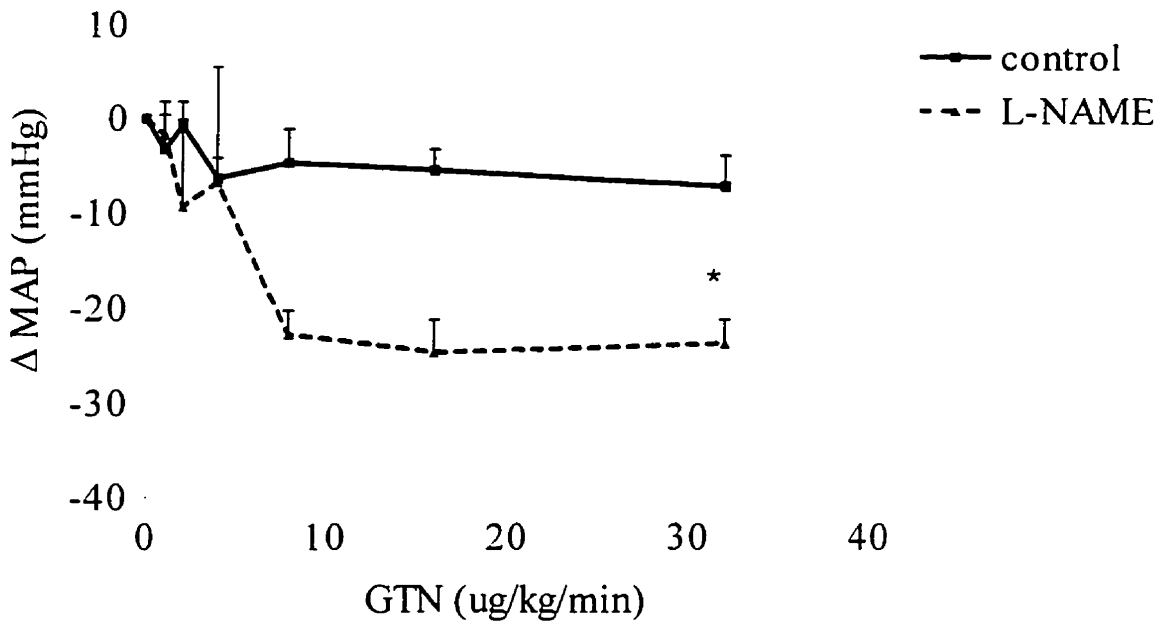

Figure 8-3: Enhanced glyceryl trinitrate dose response curve in rats treated with control and L-NAME, acutely.

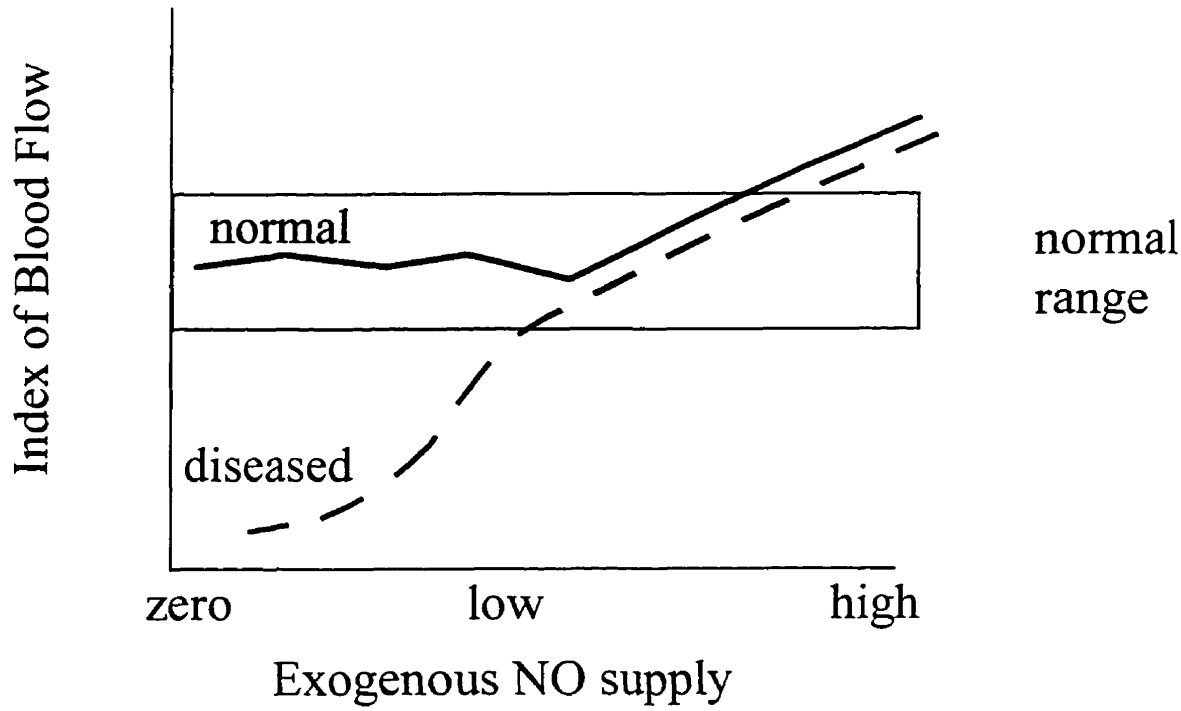

Figure 8-4: Schematic depicting the range of 'normal' vascular controlling window of NO with comparisons of control and L-NAME treated rats.

arterial pressure in the controls, almost completely reversed the NO synthase blockade induced hypertension (Figure 8-3).

Discussion:

The major findings of the present preliminary study were that: (i) the amount of NO required to completely restore mean arterial pressure in L-NAME treated rats did not lower mean arterial pressure in controls, (ii) specifically, this dramatic increase in the sensitivity to NO donors was revealed to have a 5-10 fold shift in the concentration required to provide any vasodilator response in the NO synthase blockade rats, (iii) the levels of NO required to reverse the hypertension in the chronic phase of L-NAME induced hypertension was the same levels required to reverse the hypertension following acute NO synthase blockade and (iv) the similar mean arterial pressure lowering, in both treated and controls, that occurred with the administration of 'high' concentrations of sodium nitroprusside suggests that the signaling processes were normalized at approximately 10-12 ug/kg per minute of sodium nitroprusside infusion.

Previously, the *in vivo* role of NO has been described as that of a chronic vasodilator, based largely on the development of a pressor response that occurs following NO synthase blockade. Banting *et al* (1996) has suggested that NO does not function as a chronic vasodilator, but rather a chronic inhibitory regulator of endothelin-mediated vasoconstriction. In their study, it was demonstrated that endothelin-mediated vasoconstriction does not contribute to the maintenance of vascular tone. Also, they demonstrated that almost the entire L-NAME induced hypertension was both prevented and/or reversed via the administration of an endothelin receptor antagonist. The present

study was quantitated the exogenous level of NO required to restore this balance. Taken together, we propose that NO functions to suppress endothelin-mediated vasoconstriction within a physiological 'window' (Figure 8-4).

The next phase of the investigation will entail characterizing the quantities of NO required to restore 'normal' vascular tone in L-NAME treated rats that have had tolerance induced to gylceryl trinitrate (via pretreatment with 2 x 0.2 mg/hr, changed every 24 hours, implanted subcutaneously). The result from a single trial of this protocol (results not presented) revealed (i) no blunting of the L-NAME induced pressor response, (ii) glyceryl trinitrate did not induce a lowering of mean arterial pressure (either before or after L-NAME treatment) at concentrations up to 124 ug/kg per minute (higher concentrations were not performed) and (iii) the mean arterial pressure lowering induce by infusions of SNP were identical to the non-GTN-tolerant rats, where there was a 5-10 fold shift in the quantity of SNP required to lowering mean arterial pressure in the L-NAME treatment phase in comparison to the control phase. Obviously it is difficult to speculate on an n=1, but this finding may indicate that the induction of tolerance to GTN does not alter the cellular NO requirements for 'normal' vascular function.

Chapter 9: General Discussion

The development of cardiac and vascular structural changes represents a significant independent risk factor with respect to cardiovascular associated morbidity and mortality. To date the majority of research efforts have focused on the contribution of neuro-humoral systems to the development of cardiac and vascular structural alterations, most noteably the sympathetic nervous system and the renin-angiotensin system. The present thesis embarked upon a series of experiments that have characterized the involvement of a locally mediated model of hypertension, the blockade of the endogenous production of nitric oxide. Also, in part, the present thesis examined the contribution of a sustained increase in mean arterial pressure alone as a causative trophic mechanism in the development of cardiac and vascular structural changes.

The experiments were modeled to fit within the conceptual framework believed to characterize the series of events that occur in the development and maintenance of hypertension (Folkow 1970). In this model, the progression of hypertension begins with an initiating event, followed by the up-regulation of a pressor and/or trophic response leading to the positive feedback interactions between pressure-dependent and pressure-independent alterations in cardiovascular structure and function (Figure 9-1). To this end, in order to fully understand the mechanisms underlying the hypertension associated with NO synthase blockade, a thorough understanding of the initiating and maintenance factors (vasoconstrictor mechanisms or structural alterations) involved in the hypertension

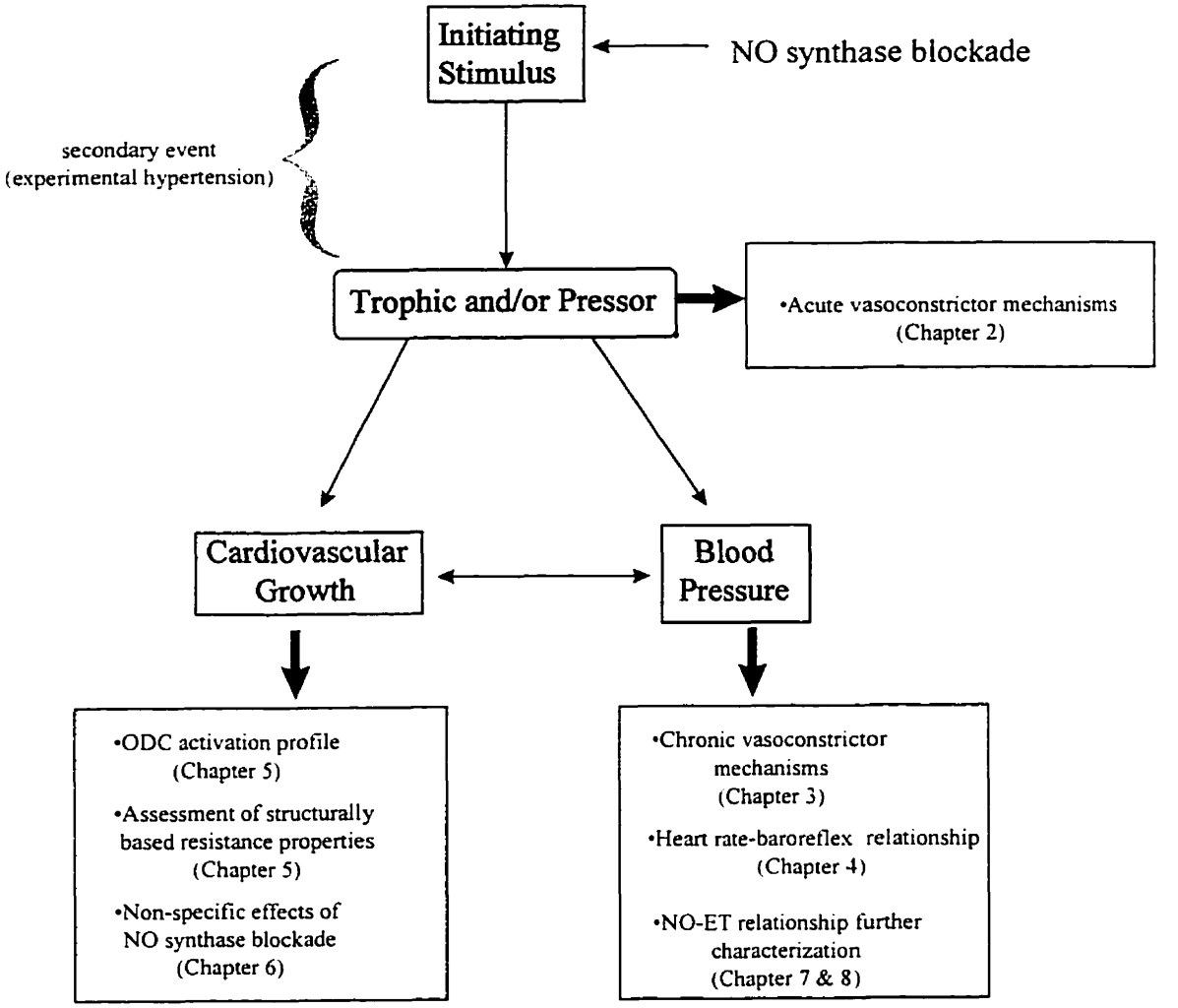

Figure 9-1: Summary of the studies completed with respect to the functional and structural changes that occur following acute and chronic NO synthase blockade. Studies are conceptualized within the framework developed by Folkow (1970).

represents a fundamental starting point in the characterization of this new model of hypertension.

Prior to specifically assessing any cardiac and structurally-based vascular resistance alterations that occur following NO synthase blockade, a series of experiments were designed to elucidate the role(s) of several functional circulatory control systems potentially involved in the initiation (Chapter 2, 4, 7 and 8) and maintenance (Chapter 3, 4, 8 and 9) of the hypertension following NO synthase blockade. These experiments were important for three reasons: (i) a general lack of consensus existed with respect to the vasoactive mechanisms underlying the hypertension induced by NO synthase blockade, (ii) the physiological role ascribed to nitric oxide has been based on 'negative' evidence (i.e. when the NO system is blocked mean arterial pressure increases, therefore NO must be a vasodilator), as opposed to specifically investigating the physiological players mediating the hypertension and (iii) an understanding of the functional systems involved in the development and maintenance of the NO synthase blockade induce hypertension allows for the targeting of specific system(s) for further investigation. In the present dissertation, the contribution of neuro-humoral and local vasoactive systems were assessed on a functional level, using a cumulative and sequential pharmacological receptor antagonist protocol (Banting *et al* 1996). This approach allowed for the calculation of both the absolute and relative contributions each of the vasoactive systems were making to the hypertension following acute (Chapter 2) and chronic (Chapter 3 & 4) NO synthase blockade.

The studies aimed at elucidating the vasoactive mechanism(s) underlying the hypertension (Chapters 2-4) clearly demonstrated a specific up-regulation of the involvement of a local system as opposed to changes in more global systems such as the sympathetic nervous system, the renin-angiotensin system and the heart rate-baroreflex relationship. These studies (Chapter 2 and 3) revealed that the pressor response following both acute and chronic NO synthase blockade was almost entirely due to enhanced endothelin-mediated vasoconstriction. These results immediately called in to question the concept that the hypertension following NO synthase blockade simply reflected a 'generalized' increase in sensitivity to the remaining vasoconstrictor systems. Banting *et al* 1996 and others (Pucci *et al* 1992, Bank *et al* 1994) have demonstrated that the contribution of neuro-humorally mediated vasoconstriction was the same or lower in the NO synthase blockade treated rats as compared to the contribution in the controls. Clearly, the hypertension following NO synthase blockade cannot be solely due to a 'generalized' increased sensitivity to the remaining systems. Further, these studies led to the development of the concept that the primary role for NO, with respect to circulatory control mechanisms was to inhibit endothelin mediated vasoconstriction (Figure 9-2).

Although this concept is supported by Richard *et al* (1995), the understanding that endothelin-mediated vasoconstriction represents the underlying mechanism of acute and chronic L-NAME hypertension has not been widely accepted. A study by Schiffrin *et al* (1995) demonstrated that the pre-pro endothelin-1 mRNA levels were not significantly

up-regulated in the blood vessels of rats treated for 3-weeks with L-NAME, as compared to controls. Also, in the present thesis, tissue measurements of endothelin-1 (via radioimmunoassay, data not presented) did not reveal enhanced levels in the blood vessels of rats treated with L-NAME in comparison to controls. These representative examples, taken together, would indicate a lack of involvement of endothelin-mediated vasoconstriction to the L-NAME induce pressor response. However, it has been demonstrated that endothelin-mediated vasoconstriction does not appear to play a role in the maintenance of mean arterial pressure under basal conditions (Banting *et al* 1996). This leads to the speculation that an increase in the activity of a system from basal to slightly above could be beyond the sensitivity limits of the current methods. As such the use of pharmacological receptor antagonists may provide the 'best' assessment of the role endothelin-mediated vasoconstriction is playing in other models of hypertension.

The specific endothelin receptors, either ET_A or ET_B or both, mediating the NO synthase blockade induce pressor response remains unclear. The ET_A receptor is known to reside on the surface of the vascular smooth muscle cell (Arai *et al* 1990), whereas ET_B receptors have been demonstrated to appear both on the surface of the vascular endothelium, as well as on the surface of the vascular smooth muscle cell (Sakurai *et al* 1990). The ET_A receptor has been demonstrated to mediate vasoconstriction via activation of diacyl glycerol and phosphadidyl inositol turnover, leading to increased activity of protein kinase C and the release of intra-cellular calcium stores (Arai *et al* 1990). The ET_B receptor has been demonstrated to potentially have a dual function, that

is ET_B receptors on the endothelium lead to the enhanced production of NO (Sakurai *et al* 1990) and ET_B receptors on the vascular smooth muscle cell may mediate vasoconstriction (the specific signal transduction mechanisms have not been investigated). In order to account for the contribution of endothelin-mediated vasoconstriction, the present studies utilized a combined ET_A/ET_B receptor antagonist, since using an ET_A antagonist alone may result in vasodilation via ET_B receptor activation and lead to an underestimation of the involvement of endothelin. A brief examination of this issue (Appendix 1) revealed that the blockade of ET_A receptors alone, did not blunt the acute NO synthase blockade in comparison to blockade of ET_A/ET_B receptors providing circumstantial evidence for a prominent role of ET_B receptor activation (an ET_B antagonist alone were not tested *in vivo* due to the prohibitive cost of these compounds). As well, some evidence has begun to surface regarding an overall differential efficacy of the endothelin receptor antagonists currently available. Minamino *et al* (1997) discussed the fact that one ET_A antagonist had the capacity to prevent the neointimal proliferation that occurs following balloon injury, while another ET_A antagonist did not. To this end, it could be proposed that blockade of endothelin receptors as assessed via infused endothelin may not reflect the appropriate method of testing the efficacy of an receptor antagonist. A potentially more physiologically relevant assessment of the efficacy of endothelin antagonism may result from assessments of the capacity of these antagonists to inhibit the pressor response that occurs following NO synthase blockade.

In the chronic phase of NO synthase blockade, endothelin-mediated vasoconstriction was demonstrated to be an important factor in the maintenance of this hypertension. In the chronic phase, the direct impact of endothelin-mediated vasoconstriction was demonstrated to be diminished, as compared to the involvement in the acute phase. Interestingly, however, endothelin-mediated vasoconstriction was shown to be playing an important indirect role in the chronic phase of this model via mediating a marked increase in sensitivity to α_1-adrenoceptor activation. As such, when the initial decrease in sympathetic drive that occurs with NO synthase blockade returns to normal levels, the net vasoconstriction becomes enhanced due to the 'priming' effects of endothelin. That is, normal levels of sympathetic drive will induce almost a 2-fold increase in sympathetically mediated vasoconstrictor activity in L-NAME treated rats as compared to control. Overall, the progression from the acute phase to the chronic phase involved a diminished direct involvement of endothelin, but an enhanced indirect involvement via a marked increase sensitivity to α_1-adrenoceptor activation.

Following the characterization of the functional control systems involved in the NO synthase blockade induced hypertension, the present thesis assessed the development of cardiac and structurally based vascular resistance changes. The activation of growth processes in the heart and blood vessels were determined using two methodological approaches: (i) acutely, via assessments of the activity profile of the growth related enzyme ornithine decarboxylase (ODC), and (ii) using left ventricle to body weight ratios

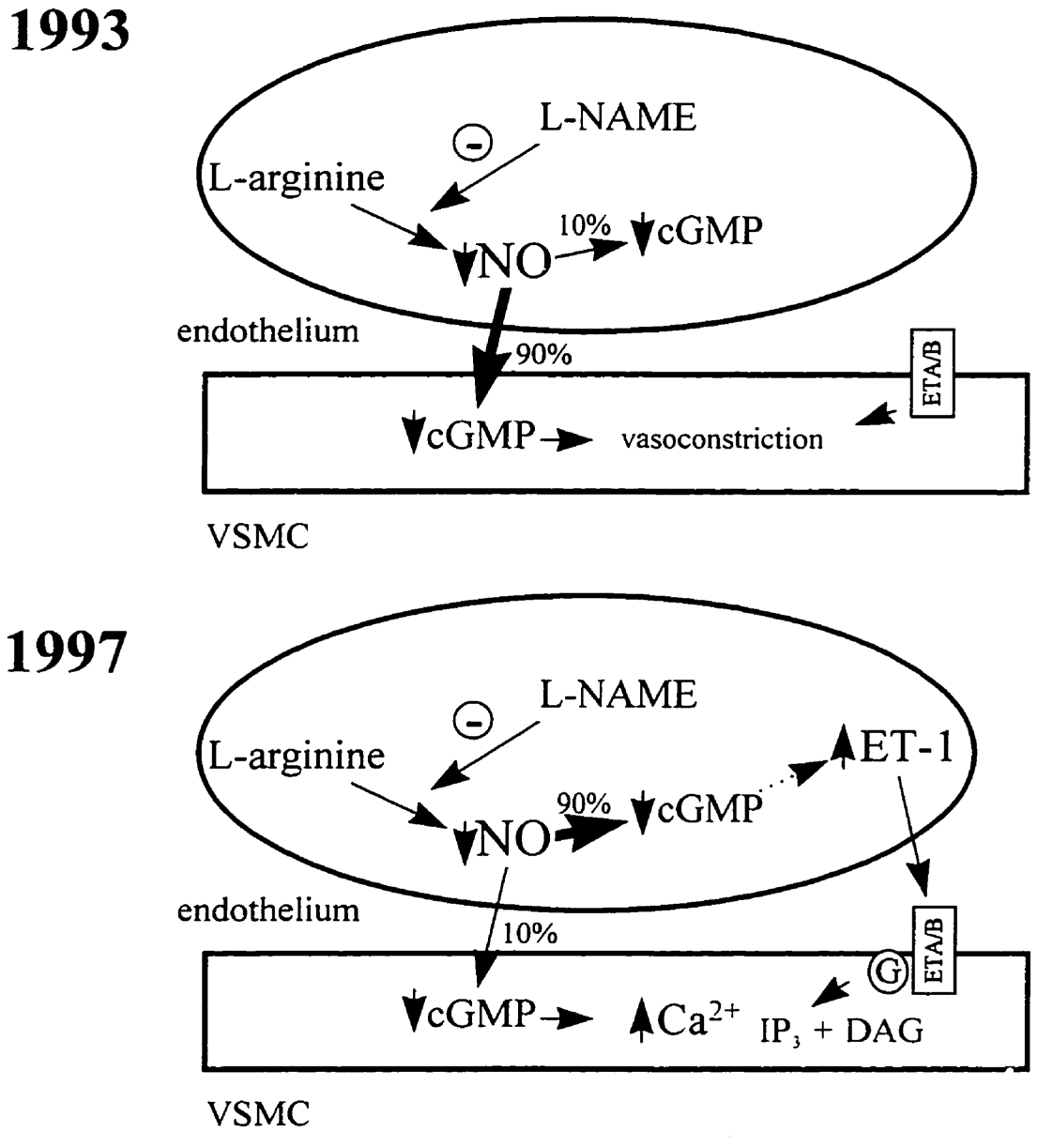

Figure 9-2: A summary of the concept proposed, regarding the chronic inhibitory role of NO on the vasoconstrictor activity of endothelin.

(heart) and hemodynamic assessment of vascular structure involving a perfusion based technique of measuring structurally-based vascular resistance properties.

The overall induction of cardiovascular growth processes, based on the activation profile of ornithine decarboxylase, was markedly blunted in the NO synthase blockade model in comparison to other equipressor models. For instance, the study by Banting *et al* (1997) plotted the ODC activation profile with L-NAME treatment together with the profile of angiotensin II infusion (Thompson *et al* 1997), where the markedly blunted profile can be easily observed. The transient increase in ODC activity (i.e. lack of sustained growth induction) observed in the present thesis (Chapter 5) is consistent with the finding that little to no cardiac and vascular hypertrophy occurred with chronic NO synthase blockade (Banting *et al* 1997). Although this result represents a 'negative' finding, it potentially provides important information with respect to the confounding effects of the presence of both hypertension and trophic factors in many experimental models, where in the present thesis these appear to have been separated. Overall, these results suggest several potential explanations for the lack of cardiovascular growth induction: (i) that a marked increase in mean arterial pressure, alone, is not a sufficient stimulus to induce cardiovascular growth processes, (ii) that up-regulation of endothelin, a known potent mitogen *in vitro*, does not induce cardiovascular growth processes *in vivo* or (iii) the NO synthase blocker L-NAME may in part have actions non-specifically to inhibit cardiovascular growth processes. Overall, the sustained hypertension that occurred with chronic NO synthase blockade produced a markedly blunted structural

adaptation as compared to other models of hypertension, such as chronic angiotensin II infusion, 2K-1C and DOCA salt (Figure 9-3). One of the potential explanations for the overall lack of cardiovascular growth induction, as mentioned above, could be the fact that the NO synthase blocker L-NAME may be inhibitory to the development of enhanced cardiovascular growth. This hypothesis was tested *in vitro* using a range of fetal calf serum concentrations as a growth stimulator on cultured aortic vascular smooth muscle cells (Chapter 6). The results of this study indicated that some level of growth inhibitory effects of L-NAME were observed, where relatively low levels (2.5% FCS) of growth stimulation markedly decreased the cellular proliferation rate, as compared to control. At higher levels of growth stimulus (5-10 %), however, L-NAME did not alter the proliferation rate of the aortic vascular smooth muscle cells. These results are similar to the findings of Schiffrin *et al* (1996), where L-NAME resulted in a decreased rate of protein and DNA synthesis following growth stimulation with IGF-1. In contrast, however, it was demonstrated that L-NAME treatment *in vivo* had the capacity to increase the activity of ornithine decarboxylase, at least acutely, indicating that the initiation phase of cardiovascular growth was not inhibited. Schiffrin *et al* (1995) has demonstrated that concomitant DOCA-salt treatment with L-NAME for 3-weeks resulted in a markedly decreased level of vascular hypertrophy as compared to DOCA-salt treatment alone. Alternatively, Moreau *et al* (1996) have demonstrated that concomitant two-kidney one-clip treatment with L-NAME for 4-weeks did not result in a blunted cardiovascular structural adaptation as compared to two-kidney one-clip treatment alone.

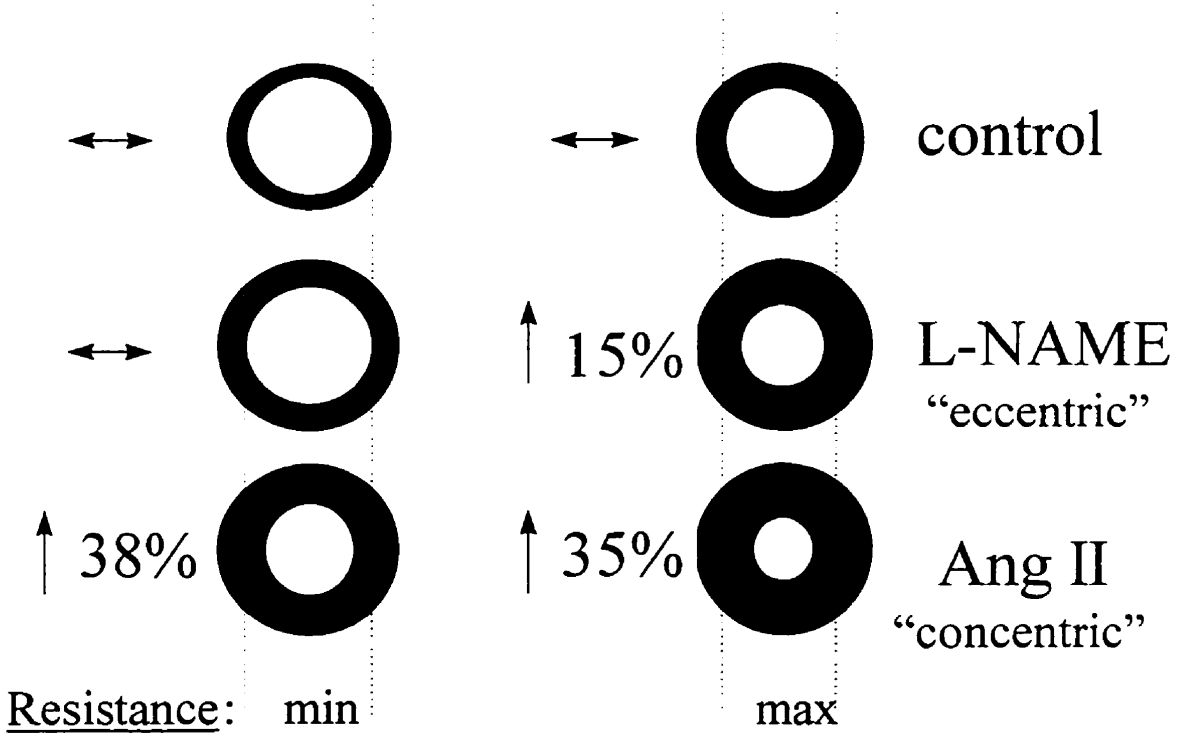

Figure 9-3: Summarizes the changes in vascular resistance properties that occur with 12 days of NO synthase blockade induced hypertension. The diagram illustrates the concept that the NO synthase blockade induced vascular were minimal as compared to 12 days of equipressor doses of Ang II (Thompson *et al* 1997).

This leads to the concept that L-NAME may be a partial inhibitor of cardiovascular growth processes, where stimulation via activation of the renin-angiotensin system (2K-1C) overrides the L-NAME inhibitory properties compared to growth stimulation via DOCA-salt treatment cannot surmount the L-NAME inhibitory properties. Although Schiffrin *et al* (1996) have suggested that the inhibitory effects of L-NAME on cardiovascular growth processes may be related to the anti-muscarinic properties of this compound, the specific mechanism(s) of this process remain equivical. Overall, the evidence from *in vitro* studies indicates that L-NAME may be an inhibitor of cardiovascular growth processes, however, it remains unclear if and to what extent this effect occurs *in vivo*.

Based on an overall lack of development cardiovascular structural alterations, the research in the present thesis turned the focus towards the further characterization of the relationship between NO and endothelin (Chapter 7 & 8). This relationship was also assessed under the controlled conditions of the isolated perfused pudendal vascular bed (Banting *et al* 1995). This technique provided access to a vascular bed in the absence of neural and hormonal influences. Chapter 7 revealed that the infusion of L-NAME, at concentrations that did not induce a change in perfusion pressure, resulted in a marked increase in sensitivity to α_1-adrenoceptor activation, consistent with the enhanced involvement observed *in vivo* (Chapter 3). Further these studies also demonstrated that sub-pressor concentrations of endothelin-1 also resulted in a similar marked increase in sensitivity to α_1-adrenoceptor activation, providing further supportive evidence for the

concept that even slightly enhanced levels of endothelin have the capacity to 'prime' α_1-adrenoceptor activation mediated vasoconstriction. The final study in Chapter 7 revealed that the markedly enhanced sensitivity to α_1-adrenoceptor activation was completely reversed following the administration of an ET_A/ET_B receptor antagonist. As such, the present series of studies (Chapter 2, 3 and 7) indicate that endothelin-mediated vasoconstriction, both directly and indirectly, plays an important role in the development and maintenance of the hypertension following NO synthase blockade.

Chapter 10: Summary and Conclusions

Overall the present series of studies demonstrated the important contribution(s) that local systems, such as endothelin, can make to the homeostasis of mean arterial pressure. The studies also indicated that a sustained increased in the level of mean arterial pressure alone, may not provide a sufficient stimulus to induce the development of cardiovascular structural alterations. In that, although a local vasoactive systems can play an important role in the regulation of mean arterial pressure, the development of cardiovascular hypertrophy likely requires that activation of a more potent growth stimulus, such as the renin-angiotensin system. Therefore, the imbalance of NO and endothelin are likely of significant importance in several peripheral vascular clinical pathologies including: renal failure, cyclosporin A induced hypertension and male erectile dysfunction, where therapies aimed at restoring the NO-endothelin balance may be able to reverse these conditions.

Application NO-ET relationship to male erectile dysfunction.

The incidence of male erectile dysfunction has been estimated to be anywhere from 20 to 70 million men world-wide. The specific etiologies of male erectile dysfunction are widely acknowledged to be multifactorial (Adams *et al* 1997). Several investigators have demonstrated that the activity of NO synthase decreases steadily with age and the onset of cardiovascular disease, persistent stress and vascular injury can enhance this degenerative process (Garban *et al* 1995, Elbbay *et al* 1995, Dunsmuir *et al* 1996, Vernet *et al* 1995). It is also widely understood that a sustained decreased blood

pO_2 levels (commonly found in the penis) can also lead to the decreased NO production (Kourembanas *et al* 1993). As such, the potential exists for multiple factors to be present in patients with erectile dysfunction that could lead to a decrease in the basal level of endothelial-derived NO production.

Based on the concept proposed in the present thesis, several mechanistic explanations for male erectile dysfunction in patients with at least partially inhibited levels of NO can be proposed: (i) as discussed above, the inhibition of endogenous NO production can lead to a marked increase in the levels of endothelin-mediated vasoconstriction, (ii) even slight enhanced levels of endothelin-mediated vasoconstriction can double or triple the net vasoconstriction from a given level of sympathetic drive (Chapter 3 and 7) and (iii) since endothelin has been demonstrated to be a potent trophic agent (Levin 1995, Luscher 1993), enhanced levels of endothelin may induce some level of vascular structural upregulation, leading to a more permanent increase in vascular resistance properties. These three events lead to a marked increase in the level of vascular tone in the pudendal vasculature. The initiation of an erection relies on a centrally mediated signal to relax the blood vessels leading to the penis, as well as relaxing the corporal smooth muscle cells. The imbalance in vascular tone created by a decrease in NO levels, based on the concomitant increase in endothelin-mediated vasoconstriction, may functionally 'cripple' the pudendal vascular bed, such that even the 'strongest' central initiated vasodilator signal will not induce an erection.

The application of the NO-ET relationship to erectile dysfunction has been tested in an animal model (Whittingham *et al* 1996, Appendix 2). Where the administration of the NO synthase blocker, L-NAME resulted in significant decrease in apomorphine-induce erections in rats. Interestingly, pretreatment with an ET_A/ET_B receptor antagonist prior to L-NAME treatment results in completely normal apomorphine induced erections. as compared to controls. As such, this preliminary data present some support of the concept that an imbalance of NO and ET may represent an important factor in developing new therapeutic strategies for the treatment of male erectile dysfunction.

Implications of the NO-ET relationship for cyclosporin A induced hypertension and nephrotoxicity.

The marked increase in the feasibility of multiple organ transplantation stems from the development of several potent immunosuppressant drugs, in order to prevent organ rejection (Opelz G *et al* 1991, Bennett and Pulliam 1983, Myers *et al* 1984). While this class of pharmacological agents have improved the success rate of organ transplantation, their widespread use has become severely limited due to serious nephrotoxic side effects (Powell-Jackson *et al* 1983). As a consequence of the renal toxicities, at least in part, a marked increase in mean arterial pressure occurs with immunosuppression treatment with cyclosporin A, which has also constrained the widespread use of these compounds (Textor *et al* 1994).

The overall mechanism(s) involved in the development of hypertension (and renal toxicity) remains equivocal, but generally accepted to be due to multiple factors. One of the side effects that has been demonstrated to occur with Cyclosporin A treatment is the inhibition of NO production. The hypertension associated with Cyclosporin A has been demonstrated to be due to markedly enhanced renal vasoconstriction (leading to a shift in the renal pressure-diuresis curve favouring volume conservation). Combining these two facts together with the concept that the inhibition of NO would lead to a marked up-regulation of endothelin-mediated vasoconstriction (Figure 10-1) may provide a mechanistic explanation for the development of hypertension. If this relationship were to hold true, two therapeutic strategies could be employed to at least partially reverse the Cyclosporin A induced hypertension. One approach would entail supplying a 'restorative' quantity of NO, as defined by Chapter 8 of the present thesis. That is, concentrations of a NO pre-cursor that do not alter normal mean arterial pressure should help to reverse the markedly enhanced renal vasoconstriction. In a preliminary study (Appendix 3), it was demonstrated that the markedly enhanced renal vasoconstriction (as observed by monitoring renal interstitial hydrostatic pressure) that occurred with cyclosporin A was completely reversed by the administration of L-arginine, at concentrations that do not alter mean arterial pressure under control conditions. Secondly, the administration of an endothelin receptor antagonist could also represent a potential strategy to reverse the hypertension following cyclosporin A treated. These studies have been performed where endothelin antagonism almost completely reversed

and/or prevented the development of cyclosporin A induced hypertension (Fogo *et al* 1992, Kon *et al* 1995, Phillips *et al* 1994).

Characterization of changes induced by substances such as L-NAME and cyclosporin A provides circumstantial evidence in support of the concept that the physiological role of NO, in terms of circulatory control, is not that of a chronic vasodilator, but rather a chronic suppressor of endothelin-mediated vasoconstriction. As well, the preliminary study on the mechanism(s) underlying cyclosporin A induced hypertension also provide further evidence in support of the concept that the quantity of NO required to restore normal vascular function are much lower than the quantities that lead to direct vasodilation under control conditions.

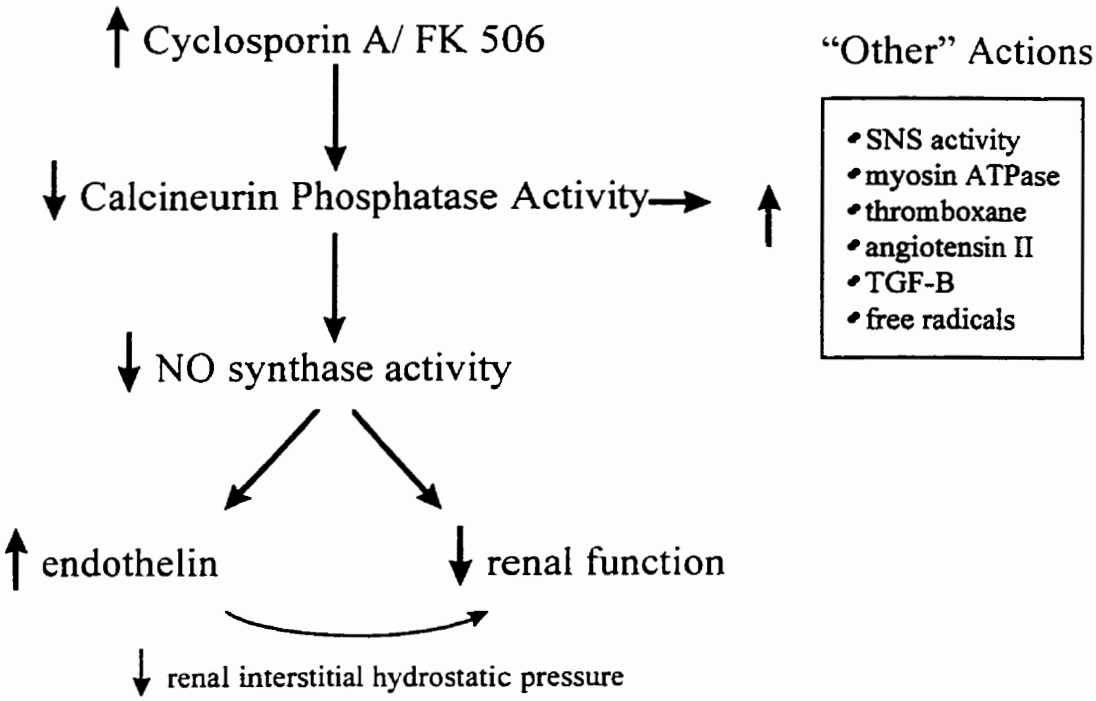

Figure 10-1: A conceptual diagram of the physiological impact of cyclosporin A or FK 506 treatment, with respect to the potential deleterious side effects such as hypertension and renal toxicity (adapted from Lundie *et al* 1997). Highlighted is the contribution, at least in part, of decreased production of NO synthase.

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Appendix 1

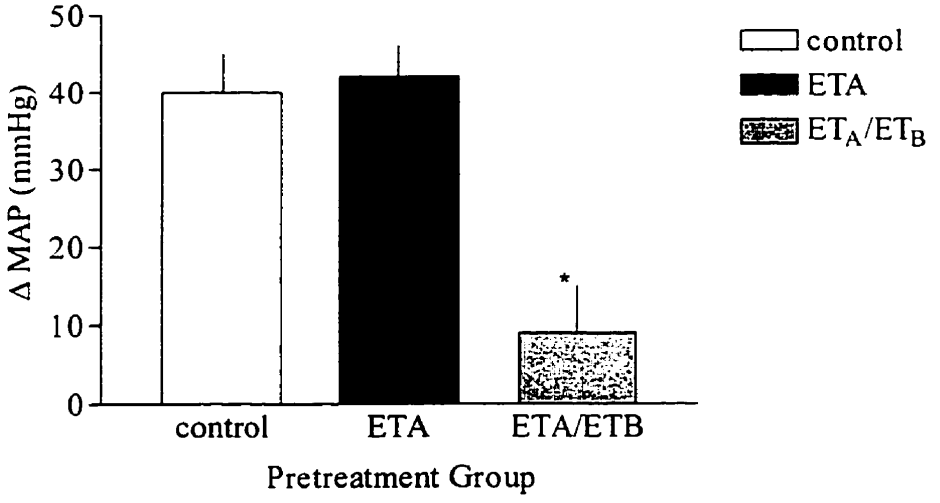

Figure A1-1: A comparison of the capacity for various endothelin receptor antagonists to inhibit the pressor response following acute NO synthase blockade with N^{ω}-nitro-L-arginine methyl ester (100 mg/kg, intraperitoneally). The ET_A receptor antagonist BQ-123 (0.15 mg/kg per minute, intravenously, solid black bar, n=3) did not attenuate the hypertension following acute NO synthase blockade. Whereas blockade of ETA/ETB receptors with PD 145065 (10 mg/kg per minute, intravenously, n=6, Banting *et al* 1996) almost completely blunted the NO synthase blockade induced pressor response. All mean arterial pressure measurements were recorded from conscious male Spraque-Dawley rats, previously instrumented with aortic and venous catheters. Values are expressed as the mean pressor response above control ± S.D., $p < 0.05$ was considered significantly different from control.

Appendix 2

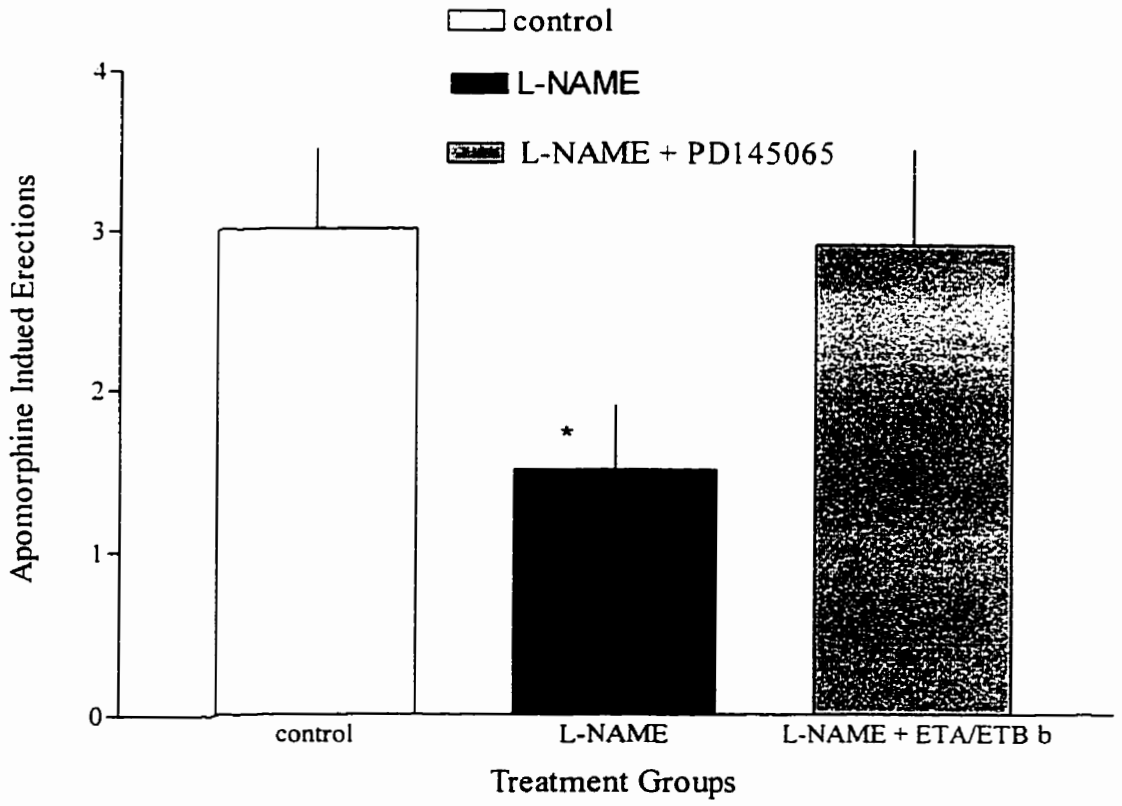

Figure A2-1: From Whittingham, Banting *et al* (1996), the reversal of N^{ω}-nitro-L-arginine methyl ester (100 mg/kg per minute, intraperitoneally) induced erectile dysfunction, as measured by a marked reduction in the number of apomorphine induced erections, as compared to control. Pre-treatment with the ET_A/ET_B receptor antagonist PD145065 completely prevented the L-NAME induced erectile dysfunction. Values are presented a mean number of erections following apomorphine administration (10 ug/kg, subcutaneously), erections were assess via a video surveillance system, ± S.D. $p<0.05$ was considered to be significantly different as compared to control.

Appendix 3

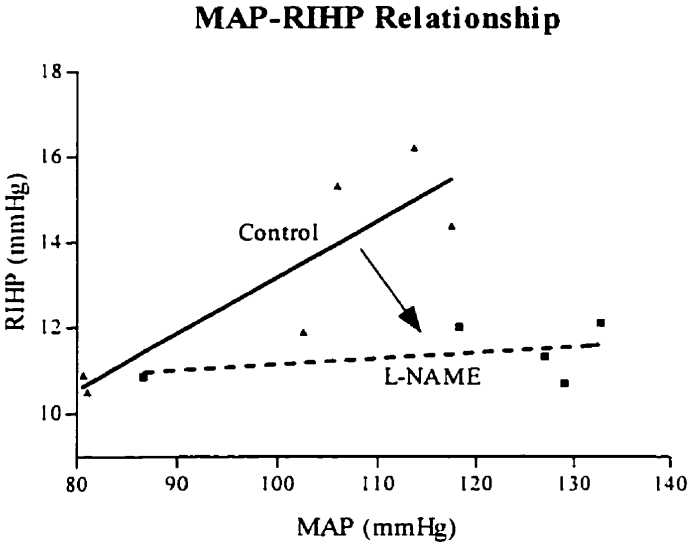

Figure A3-1: The linear regression through the mean arterial and renal interstitial hydrostatic pressure pairs that were generated via altering the level of mean arterial pressure by constriction and occluding the various arteries above and below the kidney. The administration of L-NAME (100 mg/kg per minute) resulted in a marked 'flattening' of the mean arterial pressure-renal interstitial hydrostatic pressure relationship. Based on fundamental pressure-naturesis/diuresis physiology, the 'flattening' of this curve with L-NAME treatment leads to a marked decrease pressure preventing fluid reabsorption, and as such creates a volume accumulation that likely plays at least a partial role in the hypertension following chronic (>4-weeks) of L-NAME induced hypertension.

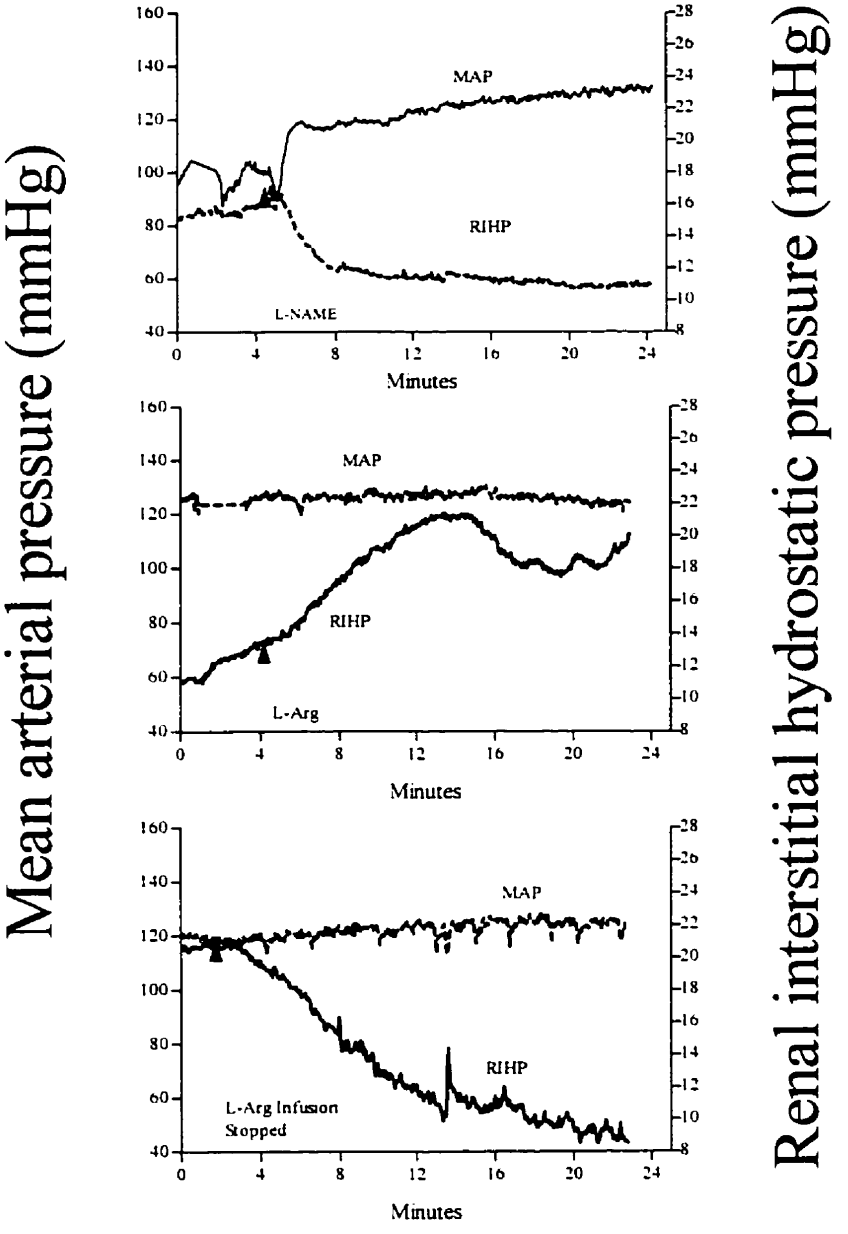

Figure A3-2: Representative tracings from a preliminary study into the mechanisms of renal vasoconstriction that occurs following NO synthase blockade or Cyclosporin A (data not shown) treatment. Upper panel reveals the marked decrease in renal interstitial hydrostatic pressure (RIHP) following blockade of NO synthase. Middle panel reveals the capacity of L-arginine, at doses that did not alter the level of mean arterial pressure, completely restored the NO synthase blockade induced decrease in RIHP. Bottom panel reveals that following the removal of L-arginine infusion, the severe decrease in RIHP returns. All recordings were made from anaesthetised male Sprague-Dawley rats.

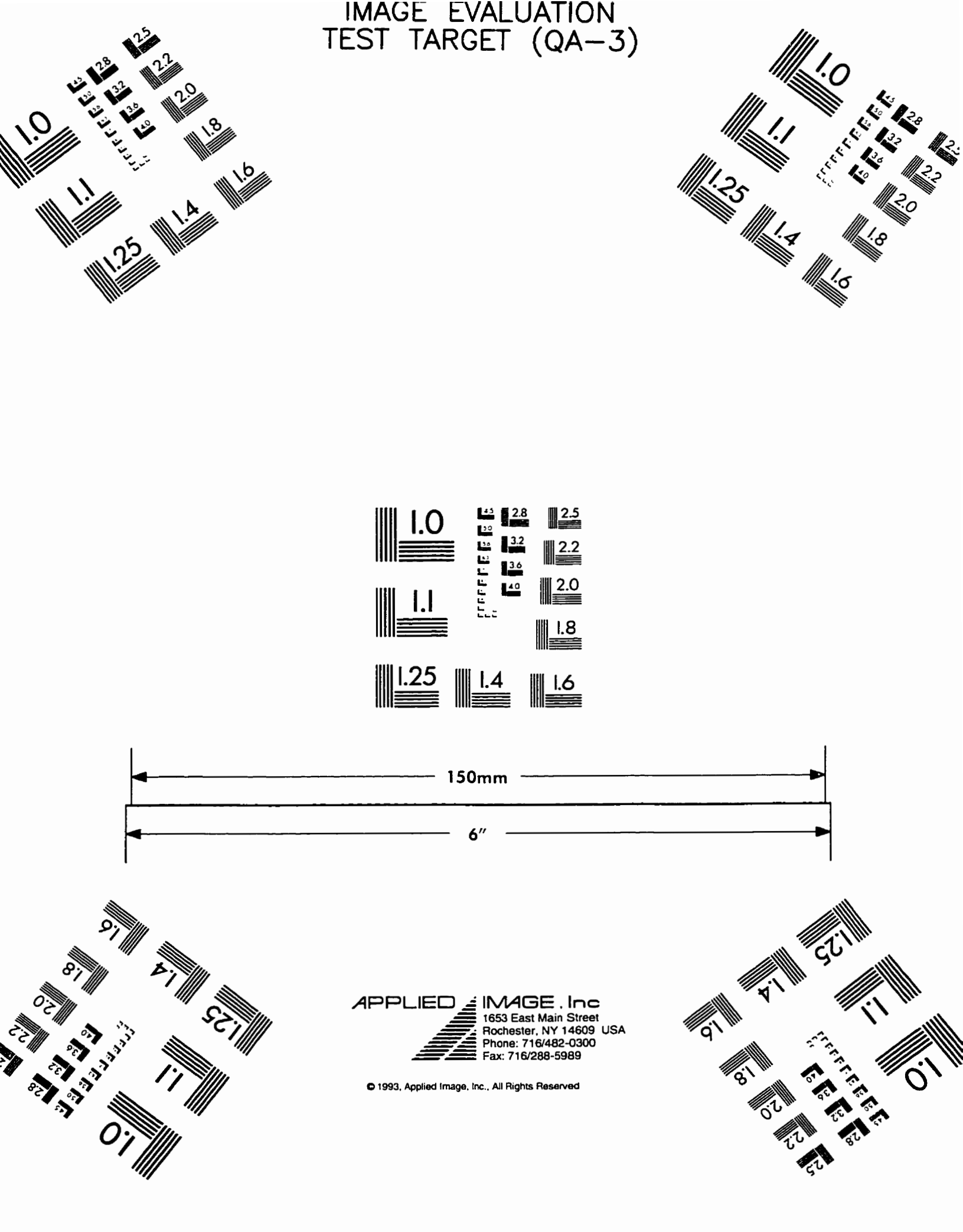
IMAGE EVALUATION
TEST TARGET (QA-3)
1.0
1.1
1.25
1.4
1.6
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