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The Role of Hyperinsulinemia, the Sympathetic Nervous System and Nitric Oxide in Cardiovascular Function During the Development of Obesity-Induced Hypertension.

Agatha T. Borne

Louisiana State University and Agricultural & Mechanical College

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THE ROLE OF HYPERINSULINEMIA, THE SYMPATHETIC NERVOUS SYSTEM AND NITRIC OXIDE IN CARDIOVASCULAR FUNCTION DURING THE DEVELOPMENT OF OBESITY-INDUCED HYPERTENSION

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in

The Interdepartmental Program in Veterinary Medical Sciences Through the Department of Veterinary Physiology, Pharmacology and Toxicology

by
Agatha T. Borne
D.V.M., Louisiana State University, 1989
May, 1998

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DEDICATION

This dissertation is dedicated to all animals used in research. They provide their bodies and lives for the benefit of mankind. May all investigators treat them with the respect and compassion they deserve: as their lives are shortened that ours may be prolonged. They cannot even understand.
ACKNOWLEDGMENTS

First and foremost, I would like to express extreme gratitude to my family, especially my parents Nettie and Leon. They instilled in their children the importance of a good education, and a desire to excel. They provided help and support throughout my extended education.

I would like to thank the Pennington Biomedical Research Center for continued support throughout my program. Their excellent facilities enabled me to accomplish my goals and perform very difficult research experiments. The Clinical Chemistry Lab (Dr. R. Tulley), the Body Composition Lab (Dr. J. DeLaney and Louis Melancon), the Biostatistics Department (Dr. J. Volaufova), and Dr. G. Smagin were always accommodating and helpful when asked to perform the various assays and procedures that made these experiments work. The aid I received from these people was invaluable.

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ABSTRACT

The interaction of hyperinsulinemia, the sympathetic nervous system (SNS) and nitric oxide (NO) was examined in lean and obese unilaterally lumbar sympathectomized dogs. Six lean and six obese dogs were studied while on a treadmill, during hyperinsulinemic euglycemic clamp (HIEC) procedures with and without concurrent nitric oxide synthase (NOS) inhibition with nitro-L-arginine (LNA). Changes in plasma insulin, femoral vascular resistance (FVR), heart rate (HR) and mean arterial pressure (MAP) were measured prior to and during a 7-week overfeeding period, and response to LNA administration was examined in the lean and obese state.

Body weight increased from 19.9 ± 0.70 to 29.7 ± 0.77kg and % body fat from 23.0% to 48.8% during overfeeding (OF). Heart rate, MAP and plasma insulin also increased during overfeeding. Femoral vascular resistance initially increased (p<0.01 in both the intact and denervated limbs), but returned to baseline levels by OF-week 3 in the denervated limb, and by week 6 in the intact limb. No significant correlation was evident between insulin levels and other cardiovascular parameters.

Hyperinsulinemic euglycemia alone did not affect MAP in either weight group. However, when hyperinsulinemia was initiated following NOS inhibition, MAP in the lean animals tended to decrease, whereas that of the obese dogs increased slightly. HR response to hyperinsulinemia (alone or concurrent with NOS inhibition) was not different between weight groups. The FVR response to hyperinsulinemia alone or with NOS
inhibition was not different between the weight groups. However, heart rate and vascular response to LNA was reduced in the obese dog.

In summary, sympathetic activity may influence the vascular response to weight gain. Also, hyperinsulinemia does not affect MAP, HR or FVR in lean or obese dogs, while walking slowly on a treadmill. However, when NOS is concurrently inhibited, lean and obese dogs show a tendency toward differential MAP and HR responses to hyperinsulinemia. Thus, the insulin and nitric oxide interaction may differ in lean and obese dogs, and may mediate development of hypertension in the obese population. Finally, the obese have a reduced cardiovascular response to NOS inhibition compared to the lean.
CHAPTER 1: INTRODUCTION

Obesity-induced hypertension is a significant clinical health risk. Approximately 25% - 50% of hypertensive people are obese (Hseuh and Buchanan, 1994), with the majority demonstrating significant reduction in blood pressure following weight loss (e.g., Weinsier et al., 1991). However, the exact mechanism by which obesity-induced hypertension develops has not been fully elucidated. In recent years, studies examining the mechanisms through which hypertension develops with obesity have concentrated on the role of hyperinsulinemia and insulin resistance (For reviews see Modan and Halkin, 1991; Hall et al., 1994; Landsberg, 1991). Both obese and non-obese hypertensive individuals are insulin resistant (Ferrannini et al., 1987), with significant correlation observed between circulating insulin levels and blood pressure. Despite these data, more recent studies report that hyperinsulinemia alone does not elevate blood pressure in either normal or insulin resistant dogs (Brands et al., 1991; Hall et al., 1990 & 1995). However, hyperinsulinemia does elevate arterial pressure in the rat (Meehan et al., 1994), and activation of the sympathetic nervous system (SNS) may be one mechanism through which insulin may promote elevations in arterial pressure (Daly and Landsberg, 1991). Thus, controversy remains as to the involvement of insulin/insulin resistance in obesity-induced hypertension.

There is a growing body of evidence that the obese state results in an altered pattern of sympathetic activity. Increased norepinephrine levels
have been observed in overweight hypertensive individuals (Rocchini et al., 1989), which could potentially mediate elevations in arterial pressure through increases in peripheral resistance and/or altered renal function precipitating fluid and sodium retention (DiBona, 1992). Acute elevations in insulin stimulate sympathetic activity in several species (Young et al., 1988; Liang et al., 1982; Rowe et al., 1981), and may be one mechanism through which elevations in arterial pressure occur (Daly and Landsberg, 1991). Still, others have shown vasodilation in skeletal muscle by insulin (for review see Baron, 1994). However, in insulin resistant subjects (e.g. obese), this response is reduced (Laakso et al., 1990). In addition, the response to vasoconstricting agents is enhanced in insulin resistant rats (Townsend et al., 1992). Thus, the obese insulin resistant state could predispose to changes in vascular resistance consistent with elevations in arterial pressure through alterations in SNS activity. Recent evidence suggests that altered sympathetic activity is essential for the development of obesity-related hypertension, via renal mechanisms (Kassab et al., 1994). However, the exact role sympathetic activity plays in the development and maintenance of obesity-induced hypertension remains obscure.

Another factor which may play a crucial role in the etiology of obesity-associated hypertension is nitric oxide. Nitric oxide (NO) has come under much scrutiny for its ability to cause vascular relaxation and reductions in peripheral resistance. It is also a potential modulator of nervous, renal and immune system function. Initial studies suggest that
the nitric oxide system may be important in various organ systems for normal physiologic functioning as well as in some disease states.

Since acute and chronic NO inhibition results in increased arterial pressure in several species, the role of NO as a possible mediator of cardiovascular hemodynamics and vascular responses in various models of hypertension has received much examination (Panza et al., 1993; Lockette et al., 1986; Koller et al., 1994). In 1990, Panza et al. described abnormal vascular relaxation in patients with essential hypertension, which was later found to be, at least in part, due to a defect in the NO system (Panza et al., 1993a, b). A potential role for the nitric oxide system in nervous tissue function has been explored. Studies suggest that in addition to modulating vascular resistance through peripheral mechanisms, NO may play a role in central regulation of sympathetic tone and/or renal nerve activity, while other experiments suggest that sympathetic activation is a modulator of NO release (Lacolley et al., 1991).

In addition to interactions with the nervous system, nitric oxide activity has also been studied with regard to its actions on insulin release, activity and sensitivity. Recent studies report that the vasodilating effects of insulin are mediated through the stimulation or synthesis/release of nitric oxide. (Scherrer et al., 1994, Steinberg et al., 1994). There is a suggestion in the literature that a positive relationship exists between endothelial nitric oxide production and insulin sensitivity (Petrie et al., 1996). This finding is supported in the study by Higashi, et al. 1997, in
which the increase in renal plasma flow, the decrease in renal vascular resistance and filtration fraction and the increase in cGMP in response to arginine infusion was reduced in essential hypertensive subjects. Also, these insulin resistant individuals showed a higher insulin response to L-arginine infusion compared to their normotensive, insulin sensitive counterparts. Thus, a relationship exists between insulin, insulin sensitivity and endothelial nitric oxide, and defects in any aspect of this system could be a potential mediator of hypertension observed in insulin resistant states such as obesity. However, these interactions have yet to be described in the obese.

Thus, our objectives were to examine the interaction between insulin, the SNS and nitric oxide in mediating femoral blood flow and vascular resistance in conscious lean and obese dogs. Also, since little information on the time course of vascular resistance changes and the onset of insulin resistance occurring with obesity is currently available in the literature, we followed these parameters over seven weeks of overfeeding and weight gain.

To study the interaction of insulin and sympathetic activity in mediating vascular resistance in the lean state and with the development of obesity, the hyperinsulinemic euglycemic clamp (HIEC) technique was utilized in lean and obese dogs with unilateral lumbar sympathetic denervation. To determine whether the nitric oxide system is also involved in the development of obesity-related hypertension through either interactions with the SNS and/or insulin, we examined the effects of nitric
oxide synthase inhibition on the cardiovascular responses during the HIEC in the same lean and obese dogs. Last, we examined whether changes in vascular resistance and hyperinsulinemia develop concurrently during the course of the development of diet-induced obesity, and whether the vascular changes were affected by sympathetic innervation. To procure these data, weekly standing and walking femoral blood flow and fasting insulin and glucose levels were monitored over the course of development of obesity in lumbar sympathectomized dogs. Thus, the time course for development of vascular resistance changes, hyperinsulinemia, and correlation between the two parameters could be determined.

We hypothesize that as insulin resistance develops with weight gain, insulin's effect to reduce vascular resistance is decreased. At this time, increases in SNS activity associated with obesity alter peripheral vascular resistance, laying a foundation for hypertension to develop. We also hypothesize that the increase in SNS activity which develops with weight gain inhibit the activity of vascular nitric oxide synthase (NOS), thereby predisposing to hypertension in the obese. Additionally, this reduction in nitric oxide activity may influence cardiovascular responses to insulin and influence arterial pressure, since there is suggestion in the literature that nitric oxide may mediate the vascular effects of insulin (Steinberg et al., 1994, Scherrer et al., 1994).
CHAPTER 2: REVIEW OF LITERATURE

Along with atherosclerotic vascular disease and certain cancers, the risk of hypertension is increased with obesity (Kannel et al., 1967). In addition, obesity is reported as an independent predictor of stroke, cardiovascular death, coronary artery disease and congestive heart failure (Hubert et al., 1983). Hypertension itself is a major risk factor for coronary heart disease, stroke and congestive heart failure (Kannel et al., 1972 and 1976; Pooling Project, 1978). Thus, obesity-induced hypertension is a significant clinical health risk afflicting humans.

Approximately 25% - 50% of obese individuals are hypertensive (Hseuh and Buchanan, 1994), with the majority demonstrating significant reduction in blood pressure following weight loss (for example, Weinsier et al., 1991). Although the exact mechanisms by which hypertension develops in the obese remain obscure, increases in cardiac output and blood volume, insulin resistance and renal sodium retention are reported to occur concurrent with weight gain and the development of hypertension in both humans and the canine model (Alexander et al., 1962; Mujais et al., 1982; Rocchini et al., 1987 and 1989a, b, c). Also, the acute response to sodium loading, the pressor response to angiotensin II, and catecholamine levels are altered in obese hypertensive human patients and in animal models (Rocchini et al., 1989a, b, c and 1990; West et al., 1991; Rowe et al., 1981; Tuck et al., 1992).

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For many years, studies examining the mechanisms of obesity-related hypertension concentrated on the role of hyperinsulinemia and insulin resistance (For review see Modan et al., 1991; Hall et al., 1994; Landsberg, 1986). Both obese and non-obese hypertensives are insulin resistant (Ferrannini et al., 1987), with significant correlation between circulating insulin levels and blood pressure. In addition, strong correlation has been identified between hypertension and insulin resistance in several disorders (non-insulin dependent diabetes mellitus [NIDDM], obesity), and insulin infusion in rats elevates arterial pressure (Qadir et al., 1996). Thus, hyperinsulinemia and insulin resistance were hypothesized to be primary factors in the development of hypertension in the obese, through either direct effects on renal Na\(^+\) absorption or via insulin-induced activation of the sympathetic nervous system (SNS) with resultant changes in vascular resistance and/or renal sodium handling. However, a growing body of evidence suggests that the obese state results in an altered pattern of sympathetic activity to peripheral resistance vessels and the kidneys, which is essential for the development of obesity-related hypertension (Kassab et al., 1994). Still, the specific mechanisms through which hypertension develops with weight gain remain obscure, and investigators now are considering the involvement of the nitric oxide system as an additional piece to the insulin and hypertension puzzle.

Over the time course of research into the cause of obesity-induced hypertension, the hyperinsulinemia and insulin resistance associated with
this disorder has received a vast amount of interest. This interest was sparked by observations that insulin resistance and glucose intolerance were correlated with the development of hypertension in both obese and non-obese subjects (Bonora et al., 1987; Lucas et al., 1985; Modan et al., 1985; Ferannini et al., 1987; Manicardi et al., 1986; Rocchini et al., 1987 and 1989a). In 1986, Lewis Landsberg published a schematic of direct and indirect mechanisms through which hyperinsulinemia/insulin resistance could potentially result in hypertension with obesity (Figure 1).

In short, with weight gain, insulin levels increase during the development of insulin resistance (primarily skeletal muscle insulin resistance). As this occurs, elevated insulin stimulates the SNS resulting in increases in peripheral and/or renal vascular resistance and heart rate, predisposing to hypertension. Along with these changes, the increase in insulin could directly alter renal sodium handling, thereby altering whole body fluid balance and potentiating increases in blood pressure. Thus, in this model, there are several mechanisms warranting experimentation to decipher the pathway(s) responsible for the elevation in arterial pressure associated with weight gain. However, all revolve around hyperinsulinemia/insulin resistance as the primary/inciting factor.

Contradictory to this hypothesis, insulin has been shown to produce vasodilatation in skeletal muscle vasculature, with associated increases in blood flow (For review see Baron, 1994). This, intuitively, should tend to reduce MAP. However, in obesity this response is impaired (Laakso et al.,
In 1993, Feldman and Bierbrier reported decreasing vascular sensitivity to insulin with increasing body mass in men. Also, the potency of insulin as a venodilator was reduced 79% in the mildly hypertensive subjects of this study. In addition to the above findings, the vascular response to vasoconstrictive agents has been shown to be enhanced in insulin resistant rats (Townsend et al., 1992). Thus, although insulin has been shown to increase blood flow and reduce vascular resistance, changes
in tissue response to insulin in obesity, in conjunction with altered
sympathetic activity, could mediate the development of obesity-induced
hypertension. In addition, the influence of insulin and the SNS in the
control of peripheral vascular resistance or renal function may vary over the
time course of the development of obesity-induced hypertension (Kraegen et
al., 1991; Penicaud et al., 1987). Insulin sensitivity of the kidneys and
skeletal muscle changes during the development of obesity. Thus, as
insulin resistance develops in the vasculature and kidneys, sympathetic
activity may become the primary factor mediating increased peripheral
vascular resistance and/or renal sodium retention during the
development/maintenance of obesity.

In studies to further describe mechanisms through which insulin may
influence arterial pressure, the sodium retaining effects of insulin have been
clearly demonstrated in vitro using isolated dog kidneys (Baum et al., 1987;
Nizet et al., 1971) and renal micropuncture techniques (DeFronzo et al.,
1976). These studies suggest that insulin has direct effects in vitro to
promote sodium reabsorption across the thick loop of the ascending limb of
Henle and the proximal and distal renal tubules. In vivo, these effects could
predispose to the development of hypertension in the obese, as obesity is an
insulin resistant/hyperinsulinemic and volume-expanded state, with
increased cardiac output and sodium retention (Rocchini et al., 1987 and
1989a, b, c). Although these vascular and renal effects of insulin may be
important factors involved in the development of hypertension in the obese, Hall and colleagues (1990, 1991 and 1995; Brands et al., 1991) have shown that hyperinsulinemia alone does not result in elevated blood pressure in the dog. Thus, the development of insulin resistance and interactions with other systems such as the SNS or nitric oxide are likely to be important factors which mediate the development of obesity-induced hypertension.

The development of insulin resistance in different tissues such as the kidney, liver and skeletal muscle is thought to occur at different rates (Kraegen et al., 1991; Penicaud et al., 1987). Hence, the physiologic responses of these tissues, and their relationship to circulatory/arterial pressure control, may vary over the course of the development of hypertension in the obese.

Recent evidence in the obesity-induced hypertensive dog model suggests that renal innervation is a primary mechanism required for the development of hypertension (Kassab et al., 1994). In this study, renal denervation attenuated the development of increased arterial pressure with the development of obesity. Another study by this same group (Kassab et al., 1995) suggests that the renal sympathetic activity may be important during the development of obesity-induced hypertension, since basal urine sodium excretion of innervated kidneys of obese dogs was significantly lower than that of the lean control animals. This difference was abolished using bilateral renal denervation in the obese dogs, but denervation had no effect on basal urinary sodium excretion in the lean controls. These data
support the idea that renal sympathetic activity may be activated and responsible, in part, for the development of obesity-associated hypertension. However, increases in sympathetic activity could also predispose to increased arterial pressure in the obese patient through alterations in peripheral vascular resistance (DiBona, 1992). Insulin has been shown to have multiple effects on the sympathetic and cardiovascular systems that could potentially result in hypertension. Thus, the interaction of insulin and these systems should be considered in the etiology of obesity-related hypertension.

For years the sympathetic nervous system has been suggested as a possible mechanism for hypertension. Increased SNS activity is reported to occur in obesity-related hypertension, as increased circulating norepinephrine levels have been observed (Rocchini et al., 1989a, b, c). Increased SNS activity may mediate elevations in arterial pressure in the obese through either increases in peripheral vascular resistance or renal mechanisms (DiBona, 1992). Changes in sympathetic activity may be an integral mechanism through which hypertension develops with obesity, since Kassab et al. (1994) provided evidence that renal innervation is crucial for the development of hypertension in diet-induced obesity in the canine model. Investigation into the interaction of insulin and sympathetic activity as a mechanism for hypertension has been fueled by the finding that insulin infusion causes increased heart rate and cardiac output, and elevations in circulating norepinephrine (Viberti et al., 1981; Rowe et al.,
Studies such as these lay groundwork for the thought that hyperinsulinemia in insulin resistant obese patients may stimulate SNS activity, which may then increase vascular resistance or reduce renal sodium and water excretion, predisposing to hypertension (Figure 1). Numerous studies using the hyperinsulinemic euglycemic clamp in rats (Young, 1988), dogs (Liang et al., 1982) and humans (Rowe et al., 1981) demonstrate that acute elevations of insulin will stimulate sympathetic activity. Assessment of circulating norepinephrine levels, changes in regional blood flow, or direct nerve recordings were examined as measures of SNS activity. In a recent study using microelectrode nerve recordings during euglycemic hyperinsulinemia, muscle sympathetic nerve activity increased significantly during insulin infusion at both normal and supra-physiologic insulin levels (Berne, et al., 1992). In this study, hyperinsulinemia was associated with increased vasoconstrictor nerve activity to skeletal muscle, but blood pressure was not notably affected. This suggests a direct stimulatory effect of hyperinsulinemia on vascular sympathetic activity, without a significant associated arterial pressure increase. Another recent study demonstrated that sympathetic nerve hyperactivity precedes the appearance of hyperinsulinemia and MAP elevations in a non-obese population (Masuo, 1997). Thus, the mechanisms through which hypertension is mediated, and how they are altered in insulin resistant and/or hypertensive states such as obesity, may be closely related to sympathetic activity. The importance of SNS activity in
hypertension is gaining more support, as a recent study in humans (Facchini, 1996) reported that increased heart rate and insulin resistance are highly correlated in humans, and this increase in heart rate (presumably reflecting increased cardiac output) is consistent with insulin-induced increases in sympathetic activity. However, other studies have previously reported that the increase in heart rate observed with obesity is the result of a reduction in parasympathetic nervous system activity controlling heart rate (Truett et al., 1996; Hirsch et al., 1991). Using microneurography, Anderson et al. (1991 and 1992), reported that physiologic increases in plasma insulin elevate sympathetic outflow in healthy and borderline hypertensives, but produce vasodilation and do not elevate blood pressure. Lembo et al., (1992) reported similar basal glucose uptake in normal and hypertensive subjects, with considerable increases in glucose uptake in response to insulin. However, the glucose uptake levels attained in the hypertensive group were significantly lower than those of the controls. During the hyperinsulinemic period of these studies, forearm norepinephrine (NE) release increased by 35% in normotensive patients. This was considerably greater in the hypertensive group, who exhibited a 140% increase in NE release. Nevertheless, the mechanism by which insulin induces muscle sympathetic overactivity is not yet completely understood. Suggested hypotheses include action on the central nervous system (CNS: ventromedial hypothalamic neurons), an indirect baroreflex-mediated action and a direct effect on muscle (for review, see Lembo et al.,
1996). The majority of data suggests that the effect of insulin on muscle NE is mediated through either central mechanisms or peripheral reflexes (Lembo et al, 1996 for review). However, one must consider that the clamp procedure itself, with only saline infusion, is reported to increase norepinephrine levels without elevating blood pressure (Moan et al., 1995). This questions whether changes in SNS activity described in previous clamp studies were a result of the experimental procedure, or insulin, per se. This requires further study. Regardless, the studies mentioned suggest that alterations in autonomic/sympathetic activity which occur in obesity may be a more important mediator of hypertension in the obese than originally implied, and the mechanism may involve interaction with insulin. Also, these pathways may involve interaction with other vasoactive factors such as nitric oxide, as one study reports that insulin-induced vasodilatory responses are not mediated by beta-adrenergic or cholinergic mechanisms, and are not markedly affected by alpha-adrenergic mediated mechanisms (Randin et al., 1994).

Although much study has examined the influence of insulin on sympathetic activity, the sympathetic nervous system has also been explored as a possible modulator of insulin-induced glucose uptake. This question is much more difficult to explore, since norepinephrine (NE) is a neurotransmitter, and circulating levels only poorly reflect synaptic cleft concentrations (Lembo, 1992). The idea that NE may potentially alter insulin action is suggested by the observation that in various models of
complete sympathectomy, the normalization of glucose from insulin-induced hypoglycemia is impaired (Sacca et al., 1977; Schacter et al., 1951; McDonough, 1939). This does not occur with reduced epinephrine levels, suggesting a metabolic role for NE (Gerich et al., 1979). In 1994, Lembo and colleagues directly tested whether NE release was able to provoke an impairment in insulin sensitivity measured using the insulin euglycemic clamp technique. They observed that when insulin was infused during lower body negative pressure (LBNP; a technique used to stimulate baroreflex activation of NE release by applying negative pressure to the body below the iliac crest using a pressurized airtight chamber) the increment in forearm NE release was greater than in control LBNP studies (Lembo et al., 1994a). Also, forearm glucose uptake was activated during insulin infusion in control and LBNP patients, but the values obtained during LBNP were 40% lower than during the control study. Additional data supporting these observations has been presented by others (Jamerson et al., 1993 and 1994). Possible mechanisms by which LBNP impairs insulin action on muscle glucose uptake are either via hemodynamic mechanisms leading to reduced perfusion or through direct metabolic effects of NE at the cellular level (For review see Lembo, 1996). The first mechanism is supported by a report that there is a reduced effect of insulin to stimulate skeletal muscle blood flow in obese men (Laakso et al., 1990). However, Capaldo et al., 1991 found that although skeletal muscle is a major site of insulin resistance in essential hypertension, the defect is
independent of muscle blood flow. In a separate study, impaired insulin-mediated augmentation of blood flow in obese NIDDM patients was attributed to the diabetic state rather than the obese status (Laakso et al., 1992). Other studies, however, also suggest increased sympathetic drive as a primary influence in hypertension (Egan, 1989; Julius, 1988; Reis, 1988). Thus, sympathetic activity may influence insulin-mediated vascular responses as well as contribute as a primary factor to precipitate hypertension in the obese.

Insulin modulation of sympathetic-mediated vasoconstriction is another avenue through which these systems may predispose to hypertension, and has incited much interest and scrutiny. In two separate studies, a marked increase in sympathetic activity (measured by norepinephrine levels and/or muscle sympathetic nerve activity) evoked by insulin did not produce changes in arterial pressure or forearm vascular resistance in humans (Anderson et al., 1991; Lembo et al., 1992). These observations support the hypothesis that insulin per se is able to blunt sympathetic-induced vasoconstriction. In one study, fat-fed dogs exhibited a loss of coronary artery vasodilation to local hyperinsulinemia (Rocchini et al., 1996). This is consistent with previous work showing reduced glucose uptake in essential hypertensive individuals (Natali et al, 1991), and the observation that this reduction may be due in large part to an impairment in the action of insulin to increase skeletal muscle blood flow in obesity and
hypertension (Baron et al., 1988; Laakso et al., 1992; Baron et al., 1991). Investigations using LBNP and insulin infusion found that increases in forearm vascular resistance normally observed with LBNP are blunted during insulin infusion (Lembo et al., 1993). Biochemical studies have demonstrated that insulin interferes with the $\alpha_2$-adrenergic receptor-mediated vascular effects (Devedjian et al., 1991), while additional findings seem to suggest that it selectively interferes with transduction of the $\alpha_2$-vasoconstrictor signal (Lembo et al., 1994b). A growing body of evidence proposes that insulin's hemodynamic effects may be mediated by enhanced endothelial function (Steinberg et al., 1994; Scherrer et al., 1994). Thus it is reasonable to propose that changes in the interaction of insulin, endothelial derived vasoactive factors (such as nitric oxide) and the SNS may modulate vascular effects, thereby influencing the development of hypertension with weight gain.

Nitric oxide (NO) is one vasoactive factor which may play a crucial role in the complex relationships influencing vascular changes predisposing to obesity-associated hypertension. In recent years, nitric oxide has come under much scrutiny for its ability to induce vascular relaxation and reductions in peripheral resistance, and as a potential modulator of nervous, renal and immune system function. Initial studies suggest that the nitric oxide system may be important in various organ systems for normal
physiologic functioning, as well as having an instrumental role in various disease states.

Nitric oxide has been identified as the major functional component of, or is identical to, the local vasodilator endothelial-derived relaxing factor (Palmer et al., 1987; Ingarro et al., 1987). It is a nitroso compound derived from the local oxidation of L-arginine (Palmer et al., 1988a, b; Sakuma et al., 1988) by the enzymatic actions of nitric oxide synthase. Nitric oxide synthase has been located in various body tissues in several isoforms which have been purified and characterized (Forstermann et al., 1991). Nitric oxide is reported to exist as either a constitutive or inducible enzyme in many cells and tissues, including vascular endothelial cells, macrophages, hepatocytes, the cerebellum and cerebrum, the pituitary gland, the nucleus tractus solitarius and the kidneys, to name a few (Forstermann et al., 1991; Moncada et al., 1991; Bredt et al., 1990). All isoforms require oxygen, NADPH and 5,6,7,8-tetrahydrobiopterin as cofactors, with some variation existing between isoforms as to other factor requirements and the nature of the isoform as either a particulate or soluble type of enzyme (Forstermann et al., 1991).

Competitive inhibition of NO synthesis has been achieved using L-arginine derivatives such as L-\(\mathrm{N}^\mathrm{\text{G}}\)-monomethyl arginine (Palmer et al., 1988b; Sakuma et al., 1988). The potency of inhibition obtained using L-arginine derivatives can vary, depending upon the tissue studied and the
drug used. $N^G$-nitro-L-arginine was found to be more potent than $N^G$-methyl-L-arginine in inhibiting NO synthesis in the brain and endothelial cells, but less potent in induced macrophages (Lambert et al., 1991; Gross et al., 1990). Differences in potency between inhibitors were also demonstrated in a study by Okumura et al., 1992, in which $N^G$-nitro-L-arginine (LNNA) was more potent in eliciting renal vasoconstriction than was $L-N^G$-monomethyl-arginine (LNMMA).

Endothelial NO acts via the stimulation of soluble guanylate cyclase to increase intracellular guanosine 3′, 5′-cyclic monophosphate (cGMP), thereby inducing smooth muscle relaxation (for review see Bassenge and Heusch, 1990; Moncada et al., 1991). Basal/continuous release of NO occurs in the vascular endothelium, but a stimulated release may be initiated by several vasoactive substances such as acetylcholine or bradykinin, or by mechanical stimuli such as pulsatile flow or shear stress (Bassenge/Heusch, 1990; Moncada et al., 1991; Rubanyi et al., 1986; Rees et al., 1989). These characteristics of NO make it ideally suited for a role in acute and/or chronic modulation of blood pressure and vascular resistance.

The vasorelaxing properties of NO have been directly demonstrated in several in vitro preparations (Moncada et al., 1991; Komori et al., 1988). Recent in vivo studies have shown that NO synthesis inhibition using L-arginine derivatives results in elevations of arterial pressure in dogs (Manning et al., 1993 and 1994; Elsner et al., 1992), rats (Perrella et al.,
1991; Ribiero et al., 1992) and rabbits (Rees et al., 1989). With NOS inhibition total peripheral resistance increases as does renal vascular resistance, with decreased renal plasma flow, urine flow and sodium excretion, but little change in glomerular filtration rate (Elsner et al., 1992; Baumann et al., 1992; Majid and Navar, 1992). However, in studies by Salazar et al. (1992 and 1993), low doses of the nitric oxide synthase inhibitor $N^\sigma$-nitro-L-arginine-methyl ester (L-NAME) infused in dogs for 3 or 5 days caused renal vasoconstriction, but did not result in hypertension unless sodium intake was increased (Salazar et al., 1993). The results of a study by Granger and colleagues (1992) comparing intrarenal versus systemic effects of NO blockade indicate that the renal effects of L-NAME are largely secondary to extrarenal effects of NO inhibition, and speculations were made as to a possible role of SNS activation. Thus, since reduced sodium excretion and increased extracellular fluid volume have been reported to occur with obesity-induced hypertension in dogs, and increased catecholamine levels have also been suggested to occur with obesity (Tuck et al., 1992; Rocchini et al., 1989b), the function of the nitric oxide system deserves study as a possible mediator of these changes during the development or maintenance of hypertension in the obese.

The role of NO as a mediator of cardiovascular hemodynamics and vascular responses in various models of hypertension has received much examination, as acute and chronic NO inhibition results in increased arterial pressure in several species, (Lockette et al., 1986; Panza et al.,
1993a, b; Koller and Huang, 1994). Using three models of hypertension in the rat, Lockette et al. (1986) showed a loss of endothelium-dependent relaxation during development of hypertension, which they felt was related to the elevated blood pressure. In 1990, Panza et al. described abnormal vascular relaxation in patients with essential hypertension, whose forearm response of blood flow and vascular resistance to acetylcholine (ACH), but not sodium nitroprusside, were significantly lower than controls. This impaired response to endothelial-derived vasodilators and the increased basal vascular resistance in essential hypertension was later found to be due, at least in part, to a defect in the NO system (Panza et al., 1993a, b), but not related to decreased availability of substrate for NO synthesis (Panza et al., 1993b). Diabetic subjects (NIDDM) are reported to have attenuation of blood flow, which is reportedly due to either increased inactivation of nitric oxide or decreased reactivity of the vascular smooth muscle to NO (Williams et al., 1996). In the spontaneously hypertensive rat (SHR), reductions in arteriolar dilation in response to increases in flow are attributed to impairment of the nitric oxide-mediated portion of flow-dependent arteriolar dilation (Koller and Huang, 1994). Thus, data are accumulating which link the function of the nitric oxide system to the development of hypertension, although data are lacking which link altered function of the nitric oxide system to obesity-associated hypertension.

In addition to the reports of abnormal responses to nitric oxide blockade, essential hypertensive patients have decreased endothelial nitric
oxide synthesis (Calver et al., 1992). Also, a positive relationship has been reported between insulin sensitivity and endothelial nitric oxide synthesis by Petrie et al., 1996 in a study examining forearm vasoconstrictor responses to hyperinsulinemic euglycemia and NOS inhibition. Thus, another mechanism through which NO may be associated with obesity-induced hypertension is through interaction with insulin. In a study by Walker, et al. (1997) insulin attenuated the contractile response to noradrenaline in rat mesenteric arteries. This effect was blunted by L-NAME, supporting the idea that insulin's effect to reduce the vasoconstriction induced by noradrenaline is nitric oxide dependent. This group proposed several mechanisms through which L-NAME could work to oppose the ability of insulin to blunt norepinephrine-induced vasoconstriction:

1) inhibiting NO synthase - probable
2) altering insulin binding
3) interfering with insulin metabolism

It is possible to postulate that insulin-induced release of NO occurs at physiologic insulin levels, resulting in tonic vasodilation. Insulin resistance is known to occur in essential hypertension (Ferrannini et al., 1987). Thus, developing insulin resistance may be associated with impaired insulin-induced NO release and a loss of this tonic vasodilating stimulus, with subsequent development of hypertension (Baron, 1996).

An alternate interaction between NO and insulin is suggested by recent studies in dogs (Bilski et al., 1995a and b) which report that NO may
be an important neurotransmitter in the pancreas. In these studies, L-arginine infusion resulted in a significant increase in pancreatic protein output (a reflection of exocrine pancreatic function), and LNNA caused significant reductions in insulin and glucose. Their paradigm of LNNA use almost abolished the increment of insulin and glucagon in response to sham and ordinary feeding. However, this LNNA infusion did not change basal pancreatic protein secretion. They proposed that these effects were probably mediated through cholinergic mechanisms, since direct cholinergic stimulation of insulin secretion was blocked with the LNNA treatment.

Other studies support the role of NO in both exocrine and endocrine pancreatic secretion in humans (Konturek et al., 1997). In this study, secretin and caerulein-stimulated pancreatic enzyme secretion was reduced by LNMMA, without alterations in volume flow and bicarbonate outputs. Addition of LNMMA to the secretagogue infusion was associated with a reduction in plasma insulin and pancreatic polypeptide (PP) levels, while addition of L-arginine raised insulin and PP levels above normal. This supports a role for NO in pancreatic insulin secretion, another avenue through which these two factors may interact. Thus, there are several pathways through which the nitric oxide system and insulin may interact to influence arterial pressure in hypertensive disorders. These require examination in the obese model of hypertension as mediators of this disease syndrome.
In addition to its involvement in cardiovascular hemodynamic function, the role of the nitric oxide system in nervous tissue function has been explored. Nitric oxide synthase activity has been identified in several CNS locations (Bredt et al., 1990; Forstermann et al., 1991), and studies exploring a regulatory role for NO in functioning of the sympathetic nervous system have been performed (Harada et al., 1993; Sakuma et al., 1992). In 1991, Vo et al. demonstrated that central inhibition of NO synthesis enhances the vasoconstrictor responses to peripheral sympathetic nerve stimulation and adrenaline in the rat tail artery. In 1993, Harada, et al. examined the effects of microinjections of L-arginine into the nucleus tractus solitarius (NTS) on blood pressure, heart rate and renal nerve activity. The results of this study suggests that NO is involved in NTS mechanisms that mediate tonic renal nerve activity in rabbits, providing support for central NO synthase activity in the regulation of renal sympathetic nerve activity. Intravenous LNMMA administration in sinoaortic-denervated and vagotomized rats increased renal sympathetic nerve activity (Sakuma et al., 1992), while Togashi and his colleagues, elicited elevations in arterial pressure and renal sympathetic nerve activity with intra-cisternal injections of LNMMA (Togashi et al., 1992). These studies further support central regulation of peripheral SNS activity by NO. Thus, in addition to modulating vascular resistance by peripheral mechanisms, NO may play a role in central regulation of sympathetic tone and/or renal nerve activity.
Other experiments have suggested that sympathetic activation is in turn a modulator of NO release (Lacolley et al., 1991). In 1991, Lacolley suggested that the SNS plays an important role in modulating the synthesis or release of vascular NO through the effects of normal sympathetic discharge, humoral activation of α-adrenergic receptors and vascular tone per se, since ganglionic blockade with chlorisondamine lowered mean arterial pressure and vascular resistance, abolished the LNA-induced pressor and renal vasoconstrictor responses, and attenuated the increase in mesenteric and hindquarter resistance in rats. In 1992, Pegoraro et al. suggested that NO synthesis may be stimulated by alpha-1 receptors due to a greater response to NO synthesis inhibition in phenylephrine-pretreated rats.

Thus, the role of NO synthase activity in central and peripheral regulation of vascular, renal and nervous system control is complex. There is clear evidence that central NO production can modulate sympathetic nervous system activity as well as evidence that peripheral sympathetic activity can modulate both the production and the action of NO in peripheral vessels. Although it is clear that these two systems interact, it is not clear how, or if, they interact in the pathogenesis of hypertension in either the lean or the obese state. The involvement of these two systems with insulin and insulin resistant disease states further complicates the mechanisms through which hypertension could develop with obesity.
Further study of this triad as modulators of obesity-hypertension is thus warranted.

The studies described in this dissertation were designed to examine the interactions of insulin, the SNS and nitric oxide in lean and obese-hypertensive dogs, further characterizing the mechanisms responsible for the development of hypertension with obesity. This was accomplished using the canine model of obesity-induced hypertension, the hyperinsulinemic euglycemic clamp technique, nitric oxide synthase inhibition and unilateral lumbar ganglionectomy to examine the following hypotheses.

1) The interaction of hyperinsulinemia and sympathetic activity were examined over the course of weight gain to determine whether changing vascular resistance is influenced by insulin or SNS activity, predisposing to arterial pressure increases. We hypothesize that as insulin resistance develops with weight gain, insulin's effect to reduce vascular resistance is decreased. At this time, increases in SNS activity associated with obesity alter peripheral vascular resistance, laying a foundation for hypertension to develop. Thus, in animals with nervous activity removed to one limb (while the SNS of the opposite limb remains intact), we monitored insulin levels and femoral vascular resistance (FVR) throughout weight gain to determine whether the vascular response to weight gain correlated with insulin levels, and were influenced by SNS innervation.

2) Local NOS activity reduces peripheral vascular resistance, but this function may be reduced in the obese. Peripheral sympathetic activity may
be important in modulating vascular responses to nitric oxide, and this interaction may be altered in the obese animal. We hypothesize that the increase in SNS activity which develops with weight gain may inhibit the activity of vascular NOS, thereby predisposing to hypertension. Thus, the vascular response to nitric oxide synthase inhibition in intact and denervated femoral arteries in the lean animals should vary slightly, due to differences in basal tone resultant from nerve input. However, as sympathetic activity increases in obesity, there should be a reduction in NOS activity in the intact femoral artery. Thus, inhibition of NOS should induce a smaller vascular response in the obese intact limb compared to the denervated limb, and widen any difference in FVR present between innervated and denervated limbs observed prior to weight gain.

3) Since interaction between insulin and NOS is also recognized, and reduced response to both are observed in the obese, abnormalities in this interaction may occur in the obese and predispose to hypertension. If NOS activity mediates vascular relaxation in response to insulin in lean animals, an abnormality in this interaction may occur in obesity. We hypothesize that a reduction in NOS activity occurs in the obese, reducing insulin's ability to induce vascular relaxation, and possibly predisposing to hypertension. Hyperinsulinemia in lean animals with normal NOS activity should reduce vascular resistance, and NOS inhibition in this population should decrease the vascular response to hyperinsulinemia if it is indeed mediated through NO activity. However, in the obese, who have reduced
vascular NOS activity, insulin may be unable to cause vascular relaxation when NOS is inhibited, thereby predisposing to pressure/vascular resistance increases. Sympathetic activity may also influence this response since it may reduce NO activity. Thus, unilateral denervation technique during these studies allows the examination of the SNS interaction.

The above hypotheses were studied in canine model of diet-induced obesity (Rocchinni et al., 1987) by using the hyperinsulinemic euglycemic clamp technique (HIEC; DeFronzo et al., 1979) and NOS inhibition in lean and obese animals. The HIEC technique uses a constant rate insulin infusion and a variable rate glucose infusion to examine insulin sensitivity while maintaining euglycemia (DeFronzo et al., 1979). This procedure raises plasma insulin levels acutely and then maintains them at a new plateau without the complication of hypoglycemia (DeFronzo et al., 1979). Thus, the HIEC technique allows one to study physiologic effects of elevated insulin levels (Liang et al., 1982) and determine sensitivity to insulin-mediated glucose disposal. The HIEC procedure was performed alone and after NOS inhibition to determine whether interaction of insulin and NOS is an important predisposing pathway for hypertension in the obese, and whether SNS activity influences the response to insulin.

The canine model of diet-induced obesity was used in these studies. This model was described by Rocchinni et al. (1987) in a study in which a high fat diet was fed for 5 weeks. During overfeeding, the animals exhibited significant weight gain which was associated with elevations in arterial...
pressure, heart rate, plasma volume, cardiac output and systemic vascular resistance, as is also described in humans (Alexander et al., 1962; Mujais et al., 1982, Rocchini et al., 1989a). Weight gain in the dogs was also associated with hyperinsulinemia/insulin resistance (Rocchini et al., 1987), as described in both obese and non-obese hypertensive humans (Ferrannini et al., 1987). Thus, this is an appropriate model to study the development of hypertension with weight gain (Rocchini et al., 1987 and 1989b). In our laboratory, this technique has been modified to include a specially formulated diet with consistent ingredients, which can be prepared in both a pelleted and powdered formulation. Also, once an animal refused to consume the entire quantity of diet presented, the liquid mixture was fed via a gastrostomy tube to ensure the diet was received in its entirety.

Unilateral denervation of one hind limb (left) was utilized in these experiments to study the interaction of the SNS and insulin on vascular activity, and to provide an intact control within each individual. Removal of nerve activity to one limb also allowed us to examine the role of sympathetic activity in mediating the response to NOS inhibition and/or hyperinsulinemia. Since the studies were performed in both lean and obese dogs, we were able to examine whether the interaction of vascular SNS activity, insulin and NO activity differed between lean and obese individuals.
CHAPTER 3: MATERIALS AND METHODS

Animal care and preparation:

The interaction of insulin, the sympathetic nervous system and nitric oxide (NO) in controlling femoral blood flow, arterial pressure and heart rate were examined in the conscious dog using chronically instrumented and unilaterally lumbar denervated animals. These experiments were performed in conscious female dogs, spayed, lean and obese, walking slowly on a treadmill (<1.61 km/h; 0.44 m/s). All procedures and protocols were approved by the Institutional Animal Care and Use Committee prior to initiation.

The canine model of diet-induced obesity-related hypertension was used in these studies, since these animals display metabolic, hormonal and physiologic changes consistent with those observed in obese hypertensive humans. These animals, although expensive to instrument and maintain, are an excellent model for studying relationships between hypertension and obesity (Rocchinni et al., 1987). Their size allows long term vascular access and telemetry recording with minimal stress.

Animals: Ten 15-21 kg spayed female dogs were purchased from the Louisiana State University School of Veterinary Medicine breeding colony. This facility is approved for the breeding of animals for research by the United States Department of Agriculture. All animals were clinically healthy prior to use, having negative heartworm antigen tests (Dirofilaria
*immitis*) and normal clinical chemistry profiles and hematology. The dogs were housed in individual stainless steel metabolic cages with epoxy-coated aluminum flooring. Each cage was fitted with an individual radiotelemetry receiver (Data Sciences International, St. Paul, MN) for remote telemetry data collection and storage via computer (IBM 386 computer using DataQuest IV system software, Data Sciences Intl., St. Paul, MN). The cages were located in a temperature controlled room (22 ± 1°C) with a twelve hour light cycle (7:00A.M.-7:00P.M.).

**Treadmill acclimation:** The method of walking during the acute studies was instituted to minimize variability between the arterial flow of the 2 pelvic limbs, without the need for anesthesia or tranquilization. Several different regimes of sedation/tranquilization or general anesthesia were explored prior to the use of this method. However, none of these protocols provided adequate animal restraint, while allowing the measurement of stable femoral blood flow or arterial pressure over the time required for completion of the acute HIEC studies. Thus, the stability of femoral artery blood flow was assessed while walking, as were heart rate (HR) and mean arterial pressure (MAP). Since these parameters were found to remain stable while walking, the acute studies were performed with the animals slowly walking on a treadmill at ≤ 1.6 km/h (0.44m/s).

The dogs were acclimated to standing and walking on a treadmill (model #1849-01, Quinton Instrument Co., Seattle, WA) prior to beginning
the studies. The animals were placed on the treadmill, allowed to stand quietly, then the treadmill started (<1.6 km/h; 0.44 m/s). The length of time spent walking was slowly increased as the dogs began acclimating/relaxing. The amount of time required for acclimation depended upon individual personality and behavior. The acute studies were not performed until each animal walked comfortably on the treadmill.

**Chronic Instrumentation and surgical procedures:** All animals had instrumentation surgically implanted in the following manner:

1. A chronic indwelling jugular catheter was surgically placed for vascular access. A hand made sialastic® (Dow Corning, Midland, MI) catheter was inserted into the left jugular vein, with the remaining tubing tunneled subcutaneously to the cranial-dorsal thoracic region where it was connected to a subcutaneous access port.

2. A remote telemetry catheter (Data Sciences International, St. Paul, MN) was placed into the left carotid artery of each animal for continuous monitoring of arterial pressure and heart rate. The associated battery unit (model # TA11PA-D70) was placed subcutaneously in the lateral cervical region. Each animal was housed in a metabolic cage equipped with its own telemetry receiver through which data was collected and transmitted to an IBM 386 computer for continuous data collection.

3. A stainless steel peg gastrostomy tube (Associated Technologies and Manufacturing Inc., Baton Rouge, LA) was placed into each animal
through a ventral midline celiotomy. The tube was exteriorized through the ventro-lateral abdominal wall, caudal to the thoracic rib margin.

4. Bilateral ultrasonic transit time perivascular flow probes (model 4RS, with a CM4B connector, Transonic Systems Inc., Ithaca, NY) were positioned around the proximal femoral arteries. The attached cable and connector were tunneled subcutaneously to the dorsal mid-cervical region and placed in a subcutaneous pocket until exteriorized just prior to the first acute experiment. Following exteriorization the cables and connectors were placed in a denim collar to prevent destruction. The collar was changed and the os flushed routinely with dilute nolvasan solution or antibiotics to minimize infection.

During acute protocols, the probe connectors were attached to a T206 flow meter (Transonic Systems, Inc., Ithaca, NY) and the femoral blood flow (FBF) data stored on an IBM S33 personal computer using DATAQ data acquisition software (Dataq Instruments, Inc., Akron, OH). Femoral vascular resistance was calculated from flow using \( FVR = \frac{MAP}{FBF} \).

5. Via a ventral midline celiotomy, the animals underwent a unilateral surgical lumbar sympathectomy (left side, L3-L6) as described for humans by Callander et al., 1958. Removal of the L3-L6 lumbar ganglia in dogs has been shown to provide complete sympathetic denervation of the pelvic limbs (Carey et al., 1967), and has been used in the dog to study femoral blood flow (Crononwett et al., 1977; Galt, et al., 1991).
The animals underwent a total of 3 surgical operations. The jugular catheter and the telemetry unit were placed during one surgery. The L₃-L₆ ganglionectomy and gastrostomy tube placement were performed together during a separate surgery, and bilateral femoral flow probes were placed on yet another occasion. Each pre-surgical and surgical procedure was conducted as follows. Following a 12-18 hour fast, each animal was premedicated with acepromazine maleate (Promace®; Fort Dodge Laboratories, Inc., Fort Dodge, IA: 0.02-0.2 mg/kg, i.m.; 0.3 mg maximum) then anesthesia was induced using thiopental sodium (Pentothal®; Abbot Laboratories, North Chicago, IL: 4-6 mg/lb, i.v. to effect). Throughout surgery the animals were maintained on 1.5-3% isoflurane inhalant anesthesia (Aerrane®; Ohmeda Caribe Inc., Guagama, PR) as individual requirements dictated. The animals were placed on circulating water heating pads to maintain body temperature during surgery and recovery. Intra-operative polyionic fluids and antibiotics (Cefazolin®, 1 gram in fluids) were administered.

Analgesics were not required in the postoperative period by any animal. However, flunixin meglumine (Banamine®; Fort Dodge Laboratories, Inc., Fort Dodge, IA: 1.1 mg/kg, i.m.) was empirically administered during the recovery period of the combination denervation/gastrostomy surgery, to reduce postoperative inflammation and discomfort which may have resulted from visceral manipulations required to access the ganglionic chain and perform the L₃-L₆ ganglionectomy and the
tube-gastrostomy. Antibiotics were administered (TMPS®; Sidmark Laboratories, Inc., East Hanover, NJ: 30 mg/kg; *per os*, BID) for a minimum of three days following surgery. A minimum of 1-2 weeks of recovery was allowed, depending upon the procedure performed, the sequence of the surgeries for each animal and their individual recovery rates. All surgical manipulations were performed at least one week prior to any acute infusion study, and a minimum of 2-2.5 weeks was allowed following denervation prior to experimentation. Indwelling catheters were flushed weekly with heparinized saline (100U/ml) to maintain patency.

**Diet manipulations:** Water was available, free choice, at all times. The animals were initially fed Purina Dog Chow® (21% protein, 49.2% carbohydrate, 8% fat, 4.5% fiber) once a day to maintain stable body weight. Following weight maintenance on Purina Dog Chow®, and prior to beginning the acute studies, a specially formulated pelleted diet was introduced (Table 1; total protein 26%, total carbohydrates 62%, total fat 12%). This pelleted diet was fed at the quantity calculated to provide the equivalent number of Kcal of energy provided by the Purina® dog chow required during stable weight maintenance. If necessary, the quantity of pelleted diet fed was adjusted to maintain stable body weight. The animals were weight stable for a least 1 week prior to the acute lean experiments. The lean animals were maintained weight stable on the custom pelleted diet (Table 1) without additional fat. Following completion of the acute lean
Table 1: Custom canine diet #D72204. Diet #D72204 was formulated by Research Diets Inc., New Brunswick, NJ.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>gm</th>
<th>kcal</th>
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<td>Soy protein</td>
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<td>Dextrose</td>
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</tr>
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</tr>
<tr>
<td><strong>TOTAL</strong></td>
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<td>3830</td>
</tr>
</tbody>
</table>

experiments, the lean animals were overfed a powdered formulation of the custom diet, to which melted lard was added to increase the energy intake to 150% above maintenance. The animals were acclimated to the higher fat food by increasing the fat content to 50%, 100% and 150% above maintenance levels over several days. The animals initially were allowed to eat the diet at will, then fed via the tube-gastrostomy once their appetite waned. This overfeeding paradigm produces significant increases in body fat over 5-7 weeks (approximately 25%-50% increase in body weight), without the development of intractable diarrhea, vomiting, or pancreatitis. All lean animals were weight stable for at least one week prior to the acute studies. At the end of the 7-week overfeeding period (during the acute obese experiments) each animal was maintained weight stable by feeding the powdered-diet and lard mixture to provide 50% above calculated
maintenance energy requirements. This individual feeding regimen was adjusted by adding or reducing the lard content to ensure weight stability during the acute post-obesity protocols.

**Body weight, body composition and MAP/HR monitoring:**

Body weight was measured in the fasted animal three times per week throughout the studies. Dual-energy x-ray absorptiometry (DEXA; Hologic model QDR-2000, Waltham, MA) was performed in anesthetized animals at the end of the lean studies and again prior to the obese protocols. DEXA is a technique for directly assessing body composition using dual x-ray beams of differing energy levels which are differentially absorbed by tissues varying in density (Jensen et al., 1993). Arterial pressure and heart rate were monitored continuously via telemetry throughout the pre-obesity experimental study period and during the overfeeding period. However, to minimize external environmental influences on blood pressure and heart, only data collected between 7:00 p.m. and 7:00 a.m. (over-night) was used for daily averages.

**Analytical procedures:**

**Plasma insulin radioimmunoassay:** Insulin levels in plasma were measured using radioimmunoassay kits purchased from Linco Research, Inc. (St. Charles, MO). Canine standards were run in triplicate with each assay, and all subject samples and canine quality controls were run in duplicate. All samples from both the lean and obese hyperinsulinemic euglycemic clamp (HIEC) and the hyperinsulinemic
euglycemic clamp performed following nitric oxide synthase inhibition (LNA-HIEC) of each animal were run within one assay (n=2 assays with three animals per assay) and weekly samples for all animals and all weeks were run in a single assay. Intra- and inter-assay coefficients of variation were 4% and 7% respectively.

The protocol followed for plasma insulin determination was the standard insulin RIA procedure followed for canine samples in our laboratory. In short, 100 µl of standards or samples were separately pipetted into borosilicate culture tubes and 100 µl of assay buffer, then 1st antibody (guinea pig anti-porcine insulin serum) were added. One hundred µl of T²⁵ insulin trace was added to each tube. The samples were incubated overnight, then 100 µl of the 2nd antibody (goat anti-guinea pig IgG) and carrier (normal guinea pig IgG) were added and the samples incubated for 2 hr at 4°C. One ml of wash buffer was then added to stop the reaction and all samples were centrifuged at 3,000 RPM for 25 min at 4°C. The supernatant was carefully poured off and the samples were allowed to dry and the activity was determined using a gamma counter (model 5500B, Beckman Instruments Inc., Fullerton, CA).

Plasma glucose analysis: Plasma glucose concentrations were determined using a YSI model 2700 glucose analyzer (Yellow Springs Instruments, Yellow Springs, OH), which utilizes the glucose oxidase method. The normal range of plasma glucose concentrations for our animals was 90-108 mg/dl, for studies 3 and 4 combined.
**Tissue catecholamine levels:** At the termination of all studies, femoral artery sections (3-4 artery sections per limb) were removed and tissue catecholamine levels assessed. Femoral artery tissue samples were recovered immediately from the animals following euthanasia. Left limb (denervated) samples were taken first to reduce the chance of lowering quantities of catecholamines due to the rapid metabolism. The samples were placed into chilled 0.1 M perchloric acid and immediately frozen in liquid nitrogen. During the homogenization procedure a total of 1.5 ml of perchloric acid was added to the volume during flushing of tubes. Tissue homogenization was performed on the same day as collection in all but one animal, using a tissue homogenizer from Omni Inc (Warrenton, VA). In this animal the homogenizer broke at the start of the first sample so homogenization was postponed until a replacement was obtained (8 days).

Tissue homogenates were assayed using high performance liquid chromatography (HPLC) to determine tissue catecholamine levels. These assays were performed by Dr. Gennady Smagin using the standard techniques of his laboratory. In short, tissue homogenate supernatant (0.5 ml) was extracted using 50 mg acid washed alumina and TRIS buffer/EDTA (1.5 M, pH 8.6). Samples were analyzed (50 µl injection volume) for neurotransmitter (NE and E) concentration using an HPLC system consisting of a pump (model 420), autosampler (model 460), Coulochem detector (model 5100A), high performance analytical cell (model 5014), and MD-150 column (all from ESA, Inc. Bedford, MA). The mobile phase
consisted of 75 mM NaH₂PO₄, 1.4 mM l-octenesulfonic acid sodium salt monohydrate (OSA), 10 μM EDTA, 10% acetonitrile adjusted to pH 3.1 with H₃PO₄. The flow rate was maintained at 0.9 ml/min. Minor changes of pH and OSA concentration were made to obtain optimal separation. Detector potentials were set at -0.04 V for electrode 1, +0.35 V for electrode 2 and at +0.4 V for the guard cell. Data were collected by a Kontron IBM-PC-based integrator (ESA, Inc, Bedford, MA), and the integrated data were transferred directly to a spreadsheet program for data analysis.

Appropriate extracted catecholamine standards were prepared (NE/arterenol and E/adrenalin, Sigma, St. Louis, MO) and run (150 μl injection volume containing 2.14 ng of NE and E) with the samples. All samples for each animal were run within the same assay. The laboratory inter-assay coefficient of variation for the catecholamine HPLC assay is <5%. Before samples were processed, assay validation was performed. This validation was performed by extraction of 100, 50, 25, 10, 5, and 0 ng/ml of NE. N-methyl-D-aspartate (NMDA) was used as the internal standard. The peak height of NE was measured and the ratio of NE to the internal standard was determined. This ratio should remain constant at varying concentrations, and the linearity of the ratio was checked for validation.

Once linearity was validated, samples were analyzed, with single point calibrations (using 10ng/ml of NE) used for daily quantitation of samples as long as no system components were changed. The concentration of NE in
the samples were in the range of 1-10 ng/ml of extract, and within the validated linear range.

**Acute experimental protocols:**

All acute experiments were performed in lean fasted animals (12-18 hour fast) and repeated in the same animals after seven weeks of overfeeding. Heart rate (HR), arterial pressure (BP) and femoral blood flow (FBF; used to calculate femoral vascular resistance, FVR) were measured throughout the studies. Following a 5-10 minute acclimation period, twenty minutes of walking baseline data were obtained at the beginning of all experimental studies. The drug doses calculated for these studies were based upon the weight of each dog on the date of the study (i.e. lean wt. or obese wt.). If necessary, a study was repeated based on data work-up, or if stable glucose levels could not be achieved within 120 min of initiating the hyperinsulinemic euglycemic clamp (HIEC) for studies 3 and 4.

**Study 1: Time course for the development of vascular resistance changes and insulin resistance with obesity.** To determine the time course of vascular resistance changes with the development of obesity, and whether these changes occur concurrent with changes in insulin levels, we monitored fasting insulin levels and vascular resistance changes throughout the development of obesity. Prior to and during the 7-week period of overfeeding, 10 ml of blood was obtained weekly from each animal, in the fasted state. On the same day blood was drawn, the animals were placed onto the treadmill (<1.6 km/h; 0.44 m/s) and 30 min of BP/HR/FBF data
were recorded (10 min acclimation and 20 min of stable recordings).
Changes in arterial pressure, heart rate, FVR and fasting insulin were
compared prior to the start of overfeeding and weekly throughout the
development of diet-induced obesity.

Study 2: Cardiovascular parameters following NOS inhibition with
LNA. Once 20 min of stable baseline data was collected, 10 mg/kg of nitro-
L-arginine (LNA; a competitive inhibitor of nitric oxide synthase; Aldrich
Chemical Co., Inc., Milwaukee, WI) was administered through the
indwelling jugular vein port. Walking on the treadmill (<1.6 km/h; 0.44
m/s) was continued for 2 hours following LNA administration while
continuously recording BP/HR/FBF. At the end of this study, L-arginine
(bolus dose of 150 mg/kg, iv) was administered to reverse the lasting effects
of LNA (Elsner et al., 1994). Nonetheless, a minimum of 5-7 days was
allowed for recovery of BP/HR/FBF response subsequent to LNA studies.

Study 3: The hyperinsulinemic euglycemic clamp (HIEC) in lean
and obese dogs. Once stable baseline walking cardiovascular data was
collected, a hyperinsulinemic-euglycemic clamp procedure (HIEC; DeFronzo
et al., 1979) was performed in each animal in the following manner. During
the 20 min walking baseline period blood samples were obtained at -15, -10
and -5 min (1-1.5 ml obtained through the jugular access port), placed into a
heparinized micro-centrifuge tube and centrifuged to separate plasma and
cells. The plasma was immediately analyzed for glucose concentration (YSI
glucose analyzer model 2700, Yellow Springs Instruments, Yellow Springs,
OH), and the remaining plasma was stored for insulin assay by RIA. The average of the -15, -10 and -5 min glucose samples was used as the baseline value for that animal, and the ± 5% glucose range was calculated. The infusion lines were primed with both insulin and glucose. At t=-5 min the insulin infusion was begun at a priming rate of 1.5 ml/min for 5 min. At the t=0 point, the insulin infusion rate was reduced to 1.0 ml/min for an additional 2 min, and at t=2 min, the rate was set to 0.5 ml/min insulin (40 mU/min) for the remainder of the study. Glucose (30% solution in sterile water) was concurrently administered at an initial priming rate of 1.0 ml/min for 1 min (starting at t=-1 min). At t=0, the glucose rate was reduced to 0.5 ml/min and adjusted as necessary thereafter to maintain plasma glucose levels within a ± 5% range of the baseline average. Both insulin and glucose infusions were administered through a cephalic vein catheter using syringe pumps (model #22, Harvard Apparatus, Southnatick, MA). Blood samples (1.0-1.5 ml) were obtained every 5 minutes from the jugular access ports to monitor plasma glucose concentration, allowing adjustment of the glucose infusion rate to maintain euglycemia (90-108 mg/dl). The remaining plasma was saved for insulin assay.

Once glucose was stabilized within 5% of baseline, FBF/BP/HR data were collected for 20-30 min while the animal continued a slow walk (<0.44 m/s). Each study ended when 5-7 consecutive stable glucose samples were obtained that were within the ± 5% baseline range. This allowed a minimum of 30-35 min of data collection while glucose was within 5% of the
baseline. Thus, the most stable 20 min could be used for statistical comparison.

During the HIEC protocols (alone and under NO inhibition) 25 IU/ml of heparinized saline (2.5 ml) was flushed through the sampling lines following sample withdrawal. Prior to all sample withdrawals, 2.0 ml of flush/blood mixture was withdrawn from the sampling line and discarded. Another 3 ml of blood was removed to ensure that no flush remained in the collection line. The 1.5 ml blood sample to be used for glucose and insulin analysis was then withdrawn, the initial 3 ml was returned to the animal and the line was flushed with 2.5-3.0 ml of heparinized-saline solution (25 IU/ml). Thus, in these extended length protocols, the maximum amount of heparin administered was approximately 25-40 mg for the 2+ hour studies. The higher level is within the recommended initial loading dose for anticoagulant therapy with heparin in dogs. Removal and discarding of the initial 2.0 ml of blood before sampling made the actual amount of heparin remaining within the animal significantly less than that calculated. These steps were taken to reduce any possible effects that may occur in fatty acid metabolism or hepatic clearance resulting from elevated levels of heparin.

No signs of coagulation defects were observed following any protocol. The maximum volume of blood withdrawn from any individual was approximately 40-50 ml (for a study lasting a full two hours). This volume was negligible in this size dog, especially in light of the fluids being replaced with the insulin and glucose infusions.
Both glucose and insulin infusions were administered through a cephalic vein catheter, and the blood samples were obtained through the jugular access port. At the termination of each study, the plasma samples were frozen at -20°C until assayed for insulin. A minimum of 48 hr was allowed between any HIEC study and another study.

**Study 4: The HIEC following nitric oxide synthase inhibition (LNA-HIEC) in lean and obese dogs.** These studies were carried out exactly as for the HIEC previously described in Study 3, with the following additions. Once baseline walking cardiovascular data were collected, the treadmill was halted and nitro-L-arginine (LNA; a competitive inhibitor of nitric oxide synthase; Aldrich Chemical Co., Inc., Milwaukee, WI; 10 mg/kg, iv) was administered. The dogs were allowed to stand or sit at will for 45 min, while waiting for cardiovascular parameters to change and stabilize following LNA administration. The treadmill was then restarted (<1.6 km/h; 0.44 m/s) and another walking baseline was obtained under the influence of NOS inhibition. During this stable 20 minute baseline of LNA-walking, the -15,-10 and -5 min baseline glucose/insulin blood samples were collected from the jugular catheter access port, as described in the original clamp protocol. Thereafter, the HIEC protocol was performed exactly as described for Study 3 [i.e. initial priming of both insulin and glucose followed by a constant insulin infusion of 0.5 ml/min (40 mU/min) and a variable glucose infusion rate adjusted to maintain euglycemia]. A
minimum of 5-7 days was allowed between any LNA study and another study.

Statistical analysis:

All statistical analyses were performed by the Biostatistics Department of the Pennington Biomedical Research Center, using PROC MIXED or PROC GLM in SAS, version 6.12. In all multiple comparisons, p-values were adjusted with respect to Tukey-Kramer or Bonferroni inequalities as specified below.

Body composition was analyzed using univariate analyses (t-test on the lean:obese difference) comparing lean and obese DEXA scan results. Change in body weight with overfeeding was analyzed with a mixed statistical model and autoregressive covariate structure (assuming equal week to week variances and thereby reducing the number of unknown parameters in the model), with baseline weight as a covariate in the model. Body weight was the dependent variable in the model, regressed over time in weeks. Additionally, the weekly change compared to baseline was assessed using the same model structure, with the difference in weight between each week and the baseline as the dependent variable and time (week) as the fixed effect.

The change in insulin during the overfeeding period (main effect of time in weeks) was analyzed using a mixed model with weekly insulin concentration as the dependent variable regressed over time (in weeks), for 7 dogs and over 8 weeks. In addition, the change in insulin concentration
from baseline for each week of overfeeding was analyzed using a mixed model with an autoregressive covariate structure (assuming equal week to week variances to reduce the number of unknowns in the model) for 7 dogs and 7 baseline to week differences (the dependent variable was the difference in insulin between baseline and each week of overfeeding, with week as the fixed effect in the model). Other weekly data (MAP, HR and FVR) were analyzed in a similar manner, determining the main effect of time as well as weekly change. For all of these weekly parameters, if the baseline parameter measure was determined to be a significant covariate in the model, it was included as such. For FVR, combined data were analyzed to determine whether differences between the limbs (denervated and intact) developed over time for either position (standing and walking). Then, each limb was analyzed separately, for each position, for main effect of time and weekly difference from baseline. The positions and limbs were analyzed in this way (separately) due to the complexity of the model when all variables were included together. Correlation analyses (insulin with weight, HR, MAP and FVR [intact and DNX]) were performed for each animal and the group.

In the study examining cardiovascular parameters and stabilization following LNA (Study 2) data were analyzed using the mixed model with autoregressive structure and baseline parameter level as a covariate, to determine whether each parameter showed a significant time effect after LNA administration (model variables of weight group-lean and obese).
Once it was determined that the cardiovascular parameters remained stable following acclimation to treatment with LNA, all post-LNA data points were combined for pre-LNA and post-LNA comparisons and for the analysis of differences in response between weight groups. The dependent variable in this mixed model was the difference between pre-LNA and post-LNA parameters of HR and MAP for 6 dogs and comparing two weight groups (lean and obese). This model used an unstructured covariate design (does not assume equal variances of the covariate matrix thus) with weight group and treatment period as the fixed effects, covariate parameter estimation and Tukey-Kramer adjustment for multiple comparisons. For FVR, the difference between the two limbs was assessed, then the individual limb response to treatment was separately analyzed and weight groups compared, due to the complexity of the model and the number of variables/sources of error. In these analyses, weight group, limb (intact or DNX) and treatment period were the fixed effects in the model.

In the HIEC and LNA-HIEC studies (Studies 3 and 4), the difference in response between the two studies in both weight groups was analyzed with each parameter as a dependent variable (HR, MAP, DNX-FVR, intact-FVR), and an unstructured covariate structure with fixed effects and covariate parameter estimation. Data were analyzed for two weight groups, LNA-HIEC and HIEC studies and n=6 dogs. In addition, the differences in response to treatment (difference between baseline and hyperinsulinemia) between weight groups were analyzed for each study separately, again...
using a mixed model with unstructured covariate design (as described above), covariate parameter estimation and Bonferroni adjustment for multiple comparisons.
CHAPTER 4: RESULTS

Ten animals were originally instrumented for inclusion in these studies. However, only 7 animals finished the overfeeding regimen, and 6 finished all obese acute experiments, due to unilateral or bilateral femoral flow probe failure.

The weekly body weight of the dogs increased significantly during the 7-week overfeeding period (Figure 2; Table 2). Although lean body mass (LBM) did not change significantly with overfeeding, the total grams of fat, % fat and bone mineral content (BMC) increased significantly (Table 2).

*Data are group means ± standard error of the mean (SEM) for n=7 dogs.
* Significantly different from baseline, p<0.01.

Figure 2: Change in body weight over the 7-week overfeeding period. a
Table 2: Body composition prior to (Lean) and following (Obese) 7 weeks of overfeeding. *

<table>
<thead>
<tr>
<th></th>
<th>Lean</th>
<th>SEM</th>
<th>Obese</th>
<th>SEM</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>19.9 ± 0.7</td>
<td>29.7 ± 0.77</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body fat (kg)</td>
<td>4.53 ± 0.84</td>
<td>14.64 ± 0.81</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Body Fat</td>
<td>23.0 ± 3.65</td>
<td>48.8 ± 1.95</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LBM (kg)</td>
<td>14.39 ± 0.63</td>
<td>14.73 ± 0.49</td>
<td>ns</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMC (kg)</td>
<td>0.53 ± 0.02</td>
<td>0.57 ± 0.02</td>
<td>p=0.01</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Data are group means (n=7) ± standard error of the mean (SEM).

ns = not significant

Postmortem femoral artery norepinephrine levels of the obese dogs were significantly different between the denervated (270.13 ± 90.5 ng/g) and intact (627.3 ± 108 ng/g; p=0.028) limb femoral arteries. This confirms reduced femoral artery catecholamine levels (as a reflection of SNS activity) in the denervated femoral artery during post-obesity studies.

Study 1: Time course for the development of vascular resistance changes and insulin resistance with obesity.

In this study, weekly comparisons of MAP, HR and FVR were made with the animals standing and walking on the treadmill. Fasting plasma insulin levels were also assessed. There was a significant increase in MAP over the 7-week period of overfeeding (significant main effect of time; Figure 3) in both the standing (p=0.022) and walking positions (p=0.022). However, using direct week to baseline comparisons, only the MAP in week 7 (standing) did the difference from baseline approach significance (p=0.056).

Heart rate also increased over the 7-weeks of overfeeding (significant main effect of time, p=0.008; Figure 4). However, only in week seven was the HR significantly increased over baseline while walking (p=0.012).
Heart rate during week 1 decreased significantly compared to baseline (p=0.03 for standing and walking).

Figure 3: MAP during the 7-week overfeeding period.

Figure 4: Heart rate during the 7-week overfeeding period.

* Significantly different from baseline, p<0.05. Values are group means ± SEM; n=7.
There was a significant overall time effect on FVR in the standing (p=0.0407) and walking (p=0.0001) positions (Figure 5) for the denervated and intact limbs. Standing FVR of the denervated limb was significantly greater than baseline during weeks 1-2 of overfeeding, whereas FVR of the intact limb was significantly greater than baseline in weeks 1-5 (Figure 5). In the walking state, both the denervated (p=0.0173) and intact limbs (p=0.0074) showed a significant time effect of high-fat feeding. However, compared with baseline walking FVR, only the intact limb showed significant increase over baseline during week 1, (p<0.0395).

* Significantly different from baseline, *p<0.05; **p<0.01. Data are group mean ± SEM, n=7.

Figure 5: Femoral vascular resistance during the 7-week overfeeding period.

Fasting plasma insulin levels increased significantly during the overfeeding period (main effect of time, p=0.04; Figure 6). Baseline insulin
concentration ranged from 5.0-12.4 IU/ml in these dogs, with a significant increase from baseline observed by week 2 of overfeeding (p=0.04). However, significant correlation between insulin and the cardiovascular parameters was not observed, and a very weak correlation of insulin with body weight was present (r=0.346; p=0.0161). Only weak correlation was evident between body weight and cardiovascular parameters (MAP: r=0.314, p=0.0281; HR: r=0.378, p=0.0075; DNX-FVR: r=-0.295, p=0.0398; intact- FVR: r=-0.295, p=0.052). The strongest correlation present was a negative correlation between heart rate and FVR of both limbs (DNX limb r =-0.71 and intact limb r=-0.75; p=0.0001 each).

* Significantly different from baseline,*p<0.05;**p<0.01. Data are group mean ± SEM, n=7.

Figure 6: Weekly fasting plasma insulin levels during the overfeeding period.
Study 2: Cardiovascular parameters following NOS inhibition with LNA.

Following the initial response to LNA, all parameters (MAP, HR and FVR) stabilized following NOS inhibition (Figures 7, 8, and 10). Thus, the post-LNA treatment data were averaged for statistical comparisons, as were data from the twenty minute pre-LNA period.

Mean arterial pressure did not differ between lean and obese dogs during the pre-LNA (20 min average for lean, 97 ± 10 mmHg; obese, 107 ± 3.0 mmHg) or post-LNA treatment periods (lean, 115 ± 1.5 mmHg; obese, 123 ± 0.65 mmHg). Following LNA administration, MAP increased significantly and stabilized in both weight groups (Figure 7). The MAP response to LNA did not differ between the weight groups.

![Figure 7: MAP response to NOS inhibition in lean and obese dogs.](image)

* Data are group means (n=6) ± SEM.
* Significant increase following LNA treatment (using a 20 min average of pre-LNA period and a 90 min average of the post-LNA period), *p<0.05; **p<0.01.

Figure 7: MAP response to NOS inhibition in lean and obese dogs.*
Heart rate (Figure 8) differed significantly between the lean and obese dogs during the pre-LNA period (p=0.0007; 20 min average for lean=107 ± 4 BPM, and obese=130 ± 4 BPM) and post-LNA period (p=0.0001; 90 min average for lean, 75 ± 1 BPM; obese, 107 ± 1 BPM). Although HR decreased significantly in both weight groups following NOS inhibition, p<0.01 for each (Figure 8), the HR response to LNA administration was attenuated in the obese animals (Figure 9; p=0.02 for 32 BPM average HR decrease in the lean and the 24 BPM reduction in HR for the obese).

* All values are group means (n=6) ± SEM.
** Significant decrease following LNA treatment, p<0.01.
# Significant difference between lean and obese (using a 20 min average of pre-LNA period and a 90 min average of the post-LNA period), p<0.01.

Figure 8: Heart rate response to NOS inhibition in lean and obese dogs.ᵃ

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Figure 9: Change in heart rate following LNA administration

Pre-LNA femoral vascular resistance did not differ between the lean and obese dogs (Figure 10). Also, pre-LNA FVR did not differ in the DNX and intact limbs in either weight groups. However, following LNA administration FVR differed between the weight groups (p=0.0086 for main effect of weight), and between the intact and denervated limbs (p=0.0048, for main effect of limb). Femoral vascular resistance increased significantly in both the DNX and intact limbs of the lean animals following LNA administration (DNX, p=0.01; intact, p=0.02). The increase in FVR in the obese group did not quite reach significance (DNX, p=0.06; intact, p=0.051). In the lean animals during the post-LNA period, DNX limb FVR was significantly higher compared to the intact limb (p=0.002; Figure 10).
However, this was not so in the obese. Additionally, FVR in the denervated limb of the lean dogs was higher than the obese during the Post-LNA period (p=0.02; Figure 10). Thus, the FVR response following LNA administration was significantly different between the lean and obese dogs (p=0.0135).

** Increase in FVR following LNA significantly different between intact and DNX limbs of the lean animals, p<0.01 (comparing 90 min averages of the post-LNA treatment periods for n=6 dogs).
# Significant difference between DNX limb FVR of lean and obese dogs, p<0.05 (comparing the 90 min averages of the post-LNA treatment period for n=6 dogs).

Figure 10: Femoral vascular (FVR) response to NOS inhibition in lean and obese dogs.

Studies 3 and 4: The hyperinsulinemic euglycemic clamp, alone (HIEC) and following NOS inhibition with LNA (LNA-HIEC).

Glucose levels were not significantly different between the lean and obese animals during the walking baseline period or during the stable euglycemic hyperinsulinemic period in either study (Table 4). During the stable hyperinsulinemic euglycemic period of the HIEC study, the glucose
infusion rate of the lean animals was almost twice that of the obese (GIR\textsuperscript{b}-HIEC; Table 3). In the LNA-HIEC, during which NOS was inhibited prior to the HIEC, the lean-obese difference in glucose infusion rate was not evident (GIR\textsuperscript{b}-LNA-HIEC). In the obese, the difference in GIR between the two studies was minimal (obese GIR\textsuperscript{b}-HIEC vs. obese GIR\textsuperscript{b}-LNA-HIEC; Table).

Table 3: Plasma glucose\textsuperscript{a} levels in lean and obese dogs during the HIEC and LNA-HIEC studies.

<table>
<thead>
<tr>
<th></th>
<th>Lean</th>
<th>Obese</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-HIEC</td>
<td>99.0 ± 1.63</td>
<td>98.7 ± 0.73</td>
</tr>
<tr>
<td>HIEC</td>
<td>96.9 ± 2.91</td>
<td>99.1 ± 1.28</td>
</tr>
<tr>
<td>Pre-LNA-HIEC</td>
<td>97.4 ± 1.97</td>
<td>99.4 ± 2.21</td>
</tr>
<tr>
<td>LNA-HIEC</td>
<td>98.2 ± 2.57</td>
<td>97.7 ± 2.33</td>
</tr>
<tr>
<td>GIR\textsuperscript{b}-HIEC</td>
<td>0.45 ± 0.12</td>
<td>0.26 ± 0.09*</td>
</tr>
<tr>
<td>GIR\textsuperscript{b}-LNA-HIEC</td>
<td>0.31 ± 0.09</td>
<td>0.30 ± 1.0</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Glucose levels in mg/dl; group mean (n=6) ± standard error of the mean (SEM).

\textsuperscript{b} GIR; glucose infusion rate during stable euglycemic period in ml/min

* Significant difference between lean and obese, p<0.05.

Baseline insulin, combining both the HIEC and LNA-HIEC studies, was higher in the obese animals (p=0.0228; Table 4). However, the difference in baseline (Pre-Clamp) insulin levels between the lean and obese animals in the HIEC study did not reach significance (Table 4), and the Pre-Clamp difference in lean vs. obese insulin for the LNA-HIEC study approached significance (p=0.053). Baseline insulin levels did not differ between the HIEC and LNA-HIEC studies. The increase in insulin observed during the stable hyperinsulinemic euglycemic period (Table 4; Stable-Clamp) was significant, for both weight groups and both studies (p<0.05).
Mean arterial pressure during the HIEC was not different from that of the LNA-HIEC studies, in either the lean or obese dogs (Figure 11, p=0.0857). Hyperinsulinemia did not significantly alter MAP in either weight group in the HIEC (Pre-HIEC vs. Post-HIEC) or LNA-HIEC studies (LNA-Pre-HIEC vs. LNA-Post-HIEC). However, in the LNA-HIEC study, the lean animals showed a trend toward reduction in MAP during hyperinsulinemia, while the obese dogs tended to increase MAP (p=0.03 unadjusted for multiple comparisons) when the HIEC was performed after NOS inhibition.

Analysis of the data collected during the HIEC and LNA-HIEC studies revealed that heart rate (Figure 12) was affected by weight group and NOS inhibition, but was not affected by hyperinsulinemia. The average difference in HR between the HIEC and LNA-HIEC studies was greater in the lean animals (38 BPM) compared to the obese (27 BPM), p=0.0004. Also, the difference in heart rate between the lean and obese animals was

Table 4: Insulin^ levels during the HIEC and LNA-HIEC studies.

<table>
<thead>
<tr>
<th></th>
<th>Pre-Clamp</th>
<th>Stable-Clamp</th>
<th>Pre-Clamp^b</th>
<th>Stable-Clamp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean†</td>
<td>5.8 ± 1.02</td>
<td>3.7 ± 0.82*</td>
<td>24.9 ± 8.55</td>
<td>48.6 ± 15.7#</td>
</tr>
<tr>
<td>Lean‡</td>
<td>31.9 ± 6.80#</td>
<td>39.3 ± 15.0#</td>
<td>16.72 ± 3.89</td>
<td>50.6 ± 11.6#</td>
</tr>
</tbody>
</table>

* Data are group means (IU/ml) ± standard error of the mean (SEM).
^ Pre-clamp period for the LNA-HIEC study was post LNA administration.
* Significantly different from Pre-clamp insulin value of the obese animals in same study (LNA-HIEC), p=0.053.
# Difference from Pre-clamp insulin value is significant (within the same weight group and study), p<0.05.
† Significant difference in Lean Pre-clamp insulin compared to obese Pre-clamp insulin, for both studies combined, p<0.05.
Data are groups means ± SEM obtained during a stable 20 min baseline period before the HIEC (Pre-HIEC or LNA-Pre-HIEC) and during the 20 min stable euglycemic period (Post-HIEC or Post-LNA-HIEC).

Figure 11: MAP response to hyperinsulinemia alone (HIEC) or during NOS inhibition (LNA-HIEC).

significantly influenced by whether it was measured during the HIEC study (lean-obese difference=27 BPM) or during the LNA-HIEC study (difference between lean and obese=36 BPM), p=0.0169. During the HIEC study, the difference in heart rate between the lean and obese weight groups was not significant. However, in the LNA-HIEC study, HR was significantly higher in the obese (p=0.0049; Figure 12) during both the LNA-Pre-HIEC (p=0.01) and the LNA-Post-HIEC (p=0.04) periods. Although, the HR differed...
between the lean and obese during LNA-HIEC study, this difference was evident prior to LNA administration (p=0.0444; Figure 13), and heart rate was not affected by the HIEC when performed alone or concurrent with NOS inhibition.

* Significantly different from lean animals during the same study period, p<0.01. Data are group means ± SEM during a stable 20 min baseline period before the HIEC (Pre-HIEC or LNA-Pre-HIEC) and during the 20 min stable euglycemic period (Post-HIEC or LNA-Post-HIEC).

Figure 12: Heart rate response to hyperinsulinemia alone (HIEC) or during NOS inhibition (LNA-HIEC).
Figure 13: Heart Rate response to LNA administration during the LNA-HIEC study

The difference in hind limb FVR between the HIEC and LNA-HIEC studies was influenced by weight group (DNX, p=0.055; intact, p=0.0419 main effect of weight group) and NOS inhibitory status (DNX, p=0.052; intact, p=0.017 for main effect of LNA treatment). However, there was no overall difference in FVR response to acute hyperinsulinemia between the intact and DNX limbs in either study, for either weight group. Although the obese animals had lower FVR compared to the lean dogs during both studies, the difference in FVR between the lean and obese only reached significance in the LNA-HIEC study (p=0.046; Figure 14). During the LNA-HIEC study, FVR was slightly higher than that observed during the HIEC study.
study. However, only in the denervated limb of the obese animals did the difference in FVR between the HIEC and the LNA-HIEC studies reach significance (p=0.0064; Figure 15).

Figure 14: Femoral vascular response to hyperinsulinemia alone (HIEC) or during NOS inhibition (LNA-HIEC).

**Significant difference in hind limb FVR between lean and obese during the LNA-HIEC study, p<0.05.**

Data are groups means ± SEM obtained during a stable 20 min baseline period before the HIEC (Pre-HIEC or LNA-Pre-HIEC) and during the 20 min stable euglycemic period (Post-HIEC or Post-LNA-HIEC).
Overall DNX-FVR of the obese dogs is significantly different between HIEC and LNA-HIEC studies, p<0.05. Data are groups means (n=6) ± SEM obtained during a stable 20 min period before (Pre-Clamp) or during the 20 min stable euglycemic period (Post-Clamp).

Figure 15: Difference in denervated limb FVR of the obese dogs during the HIEC and LNA-HIEC studies.
CHAPTER 5: DISCUSSION

The results of these studies suggest that, contrary to our original hypothesis, FVR increases immediately with weight gain. However, with continued weight gain, vascular resistance returns to baseline levels and may drop below baseline levels. This vascular response may be influenced by sympathetic activity, since the reduction in FVR observed with continued weight gain occurred more rapidly in the denervated hind limb. Additionally, these studies identify a difference in cardiovascular response to LNA administration (NOS inhibition) between lean and obese dogs. This response is suggestive of a reduction in NOS activity in the obese. Finally, our data suggest that nitric oxide activity mediates insulin sensitivity.

As has previously been reported in dogs (Rocchini et al., 1987; Truett et al., 1996), overfeeding of a high-fat diet caused significant weight gain in our animals. Consistent with our previous studies in which DEXA analysis was employed (Truett et al., 1996), this weight gain was associated primarily with an increase in fat deposition, although increased bone mineral content (BMC) was identified. However, it is unlikely that the marked change in BMC is the result of weight-induced bone remodeling, but rather was a limitation/artifact of the DEXA measuring technique. Similar artifacts have been reported when measuring BMC in humans, and are likely the result of beam hardening at extremes of tissue thickness and composition (Tothill and Pye, 1994; Tothill and Avanell, 1994; Jebb et al., 1993).
Study 1: Time course for the development of vascular resistance changes and insulin resistance with obesity.

Although we observed a significant time effect of overfeeding on MAP (measured weekly while on the treadmill; Figure 2), only in week 7 did the increase differ significantly from baseline. This elevation in MAP was slightly delayed in onset compared to our previous work (Truett et al., 1996), and may have been influenced by the presence of the L3-L6 ganglionectomy or exercise.

In 1991, Galt and Cronenwett reported a reduction in the MAP of anesthetized dogs in which unilateral lumbar sympathectomy procedures had been performed. However, an earlier report by this same group did not identify significant reduction in MAP with ganglionectomy (Cronenwett and Lindenauer, 1977). In neither case were the animals followed chronically in the conscious state to determine whether the change in MAP was persistent. Another chronic study using conscious dogs did not report MAP measurements (Lee et al., 1971). Thus, data in the literature is unclear, and it is possible that the unilateral paravertebral ganglionectomy in our study may have delayed the elevation of MAP with weight gain.

Another factor which may have influenced the development of hypertension in these animals (though an unlikely factor) was the weekly exercise regimen (walking ≤1.6 km/hr [0.44 m/s]; 30 min/week). Moderate exercise is known to reduce arterial pressure in humans (Jennings et al., 1984 and 1986; Kingwell et al., 1993; Pescatello et al., 1991). Most human
studies describe moderate exercise paradigms as 30-45 min of exercise three times per week, while maintaining approximately 60% \( \text{Work}_{\text{max}} \) (Jennings et al., 1984 and 1986; Kingwell and Jennings, 1993) or 70-80% \( \text{V}_{\text{O2max}} \) (Rogers et al., 1996). In the study by Rogers et al. (1996), low-intensity exercise training (40-50% \( \text{V}_{\text{O2max}} \), 3 times/wk) was reported to be a more effective stimulus in reducing resting arterial pressure and blood pressure responses to stress than was moderate-intensity exercise (70-80% \( \text{V}_{\text{O2max}} \)) in borderline hypertensive humans. Although this study reported a pressure-reducing benefit of exercise in humans, the exercise level, even in the low-intensity paradigm, was much more intense than that used in our weekly canine regimen. Thus, based upon human work, it is unlikely that the level of exercise provided by the weekly 30 min walking regimen was responsible for the delay in onset of elevated MAP in our dogs.

By comparison, O'Leary et al., (1997) used 8 km/hr with a 15% grade as a moderate exercise regimen in dogs. Mild exercise was described as 3.2 km/h at 0% grade in that study, while 4 km/h with a 13% grade, was used as a "mild" exercise regimen in dogs in a study examining systemic and regional blood flow at various exercise intensities (Pagny et al., 1986). The dogs in our studies walked slowly on a treadmill during the weekly studies at a speed less than <1.6 km/h, with zero grade. According to heart rate and oxygen consumption data previously observed in response to treadmill exercise in dogs (Cerretelli et al., 1964), the workload of our dogs, even at the highest average HR (week 7 walking HR =129 ± 8.4 BPM) corresponds
to less than 25% \( V_{O2\ max} \) if assessed using the graph presented in the Cerretelli study. Thus, the exercise level of our dogs was considerably less than that used in mild or low intensity exercise regimens in other canine studies, and one would not expect the exercise program to have influenced chronic MAP measurements.

Our study examining changes in vascular resistance with weight gain appear to indicate that femoral artery vascular resistance increases immediately following overfeeding/weight gain, and a reduction in FVR ensues with continued weight increase. These vascular changes may be influenced by autonomic nervous system input, as the reduction in FVR occurred earlier in the limb in which a lumbar sympathetic ganglionectomy was present. Systemic vascular resistance has been reported to be higher (Rocchini et al., 1987) and total peripheral resistance (TPR) reported to be lower than normal in obese dogs (Mizelle et al., 1994; Hall et al., 1995). Mizelle et al. reported an initial reduction in TPR followed by a trend toward, but not reaching, normal levels by week 4 of high-fat feeding. Our results, however, suggest that although a normal or reduced vascular resistance may be present in the obese dog, the initial stages of weight gain are associated with higher than normal FVR. As literature regarding this subject conflicts, further work in this area is warranted. Additionally, sympathetic activity may influence the vascular changes occurring during the initial period of weight gain, since the reduction in FVR to baseline levels as weight gain continued was prolonged in the intact limb. This
suggests that sympathetic activity to the limb is instrumental in maintaining vascular resistance at a level higher than that in vessels without sympathetic input. This too, warrants further study.

In examination of the relationship of insulin and weight gain in these studies, we observed increasing insulin concentrations (as a reflection of increasing insulin resistance) with weight gain. However, insulin did not appear to correlate with arterial pressure or vascular resistance. This is consistent with the work of John Hall and his colleagues (1990; 1995), who could not identify an effect of chronic insulin infusion to increase arterial pressure in lean or obese dogs. Although a correlation between insulin and the cardiovascular parameters (HR, MAP, or FVR) was not evident, the significant increase in insulin preceded the return of FVR by 1 week in the DNX limb, but by 3 weeks in the intact leg. This suggests that the increasing insulin concentration may work toward reductions in FVR and arterial pressure with weight gain were there not concurrent increased SNS activity in the obese. Previous findings that insulin infusion stimulates vasodilation (For review see Baron, 1994) and a reduction in arterial pressure (Hall et al., 1995) support this theory. Our findings suggest that autonomic activity in the intact limb influences FVR during weight gain, and increased insulin levels may help prevent continued elevation in FVR in vessels that remain sensitive to its effects. Thus, sympathetic activity may be an initiating factor predisposing to the development of hypertension associated with weight gain, and interaction with insulin may be important.
However, we did not observe a difference in response to acute hyperinsulinemia between lean and obese dogs in the HIEC portions of our studies, nor were there differences in the response to hyperinsulinemia in the DNX and intact limbs.

A recent study identified renal sympathetic activity as a primary cause of hypertension in obesity (Kassab et al., 1994). In that study, bilateral renal denervation greatly attenuated the development of hypertension with overfeeding. Thus, although sympathetic renal mechanisms appear to be a primary factor involved in the development of obesity-related hypertension (Kassab et al., 1994), our study suggests that peripheral vascular effects of increased sympathetic activity may also be important in initiating the development of hypertension, prior to measurable changes in arterial pressure. These vascular changes may play a pivotal role in the series of events leading to hypertension in the obese. They are not likely, however, to be working alone, and warrant further study.

Study 2: Cardiovascular parameters following NOS inhibition with LNA.

This study of canine cardiovascular function and stabilization following nitric oxide inhibition indicates that MAP, heart rate (HR) and femoral vascular resistance (FVR) are altered by administration of L-nitro-arginine. Also, the data suggest that the cardiovascular response to NOS inhibition differs between lean and obese dogs. Finally, once acclimated to
NOS inhibition, cardiovascular parameters remain stable for the time period required to perform the hyperinsulinemic euglycemic clamp procedures described in our primary experiments.

In this experiment, acute nitric oxide synthase inhibition with LNA significantly increased MAP, which is consistent with previous findings by Elsner et al., 1992. As in the Elsner study, a significant reduction in heart rate occurred following LNA treatment in our animals, but this response was significantly reduced in the obese. In addition, the obese dogs had a significantly lower FVR response to LNA. The obese animals in both the LNA-time course and the LNA-HIEC studies failed to show a significant change in FVR following LNA administration. However, a significant FVR response to LNA was evident in the lean dogs, suggesting greater NOS activity compared to the obese. This observation is consistent with the previous work in essential hypertensives, who exhibit reduced vascular relaxation (Panza et al., 1993a, b). The reduction in vascular reactivity in this population is thought to involve abnormalities of the NOS pathway (Panza et al., 1990 and 1993b). However, it is not due to reduced availability of the natural substrate for NO synthesis (Panza et al., 1993a), and is not isolated to a specific defect of endothelial cell muscarinic receptors (Panza et al., 1994) or a single signal transduction pathway (Panza et al., 1995). Thus, although data are growing to describe the mechanisms through which reduced vascular response to NOS inhibition occurs in a population with physiologic changes similar to that of the obese.
(essential hypertensives), the pathways remain obscure. The reduced vascular response to NOS inhibition in the obese could reflect a defect in NO synthesis, release or diffusion of NO to vascular smooth muscle.

In addition to the finding that after initial changes in MAP, HR and FVR cardiovascular parameters stabilize following LNA administration, this study suggests that obesity is a disorder of altered activity of the nitric oxide system. The obese animals of this study exhibited a reduced cardiovascular response to NOS inhibition. Also, the vascular response to NOS inhibition may be influenced by sympathetic nervous system activity, since the denervated and intact limb FVR response to LNA administration differed in the lean dogs. Additionally, since the FVR response to NOS inhibition differed in lean and obese dogs, this could be a pathway mediating the development of hypertension with weight gain. 

Studies 3 and 4: The HIEC, alone and following NOS inhibition.

In these studies examining the interaction of NOS, insulin and sympathetic activity, baseline insulin levels of the obese animals were greater than the lean. This suggests reduced insulin sensitivity in the overweight dogs, reflected as higher insulin levels. The finding of higher glucose infusion rates (GIR) during hyperinsulinemic euglycemia in the lean animals also supports a difference in insulin sensitivity between the weight groups (i.e. the lean are more sensitive to insulin-stimulated glucose disposal, thus require higher exogenous glucose infusion to maintain euglycemia). These observations are consistent with previous findings of
hyperinsulinemia and/or reduced response to a glucose tolerance test in the obese, reflecting resistance to insulin stimulated glucose disposal (Rocchini et al., 1987, 1989 and 1996; Truett et al., 1996).

When hyperinsulinemic euglycemia was performed concurrent with NOS inhibition (LNA-HIEC study), no difference in GIR between the lean and obese animals was observed. Thus, NOS activity appears to influence insulin sensitivity in the canine. This finding contradicts those of Swislocki et al. (1995), who report similar glucose tolerance in response to oral glucose tolerance tests in controls and rats administered L-NAME in their drinking water. However, our data coincide with a study by Petrie et al. (1996), who report a positive relationship between basal vascular NO production and insulin sensitivity in humans. In that study, individuals who were relatively insensitive to insulin-mediated glucose uptake using the clamp method also exhibited decreases in basal endothelial NO production.

Although glucose infusion rate within each weight group did not differ between the HIEC and LNA-HIEC studies, the lean animals tended to require a lower infusion rate of glucose when the clamp procedure was performed following LNA administration. This suggests that NOS inhibition may have a greater effect on insulin sensitivity in normal compared to obese dogs. Two mechanism through which a reduction in insulin sensitivity may have occurred in the lean versus the obese dogs in our studies are:
1) A greater increase in vascular resistance following LNA in the lean animals caused a greater reduction in blood flow in this group. This reduction in flow decreased insulin delivery to tissues, thereby reducing insulin stimulated glucose disposal and insulin sensitivity of the lean animals.

2) If insulin's effect to reduce vascular tone and stimulate glucose uptake is mediated partly through nitric oxide activity (Chen and Messina, 1996; Baron, 1996), reduced NOS activity in the obese animals would cause a baseline insulin resistance in this weight group. The lean animals, having greater baseline NOS activity would be more sensitive to insulin stimulation. If NOS was inhibited in both lean and obese animals, the change in insulin sensitivity between studies with and without NOS inhibition should be larger in the lean animals (since they should have greater baseline NOS activity). Thus, the influence of blocking nitric oxide on insulin's ability to stimulate glucose disposal should be more severely affected in the lean animals.

In support of mechanism number 1, insulin resistant subjects [obese in our study] are known to have a reduced vascular response to NO blockade (Panza et al., 1993a and b [in essential hypertensives]; Petrie et al., 1996). Thus, if vascular NOS activity is greater in the lean insulin sensitive dogs, there may have been a greater effect of NOS inhibition to reduce tissue perfusion in this group. The larger increase in FVR (lower flow) of the lean
dogs may have resulted in a reduction in insulin delivery, and a tendency toward reduced insulin sensitivity during the LNA-HIEC study. Skeletal muscle blood flow is known to modulate insulin-mediated glucose uptake (Baron et al., 1994). Thus, differences in tissue perfusion could have affected insulin sensitivity (as reflected by GIR) in these studies. In the obese dogs, with reduced baseline NOS activity, the vasculature may have been less affected by NOS inhibition. Thus, insulin delivery (resultant from changes in blood flow/FVR) may not have been as greatly altered in the obese dogs during hyperinsulinemia in LNA-HIEC study. Thus, insulin sensitivity would not appear to differ between the HIEC and LNA-HIEC studies in the obese (as observed in our dogs). In the lean animals of the LNA-HIEC study, the effects of LNA to increase vascular resistance (reduce flow) may have been greater than in the obese. This lower flow in the lean animals of the LNA study may have resulted in reduced glucose disposal (seen as increased glucose infusion rate of the lean, compared to the HIEC study alone). Thus, the difference in glucose infusion rates between lean and obese dogs of the HIEC study would no longer be evident in the LNA-HIEC study. The findings of Baron and his colleagues (1995), suggest that reduced skeletal muscle perfusion contributes to insulin resistance induced by L-NMMA in rats. In addition, the work of Bergman and his colleagues suggests that delayed insulin delivery to tissues an important factor in the insulin pathway responsible for the insulin resistant state (Yang et al., 1989; Ader and Bergman, 1994), and is thus somewhat flow dependent.
Thus, the insulin sensitivity results of our HIEC and LNA-HIEC studies could possibly reflect differing tissue perfusion.

The finding of reduced insulin sensitivity mainly in the lean dogs during the LNA-HIEC study also supports the second hypothesized mechanism of NOS-insulin interaction (NOS-mediated insulin activity mediates insulin sensitivity/glucose uptake). There is a large body of evidence supporting the fact that insulin-mediated vasodilation is nitric oxide dependent (Steinberg et al., 1994; Scherrer, et al., 1994) and the degree of vasodilation is proportional to insulin sensitivity (For review see Baron, 1996). In obese humans with and without NIDDM, a recent study found a 40-50% reduction in endothelium-dependent vasodilation under basal conditions, as well as a marked impairment of insulin's physiologic ability to enhance this response (Steinberg et al., 1996). The lean animals of our study exhibited a greater increase in FVR with LNA administration than observed in the obese (Study 2 of these experiments; the LNA-cardiovascular stabilization study). This suggests higher NOS activity in the lean versus obese dogs in the basal state. Thus, insulin (if mediated through NO activation) should have caused a greater effect on glucose disposal in the lean animals, as observed in the HIEC study. Also, if basal NO activity is increased in the lean, baseline insulin sensitivity should also be higher than in the obese. This was the case in the lean animals of our studies, who had increased insulin sensitivity compared to the obese dogs of our studies (based upon higher basal insulin levels and increased glucose
infusion rates required to maintain euglycemia during hyperinsulinemia). These data suggest that insulin action may be mediated by nitric oxide. Also, NOS inhibition appears to have a greater effect on insulin sensitivity in lean versus obese dogs, possibly due to greater nitric oxide activity in lean individuals.

In the HIEC and LNA-HIEC studies, MAP was not significantly affected by acute hyperinsulinemia, in either the lean or obese dogs. This finding is consistent with data of Hall et al., 1995, who did not find significant elevations in MAP with chronic insulin infusion in normal or obese dogs. Interestingly however, the change in MAP observed in response to hyperinsulinemia when NOS was concurrently inhibited (LNA-HIEC study) varied slightly between the lean and obese. In this study, hyperinsulinemia tended to reduce MAP in the lean animals, whereas MAP in the obese animals was slightly increased. Although these differences were not significant in our small group of animals, further study is warranted.

Although MAP increased slightly in the obese and slightly decreased in the lean following hyperinsulinemia during the LNA-HIEC study, neither the femoral vascular resistance or HR was significantly influenced by hyperinsulinemia alone (HIEC) or concurrent with NOS inhibition (LNA-HIEC). However, in the lean animals a slight reduction in FVR was observed when insulin was infused following LNA administration. This was not observed in the obese. Also, the obese dogs exhibited a slight increase in
HR during insulin infusion in the LNA study. This increase in HR suggests an increase in cardiac output (CO) occurred with insulin infusion, as previously reported (for review see Baron, 1994). In the presence of an inappropriate vascular response in the obese dogs however, this increase in CO may have led to the increase in obese MAP. In the lean group, who had a more appropriate reduction in FVR (though not significant) in response to an increase in CO, MAP remained unchanged. This hypothesis cannot be proven from our data however, since cardiac output was not measured in our studies.

Since increases in MAP reflect changes in systemic vascular resistance, cardiac output, or blood volume/renal mechanisms (Guyton, 1991), one cannot fail to mention other mechanisms through which the slight increase in MAP observed in the obese during NOS inhibition and insulin infusion may have developed. Increases in vascular resistance may have occurred in unmeasured portions of the circulatory system (other than the femoral arteries) or renal fluid retention may have precipitated increases in plasma volume and cardiac output. Alternatively, a direct change in cardiac inotropic function may have taken place, increasing stroke volume and cardiac output (CO = stroke volume \cdot HR) and precipitating the pressure elevation observed in the obese dogs during the LNA-HIEC study. However, neither renal function nor cardiac output were measured in these experiments.
Although the data do not prove that cardiac output increased with insulin infusion, and the cardiovascular changes discussed above did not reach significance, baseline data collected during these studies support the hypothesis that abnormal vascular responses were present in the obese animals. Prior to manipulation with insulin, the HR of the obese dogs in the LNA-HIEC study was greater than that of the lean animals. However, although FVR is lower in the obese group, the obese dog MAP is slightly higher than the lean. Thus, prior to insulin administration, the peripheral vascular response to the increased heart rate in the obese animals was not adequate to compensate for the assumed higher cardiac output (CO=stroke volume • HR) of the obese animals. Thus, infusion of insulin may have further increased cardiac output, and with inadequate peripheral vascular response in the obese, MAP increased. As mentioned above, CO=SV.HR, and changes in muscular efficiency of the heart can occur which may negate a rise in CO with a concurrent rise in HR (Guyton, 1991). Thus, inappropriate vascular response to an increase in CO induced by insulin may have resulted in the increase in MAP observed in the obese group. Since we did not measure cardiac output, we cannot prove that the higher baseline HR of the obese was associated with increased CO, nor that insulin increased CO during hyperinsulinemia.

Though an unlikely mechanism, MAP may have increased during acute hyperinsulinemia and concurrent NOS inhibition via renal mechanisms. Nitric oxide inhibition has been shown to increase renal
vascular resistance and reduce renal blood/plasma flow in dogs (Elsner et al., 1992; Majid et al., 1993), reducing sodium excretion (Majid et al., 1993). Additionally, nitric oxide-induced changes in the renin-angiotensin system are reported, but are not as clear (Elsner et al., 1992; Salazar et al., 1992; Schnackenberg et al., 1997; Sigmon et al., 1992). LNA-induced changes in renal function may have led to reduced sodium and water excretion in the animals of the LNA-HIEC study. Additional exogenous infusion of insulin and glucose during the clamp procedure may have compounded fluid changes occurring with LNA administration. Since insulin is known to have anti-natriuretic effects (DeFronzo, 1976, Rocchini et al 1989b), the obese are reported to have a reduced natriuretic response to volume expansion (West et al., 1992), and insulin resistant hypertensive dogs may have increased renal sensitivity to NOS inhibition (Martinez et al., 1993), performing the clamp procedure with concurrent NOS inhibition may have changed renal fluid handling enough to increase MAP (via volume expansion) in the obese but not the lean dogs. Total body water and renal function were not measured in our studies, so these hypotheses are only speculation. Renal regulation of arterial pressure is a more chronic method of pressure regulation. Thus, significant volume expansion (with associated increases in cardiac output and MAP) would be unlikely in such a short protocol (approximately 2hr). Additionally, average quantity of fluid (insulin and glucose infusions) administered to the obese dogs in the LNA-HIEC study
was approximately 60 ml, which would be unlikely to cause significant volume expansion.

Thus these studies suggest that interaction between insulin and nitric oxide may influence cardiovascular responses to weight gain. The nitric oxide system is important for the lean and obese response to insulin, and may be an important mechanism influencing insulin sensitivity. Although the results did not reach significance, differences in the insulin-nitric oxide relationship in the vasculature of the obese may occur, with a trend toward elevations in arterial pressure during acute hyperinsulinemia. However, the mechanism through which arterial pressure increases in the obese when insulin is infused during NOS inhibition remains obscure.

The small number of animals used in these studies, and the large variation in response may have overshadowed the ability to identify small significant differences (especially since unadjusted p-values suggest a trend toward increased MAP and HR in the obese). This area deserves further study, since this alteration may be important in the arterial pressure response to weight gain as MAP tended to increase in the obese but decrease in the lean.
CHAPTER 6: CONCLUSIONS

The studies described herein suggest that in the initial stages of overfeeding, there is increase in FVR, which then decreases to normal levels. The changes in FVR appear to be influenced by sympathetic activity, as the return to baseline FVR was delayed in the limb retaining normal sympathetic innervation. Interaction of insulin with the vascular responses to weight gain does not appear significant, since correlation was not evident between insulin levels and vascular resistance.

The primary studies also provided interesting data about the NOS/insulin/SNS relationship. Lean and obese dogs respond differently to nitric oxide synthase inhibition: the obese exhibited a reduced cardiovascular response to LNA administration (Study 2). As we hypothesized, SNS activity may be an important mediator (inhibitor) of NO activity, since the denervated limbs of the lean dogs showed a greater increase in FVR following NOS inhibition. In addition, this SNS/NO interaction may be abnormal in the obese, since the response of the DNX limb of the obese dogs was lower than in the lean. We speculate that higher sympathetic activity in the obese may reduce peripheral NOS activity in this population, and reduce NOS-mediated vascular relaxation. Further examination of the nitric oxide/SNS interaction is warranted in the search for the mechanisms involved in the development of hypertension in the obese.
Contrary to our hypothesis that NOS-mediated vascular response to insulin is abnormal in the obese, these studies did not identify a significant difference in cardiovascular response to hyperinsulinemic euglycemia between lean and obese dogs, when performed alone or in concert with nitric oxide inhibition. However, the trend toward increasing MAP with insulin infusion and concurrent NOS inhibition in the obese suggests that changes in the insulin-NO relationship may occur in the obese, predisposing to elevated arterial pressure. Thus, further study, using a larger sample size, is warranted to clarify changes in nitric oxide and insulin responses of the obese.

Another interesting finding of these studies is that NO activity influences insulin sensitivity in lean animals, with NOS inhibition resulting in a more 'resistant' state. This 'resistance' may involve a greater effect of NOS inhibition to reduce blood flow in the lean thereby altering insulin delivery to tissues, or may occur via reduced NOS-mediated insulin stimulated glucose disposal. Increases in SNS activity reported to occur in the obese may be a mechanism mediating this reduction in NOS activity, and thus indirectly influence insulin sensitivity.
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Animals arrive; weight maintenance
Dog Chow®

4+ weeks

Begin acute lean studies; HIEC, LNA-HIEC, response to LNA

Day 1 of 7-week overfeeding period with weekly MAP/HR/FVR/insulin measurement

Repeat acute studies in obese; HIEC, LNA-HIEC, response to LNA

Collect tissues for catecholamine levels

3-4 weeks

3-4 weeks

Animals arrive; weight maintenance
Dog Chow®
APPENDIX B: ABBREVIATIONS

ACH  acetylcholine
BMC  bone mineral content obtained using DEXA
BPM  beats per minute
cGMP guanosine 3', 5'-cyclic monophosphate
CNS  central nervous system
DEXA dual emission x-ray absorptiometry
DNX  SNS denervated limb (L3-L6 paravertebral ganglionectomy)
FBF  femoral artery blood flow
FVR  femoral vascular resistance calculated using $FVR = \frac{MAP}{FBF}$
HR  heart rate (BPM)
Intact  limb with sympathetic activity intact (right hind limb)
IU  international units
LBM  lean body mass obtained using DEXA
LBNP  lower body negative pressure
LNA or L-NAME  $N^G$-nitro-L-arginine-methyl ester
LNAMMA  L-$N^G$-monomethyl-arginine
LNNA  $N^G$-nitro-L-arginine
MAP  mean arterial pressure (mmHg)
NE  norepinephrine
NIDDM  non-insulin dependent diabetes mellitus

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<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
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<tr>
<td>NTS</td>
<td>nucleus tractus solitarius</td>
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<tr>
<td>SEM</td>
<td>standard error of the group mean</td>
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<tr>
<td>SHR</td>
<td>spontaneously hypertensive rat</td>
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<tr>
<td>SNS</td>
<td>sympathetic nervous system</td>
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VITA

Agatha T. Borne was born into the family of Dr. Leon L. Borne, Jr. and Nettie Trosclair Borne on November 7, 1964. She was the fourth of five children, all born in Thibodaux, Louisiana, and raised in the small community of Kraemer. Her elementary school education was obtained locally, with junior high school and high school levels undertaken in Thibodaux, Louisiana. During junior high and high school, Agatha worked in a family-owned grocery store. However, throughout her senior year of high school and college she also worked as a paraprofessional for mentally and physically handicapped students requiring transport to special schools. Following graduation from Thibodaux High School, Agatha attended Nicholls State University, in Thibodaux. During college, she was awarded both a T.H. Harris and an academic scholarship, based upon academic excellence. Although Agatha aspired to become a veterinarian, she explored other options, and in 1984 obtained her pilot's license. To complete the final requirements for application to veterinary school she attended Louisiana State University. While in college at L.S.U., she held a student job working for the LSU Alumni Association.

In 1985 Agatha was accepted into the L.S.U. School of Veterinary Medicine. She was allowed to participate in an honors research program and become a member of the Phi Zeta Veterinary Honors Society. Agatha spent 10 weeks of her senior year of veterinary school at Castleton Farms in Lexington, Kentucky. Here she gained experience in equine practice under
the direction of Dr. H. Steve Conboy. In 1989 Agatha completed her studies, earning a Doctor of Veterinary Medicine degree.

Following graduation Agatha completed a one-year internship at the Boren Veterinary Teaching Hospital in Stillwater, Oklahoma, and a two-year residency at the North Carolina State University College of Veterinary Medicine in Raleigh, North Carolina. Both were in large animal (equine) medicine. In 1992 Agatha entered the graduate program of the LSU-School of Veterinary Medicine in the Department of Veterinary Physiology, Pharmacology and Toxicology, working toward fulfilling the requirements for a doctoral degree in physiology. She worked in the research laboratory of Dr. David B. West, at the Pennington Biomedical Research Center, becoming intimately involved in studies examining mechanisms of obesity-induced hypertension. In 1995 Agatha was awarded a Research Service Award from the Louisiana Affiliate of the American Heart Association, to study the interaction of the nitric oxide and the sympathetic nervous systems in the development of diet-induced obesity and hypertension in dogs. In 1996 this award was relinquished, when she was awarded a two year National Research Service Award, Individual Postdoctoral Fellowship from the National Heart, Lung and Blood Institute to study the same disorder.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Agatha T. Borne

Major Field: Veterinary Medical Sciences

Title of Dissertation: The Role of Hyperinsulinemia, the Sympathetic Nervous System and Nitric Oxide in Cardiovascular Function During the Development of Obesity-Induced Hypertension

Approved:

[Signatures]

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

December 16, 1997