CARDIOVASCULAR EFFECTS OF L-ARGININE IN A RAT MODEL OF HEART FAILURE

by

Amanda Jay Fortin, B.Sc. (Hon.)

Department of Pharmacology and Toxicology

A thesis submitted in partial fulfilment of

the requirement for the degree of

Master of Science

Faculty of Graduate Studies

The University of Western Ontario

London, Ontario

May, 1997

ABSTRACT

Impaired endothelium-dependent vasodilation may contribute to increased vasoconstriction in heart failure. Studies have shown that acute L-arginine treatment restores impaired endothelium-dependent relaxation. This thesis examines the effects of chronic oral L-arginine treatment on cardiovascular function in heart failure rats.

Sprague-Dawley rats were treated with L-arginine in drinking water (12.5g/L & 50g/L) or water placebo for 8 weeks following coronary artery ligation or sham surgery. The lower dose of L-arginine treatment restored the decreased plasma L-arginine levels seen in heart failure, while the higher dose of L-arginine produced significantly higher plasma levels than seen in sham placebo. The lower dose but not the higher dose of L-arginine significantly improved endothelium-dependent relaxation of isolated aortic rings in heart failure. Neither treatment affected basal hemodynamic parameters.

Thus, chronic, low dose, oral L-arginine treatment restores plasma L-arginine levels and improves endothelial-dependent relaxation in heart failure rats but does not restore basal cardiac performance.

KEYWORDS: L-Arginine Heart Failure Nitric Oxide

Endothelial Function Rat model

ACKNOWLEDGEMENTS

I would like to thank my supervisors, Dr. J.Malcolm O.Arnold and Dr. Qingping Feng, for their help and guidance along the way. I would like to acknowledge the help of all the people in the lab, especially Xiangru, Jeremy, Pam, John, and Michelle, who freely gave their assistance and friendship whenever it was needed.

I thank the members of my advisory committee, Drs. M. Cook, M. Karmazyn, and B. Kline, for their patience and flexibility, as well as for their constructive criticism.

I also thank my parents, Bruce and Debbie, my brother Adam, and my grandparents for all their support and encouragement over the years. Special thanks to Margaret who made my summer in London much more enjoyable. And last but not least, I want to thank Jeffrey for all his love and support.

These studies were supported by a Grant-in-Aid from the Heart and Stroke Foundation of Ontario, the Victoria Hospital Research Fund, the Indian and Inuit Health Careers Program and through the Ontario Graduate Scholarship fund.

TABLE OF CONTENTS

Certificate of Examination		ii
Abstract		iii
Acknowledgements		iv
Table of Contents		v
List of Tables		viii
List of Figures		ix
List of Abbreviations		xi
1 Introduction		1
1.1	Rationale	2
1.2	Heart Failure	3
1.2.1	Prevalence of Heart Failure	4
1.2.2	Vasodilation Therapy in Heart Failure	4
1.2.3	Compensatory Responses in Heart Failure	5
1.2.4	Endothelial Function in Heart Failure	10
1.3	Endothelium-Derived Relaxing Factor	11
1.3.1	Nitric Oxide Activity	14
1.3.2	Systemic Effects of Nitric Oxide: Implications in Heart Failure	16
1.3.2.1	Basal Nitric Oxide Release	18
1.3.2.2	Stimulated Nitric Oxide Release	19
1.3.3	Endothelial Dysfunction in Heart Failure	20
1.4	L-Arginine	21
1.4.1	L-Arginine Kinetics	22
1.4.2	Physiological Effects of L-Arginine	25
1.4.3	Effects of L-Arginine and Nitric Oxide Production	27

2 Materials and Methods 35

2.1 Animal Protocol 36

2.1.1 Rationale for Use of Current Heart Failure Model 36

2.1.2 Coronary Artery Ligation Surgery 36

2.1.3 Post Mortem Examination 38

2.1.4 Measurement of Infarct Size 38

2.2.1 Rationale for Hemodynamic Study 40

2.2.2 Hemodynamic Protocol 41

2.3.1 Rationale for Organ Bath Study 43

2.3.2 Organ Bath -- Ring Preparation 44

2.3.3 Organ Bath -- Apparatus 44

2.3.4 Organ Bath Protocol 45

2.4.1 Rationale for Nitrate Assay 46

2.4.2 Nitrate Assay Protocol 48

2.5.1 Rationale for L-Arginine Treatment Dose 49

2.5.2 L-Arginine Treatment 50

2.5.3 Measurement of Plasma L-Arginine Levels 51

2.6 Statistical Analysis 51

3 Results 53

3.1 Characterization of the Rat Heart Failure Model 54

3.2 Characterizing the Chronic Oral L-Arginine Treatment 58

3.3 The Effects of L-Arginine Treatment on Cardiac Function in Sham Rats 64

3.4 The Effects of L-Arginine Treatment on Cardiac Function in Heart Failure Rats 66

3.5 The Effects of L-arginine Treatment on Endothelium-Dependent Relaxation 77

4 Discussion 85

4.1 Establishing the Heart Failure Model 86

4.2 L-Arginine Availability in Heart Failure 88

4.3 L-Arginine Treatment, Dosage, and Plasma Levels 89

4.4 Plasma Nitrate Levels 90

4.5 L-Arginine Effects on Cardiac Function in Sham Rats 92

4.6 L-Arginine Effects on Cardiac Function in Heart Failure Rats 93

4.7 Effects of L-Arginine on Endothelium-Dependent Relaxation 95

4.8 Summary and Conclusions 97

References 99

Vita 120

List of Tables

Table I: Physical and hemodynamic characteristics of heart failure rats compared to sham. 56

Table II: Physical and hemodynamic characteristics of untreated, low dose, and high dose L-arginine treated sham rats. 65

Table II: Comparison of the EC_{50} values of untreated and L-arginine treated sham and heart failure rats for relaxation responses to sodium nitroprusside and acetylcholine. 79

List of Figures

Figure 1: The biosynthetic and metabolic pathways of L-arginine including the synthesis of nitric oxide and its inhibitors. 13

Figure 2: Possible L-arginine pathways for improving cardiac performance in heart failure. 29

Figure 3: Photographs of third section of representative sham and ligated left ventricles sliced transversely from base to apex. 57

Figure 4: Average daily water consumption measured for 8 weeks following coronary artery ligation or sham surgery. 60

Figure 5: Average dose of L-arginine consumed by low and high dose L-arginine treated sham and heart failure rats. 61

Figure 6: The effect of heart failure and chronic oral L-arginine treatment on plasma L-arginine concentrations. 62

Figure 7: The effect of heart failure and chronic oral L-arginine treatment on plasma nitrate and nitrite concentrations. 63

Figure 8: Comparison of infarct sizes induced by coronary artery ligation in untreated, low, and high dose L-arginine treated heart failure rats. 68

Figure 9: Effect of L-arginine treatment on body weights of rats 8 weeks following coronary artery ligation. 69

Figure 10: Effect of L-arginine treatment on heart weights of rats 8 weeks following coronary artery ligation. 70

Figure 11: Effect of L-arginine treatment on atria and right ventricle weights of rats 8 weeks following coronary artery ligation. 71

Figure 12: Effect of L-arginine treatment on the left ventricle weights and volumes of rats 8 weeks following coronary artery ligation. 72

Figure 13: Effect of L-arginine treatment on cardiac index in rats 8 weeks following coronary artery ligation. 73

Figure 14: Effect of L-arginine treatment on left ventricular end-diastolic pressures in rats with heart failure. 74

List of Figures cont'd:

Figure 15: Effect of L-arginine treatment on left ventricular systolic pressures in rats with heart failure. 75

Figure 16: Effect of L-arginine treatment on left ventricular pressure development (+/- dP/dt) in rats with heart failure. 76

Figure 17: Cumulative concentration-response curves to acetylcholine and sodium nitroprusside done in thoracic aortic ring segments of heart failure and sham rats preconstricted to $PGF_{2\alpha}$. 80

Figure 18: Cumulative concentration-response curves to acetylcholine and sodium nitroprusside done in thoracic aortic ring segments of untreated, low and high dose L-arginine treated sham rats. 81

Figure 19: Cumulative concentration-response curves to acetylcholine and sodium nitroprusside done in thoracic aortic ring segments of untreated and low dose L-arginine treated heart failure rats. 82

Figure 20: Cumulative concentration-response curves to acetylcholine and sodium nitroprusside done in thoracic aortic ring segments of untreated and high dose L-arginine treated heart failure rats. 83

List of Abbreviations

ACE	angiotensin converting enzyme
ADMA	N^G-N^G-dimethylarginine, asymmetric dimethylarginine
ANOVA	one-way analysis of variance
ATPase	adenosine triphosphatase
bpm	beats per minute
°C	degrees Celcius
CHF	congestive heart failure
$CaCl_2$	calcium chloride
cAMP	adenosine 3',5'-cyclic monophosphate
cc	millilitre
cGMP	guanine 3',5'-cyclic monophosphate
CI	cardiac index
cNOS	constitutive endothelial nitric oxide synthase, NOS III
CO	cardiac output
CO_2	carbon dioxide
dP/dt	rate of pressure development
EC_{50}	concentration required to achieve 50% maximum effect
g	gram
IGF-I	insulin-like growth factor-I
iNOS	inducible nitric oxide synthase, NOS II
i.p.	intraperitoneal
KCl	potassium chloride
kg	kilogram
KH_2PO_4	potassium phosphate
K_m	Michaelis constant, substrate concentration to achieve half maximal reaction velocity under saturating conditions
LD_{50}	lethal dose in 50% of the population
L-NMMA	N^G-monomethyl-L-arginine
LV	left ventricle
LVEDP	left ventricular end-diastolic pressure
M	molar
$MgSO_4$-$7H_2O$	magnesium sulphate - hydrated
min	minute
mg	milligram
mL	millilitre
mM	millimolar
mmHg	millimeter of mercury
mmol	millimole
NaCl	sodium chloride
$NaHCO_3$	sodium carbonate
NED	N-(1-napthyl)ethylene diamine
PE-50	polyethylene tubing size 50

List of Abbreviations cont'd:

$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PKG	cGMP-stimulated protein kinase
RNA	ribonucleic acid
rpm	rotations per minute
sec	second
SEM	standard error of the mean
SLVP	systolic left ventricular pressure
TGFβ	transforming growth factor β
TNF	tumor necrosis factor
μM	micromolar

1. Introduction

1.1 Rationale for the Study

Vascular function is important in the control of cardiac performance as evidenced by the emergence of vasodilation therapy as an important advancement in the treatment of heart failure in recent years. Vasodilators unload the heart by decreasing peripheral vascular resistance and improving myocardial performance (Haas & Leier, 1994).

Nitric oxide is a potent endogenous vasodilator synthesized from L-arginine by nitric oxide synthase in the endothelium. Nitric oxide is released in response to many vascular stimuli, including acetylcholine (Palmer *et al.*, 1988b), however, in heart failure this endothelium-dependent vasodilation to acetylcholine is impaired (Drexler *et al.*, 1994; Feng *et al.*, 1997; Teerlink *et al.*, 1993), and is thought to contribute to increased vascular resistance in heart failure (Drexler *et al.*, 1992; Kubo *et al.*, 1991; Ontkean *et al.*, 1991). The decreased endothelium-dependent relaxation in heart failure is associated with a decreased activity of the constitutive endothelial nitric oxide synthase (cNOS) enzyme. (Feng *et al.*, 1996b; Feng *et al.*, 1996c; Ontkean *et al.*, 1991).

Recent studies have demonstrated that L-arginine availability is a rate-limiting step for nitric oxide synthase (Rubanyi, 1991). Decreased L-arginine availability results in decreased nitric oxide production in endothelial cells. As well, plasma levels of an endogenous nitric oxide synthase inhibitor, asymmetric dimethylarginine (ADMA), have been shown to be increased in heart failure (Feng *et al.*, 1996c) and to impair endothelium-dependent relaxation to acetylcholine. L-arginine competes with this inhibitor for the active site on nitric oxide synthase and dose-dependently reverses the inhibition of ADMA (Azuma *et al.*, 1995).

Supplementation of the nitric oxide precursor, L-arginine, has been shown to normalize vascular dysfunction in heart failure in acute *in vivo* and *in vitro* studies (Feng *et al.*, 1996c; Hirooka *et al.*, 1994; Ogilvie & Zborowska-Sluis, 1995; Panza *et al.*, 1993a), suggesting a decreased L-arginine availability or an imbalance between the levels of L-arginine and endogenous nitric oxide synthase inhibitors in heart failure. The success of L-arginine in acutely improving impaired endothelial function in heart failure prompted research into its contingency as a chronic treatment of heart failure. Thus, the potential for chronic L-arginine administration as a novel vasodilator treatment in heart failure was investigated using a rat model of heart failure and monitoring vascular responsiveness *in vitro* as well as hemodynamic parameters *in vivo*.

1.2 Heart Failure

Heart failure is a state of impaired cardiac function characterized by decreased cardiac output, elevated left ventricular end-diastolic pressure, and decreased contractility. As a consequence, the heart muscle is incapable of supplying the body's requirements for nutrients and oxygenated blood. Heart failure is a disease of the heart and circulation which evolves over time as a consequence of initial stress or damage to the myocardium. The etiology of heart failure may involve acute myocardial infarction, toxins such as alcohol or cytotoxic drugs, infection by viruses, or prolonged cardiovascular stress due to hypertension or valvular disease. In some cases the cause of cardiac injury is not identified and the syndrome is then termed idiopathic cardiomyopathy (Sandler & Fry, 1990).

Hemodynamic and neurohumoral mechanisms are activated to aid in preserving cardiac function and tissue perfusion after myocardial injury. The condition of heart failure develops not when the heart is first injured but when such compensatory mechanisms are overwhelmed or exhausted (Packer, 1992).

1.2.1 Prevalence of Heart Failure

Heart failure is not only one of the leading causes of death for adults in Canada (Busse *et al.*, 1994) but it is also a major public health problem in most developed countries. Patients with congestive heart failure make up approximately 1% of the total populations of Canada and the United States (Brophy, 1992; Kannel *et al.*, 1994; Schocken *et al.*, 1992). In the United States, the number of deaths in which heart failure was considered to be the underlying cause increased from 118,000 in 1970 to 270,000 in 1990 (Group, 1996). Thus, the incidence and prevalence of heart failure have been increasing in recent years despite significant advances in prevention and treatment of cardiovascular diseases (Kannel *et al.*, 1994).

1.2.2 Vasodilation Therapy in Heart Failure

Among the current approaches to the treatment of heart failure are diuretics, positive inotropic drugs, and vasodilators including angiotensin converting enzyme (ACE) inhibitors (Dargie *et al.*, 1987). Diuretics function to reduce blood volume through increased sodium excretion. Positive inotropic agents such as the digitalis glycosides and catecholamines function to increase cardiac contractility and thereby improve cardiac output, however, they also increase oxygen demand which may be detrimental (Haas & Leier, 1994).

An important advancement in the treatment of heart failure involves the use of drugs which reduce peripheral vascular resistance through vasodilation. A good example of vasodilator therapy in heart failure is the use of ACE inhibitors. This class of drugs inhibits the enzyme that produces the vasoconstrictor angiotensin II and degrades the vasodilator bradykinin (Dargie *et al.*, 1987). Through these mechanisms ACE inhibitors both inhibit vasoconstrictor actions and promote vasodilation in heart failure.

The primary objective of vasodilator therapy is to unload the volume- or pressure-overloaded heart by reducing preload and afterload, thus improving clinical status, exercise capacity, and survival (Haas & Leier, 1994). By reducing ventricular systolic wall stress through afterload reduction and diastolic wall stress through preload reduction, vasodilator therapy reduces myocardial oxygen consumption. This has a favourable effect on myocardial energetics in heart failure and improves systolic and diastolic performance (DeMarco *et al.*, 1988; Haas & Leier, 1994). Interventions that successfully unload pressure- and volume-overloaded ventricles generally result in a regression of myocardial hypertrophy (Cooper, 1987; McDonald *et al.*, 1993; Pardis *et al.*, 1984; Smith & White, 1986). All of these potential improvements may contribute to stabilization and perhaps regression of the condition of heart failure. This thesis investigates a possible role for L-arginine as a new vasodilator therapy in heart failure.

1.2.3 Compensatory Responses in Heart Failure

The major factors affecting cardiac function include preload, afterload, and contractility. Preload is the stretching force that determines the precontraction muscle length, often determined by the amount of blood in the ventricles at the end of diastole

(Little & Little, 1984). An increase in the preload of a healthy heart triggers a compensatory increase in stroke volume in accordance with the Frank-Starling Law (Sandler & Fry, 1990). Afterload is the tension that must be developed by the myocardium to overcome the forces that oppose ventricular ejection. These forces are determined by a number of factors including arterial impedance which takes into account peripheral vascular resistance and arterial compliance (Little & Little, 1984). The third factor, contractility, is the strength with which the myocardium contracts. Contractility is increased through a variety of mechanisms including activation of the sympathetic nervous system. However, increased contractility concomitantly increases myocardial oxygen consumption which may contribute to the progression of heart failure (Sandler & Fry, 1990). Patients with heart failure characteristically have both increased preload and afterload as well as depressed myocardial contractility (Little & Little, 1984).

Decreased cardiac output, a primary sign of heart failure, is a consequence of increased preload, afterload, and decreased ventricular function. The body responds to the condition of heart failure along several lines including cardiac dilatation, hypertrophy, and activation of the sympathetic nervous system, the renin-angiotensin system and other neurohumoral systems (Francis *et al.*, 1984). Each of these compensatory mechanisms attempts to improve the abnormal hemodynamics associated with heart failure.

After myocardial damage the heart becomes dilated. As the ventricle is stretched in response to increased blood volume the Frank-Starling law comes into effect and the heart responds with increased stroke volume, thus improving cardiac output. However, in heart failure, the injured heart is unable to increase cardiac output past a critical point of

ventricular stretch and as a consequence the Frank-Starling compensatory mechanism fails (Sandler & Fry, 1990).

The sympathetic nervous system is activated in response to decreased cardiac output in the heart failure state (Francis *et al.*, 1984). The sympathetic system increases both heart rate and contractility in an effort to directly improve cardiac output but it also affects cardiac output indirectly through arterial vasoconstriction, diverting blood away from less important tissues such as skin, muscles, and bowel, towards the heart and brain (Francis *et al.*, 1984). Vasoconstriction enhances venous return and increases preload in an attempt to normalize cardiac output. A negative consequence of the redistribution of blood flow away from kidneys, limbs, and splanchnic beds is a chronically diminished nutrient and oxygen supply to these tissues which manifests in the reduced exercise capacity and impaired renal function that is characteristic of heart failure patients (Wilson *et al.*, 1986).

The compensatory mechanisms elicited by the renin-angiotensin system require several days to become fully effective because of the system's endocrine nature. The actions of the primary effector hormone, angiotensin II, include not only arterial vasoconstriction with diversion of blood flow towards vital organs and away from muscle, but also stimulation of the release of aldosterone (Dzau *et al.*, 1981). Aldosterone serves to increase sodium and water retention, thereby increasing circulating blood volume. This causes increased venous return to the heart and consequently increases preload and cardiac output (Dzau *et al.*, 1981). However, excessive activation of the

renin-angiotensin system would impair cardiac function both directly and indirectly through increased preload and afterload.

Lastly, cardiac hypertrophy is a compensatory response of myocardial cells to persistently elevated atrial and ventricular pressures caused by hemodynamic overloading. Cardiac hypertrophy is characterized both by increased heart weight and re-expression of fetal isoform contractile proteins such as skeletal α-actin (Green *et al.*, 1982). The main stimuli for cardiac hypertrophic growth include stretch on the heart, adrenergic regulation, and peptide growth factors such as fibroblast growth factor and transforming growth factor which are prevalent in heart failure (Mann & Roberts, 1995).

The hypertrophic response serves to increase the strength of the overall myocardial contraction in heart failure. However, since the major determinants of myocardial oxygen demand are contractility and ventricular wall stress, increasing the size and contractility of the impaired ventricles is likely to increase ventricular wall stress and contribute to increased myocardial oxygen demand and consumption (DeMarco *et al.*, 1988). As a consequence of the increased oxygen demand the heart must actually work harder in order to provide for itself. Thus hypertrophy, in causing increased oxygen demand, contributes to the progression of heart failure. It is of interest that the fundamental cardiac biochemical, anatomical, and functional adaptations that develop in response to hemodynamic overloading during hypertension are reversible upon restoration of normal loading conditions (Cooper, 1987). Therefore, a treatment which effectively restores normal hemodynamic conditions in heart failure might also reverse the compensatory cardiac hypertrophy and increased oxygen demand.

The development of excessive peripheral vasoconstriction and sodium retention in heart failure represents a shift from a state of compensation to a state of decompensation. Whereas prior to the onset of heart failure compensatory mechanisms are directed towards supporting cardiac function, after the onset of heart failure the circulation's main objective is to support systemic perfusion pressures (Packer, 1992). Homeostatic responses which initially maintain the *status quo* become harmful and worsen the condition of heart failure, furthering the progression of the disease. Francis *et al.* hypothesized that these compensatory mechanisms designed to increase preload and afterload participate in a 'vicious cycle' which actually contributes to the progression of heart failure (Francis *et al.*, 1984).

Systemic vasoconstriction, which is a hallmark of advanced chronic congestive heart failure (Francis *et al.*, 1984), appears to be a consequence of several compensatory mechanisms including activation of the sympathetic nervous system, vasopressin, and the renin-angiotensin system (Packer, 1992; Zelis & Flaim, 1982). The neurohumoral factors involved in this increased vasoconstriction have been studied extensively while the impact of local vasoactive factors released from the vascular endothelium in heart failure should not be overlooked.

The endothelium lining the vasculature is the largest organ in the body (Pepine *et al.*, 1997). Serving both endocrine and paracrine regulatory functions, the endothelium affects hemodynamic forces, hemostasis, vascular remodelling, inflammation, as well as the changing hormonal environment of the vasculature (Pepine *et al.*, 1997).

1.2.4 Endothelial Function in Heart Failure

Vascular endothelial cells participate in many homeostatic functions, particularly in the regulation of vascular tone through the release of potent vasoactive agents such as prostacyclin (Moncada *et al.*, 1978) endothelium-derived relaxing factor (Furchgott & Zawadzki, 1980), and endothelin (Yanagisawa *et al.*, 1988). Endothelial dysfunction, specifically impaired endothelium-dependent vasodilation, has been suggested as a possible mechanism of increased vascular resistance in heart failure (Drexler & Lu, 1992; Kaiser *et al.*, 1989; Kubo *et al.*, 1991; Ontkean *et al.*, 1991). Regulation of endothelium-dependent vasodilation and constriction is altered in heart failure and may contribute to the vasoconstricted state.

The role of the endothelium as a potent modulator of vascular smooth muscle function is the subject of much study. Endothelin, first identified in 1988, is a potent vasoconstrictor produced by the endothelium (Yanagisawa *et al.*, 1988) which has multiple effects on various tissues throughout the body. Its effects on the cardiovascular system include a positive inotropic effect on the myocardium, mitogenic properties (Ito *et al.*, 1991), influences on salt and water homeostasis (Goetz *et al.*, 1988), as well as enhancement of the vasoconstrictor responses to sympathetic nerve stimulation (Wong-Dusting *et al.*, 1991). Together these actions promote vasoconstriction and increase blood pressure. Endothelin production is induced by angiotensin II which is elevated in heart failure (Hahn *et al.*, 1990). In addition, endothelin is regulated in a negative feedback manner by vasodilator substances in that endothelin both stimulates the release of dilators,

such as nitric oxide and prostacyclin, from the endothelium and has its own vasoconstrictor actions modulated by these vasodilators (Rubanyi, 1993; Vane *et al.*, 1990). The plasma concentrations of endothelin are elevated in heart failure (Cody *et al.*, 1992; Margulies *et al.*, 1990) which suggests that this vasoconstrictor could have important pathophysiological effects.

Endothelial cells also synthesize vasodilators. Prostacyclin is produced in response to shear stress and other stimuli and is most notably a potent inhibitor of platelet aggregation (Vane *et al.*, 1990). Prostacyclin and other prostaglandins are synthesized from arachidonic acid and their levels are elevated in heart failure (Dzau *et al.*, 1981). Since the production of prostaglandins is stimulated directly by vasoconstrictor hormones such as angiotensin, vasopressin, and norepinephrine (McGiff *et al.*, 1972; Zusman & Keiser, 1977), prostaglandins may function to counterbalance vasoconstriction. Vasodilators whose release is impaired may not be present in adequate concentrations to compensate for the increased vasoconstriction in heart failure. Thus, a possible mechanism for ameliorating the state of peripheral vasoconstriction, increased preload, and afterload in heart failure may lie in augmenting the release of vasodilators and restoring the balance of agents controlling vascular tone. This may be the case for endothelium-derived relaxing factor.

1.3 Endothelium-Derived Relaxing Factor

Furchgott and Zawadzki (1980) initially demonstrated that relaxation of vascular rings in response to acetylcholine requires the presence of an intact endothelium containing muscarinic receptors (Furchgott & Zawadzki, 1980). Stimulation of these

receptors causes the endothelial cells to release an endothelium-derived relaxing factor which then acts on smooth muscle cells of the artery causing them to relax (Furchgott, 1981). The chemical nature of endothelium-derived relaxing factor has since been identified as the endogenous nitrovasodilator nitric oxide (NO) (Palmer *et al.*, 1987) or a related nitroso compound (Ignarro *et al.*, 1987) which is formed from the amino acid L-arginine by the enzyme nitric oxide synthase (Palmer *et al.*, 1988a; Palmer *et al.*, 1988b).

Nitric oxide is synthesized in the vascular endothelial cells by the enzyme nitric oxide synthase from the terminal guanidino nitrogen of the amino acid L-arginine (Feng & Hedner, 1990a; Moncada & Higgs, 1993; Palmer *et al.*, 1988a), figure 1. The reaction is stereo-specific and requires a variety of co-factors including NADPH, flavoproteins, and tetrahydrobiopterin. The co-product of this reaction is L-citrulline (Mayer *et al.*, 1989; Palmer & Moncada, 1989). The stable end products of the L-arginine, nitric oxide signalling pathway are the oxidation products of nitric oxide, namely nitrite and nitrate (Ignarro *et al.*, 1987).

Three nitric oxide synthase isoforms have been identified which differ in their cellular distribution, calcium requirements, and K_m for L-arginine (Bredt & Snyder, 1994). Two of the isoforms are constitutively expressed, the neuronal nitric oxide synthase (nNOS or isoform I) and the endothelial nitric oxide synthase (cNOS or isoform III). cNOS is the enzyme responsible for the endothelium-derived nitric oxide production and functions in the regulation of basal vascular tone, blood pressure, and tissue perfusion (Vallance *et al.*, 1989). The third isoform is inducible nitric oxide synthase (iNOS or isoform II) which is induced to produce large amounts of nitric oxide in response inflammatory stimuli such as cytokines (Moncada & Higgs, 1993; Moncada *et al.*, 1991).

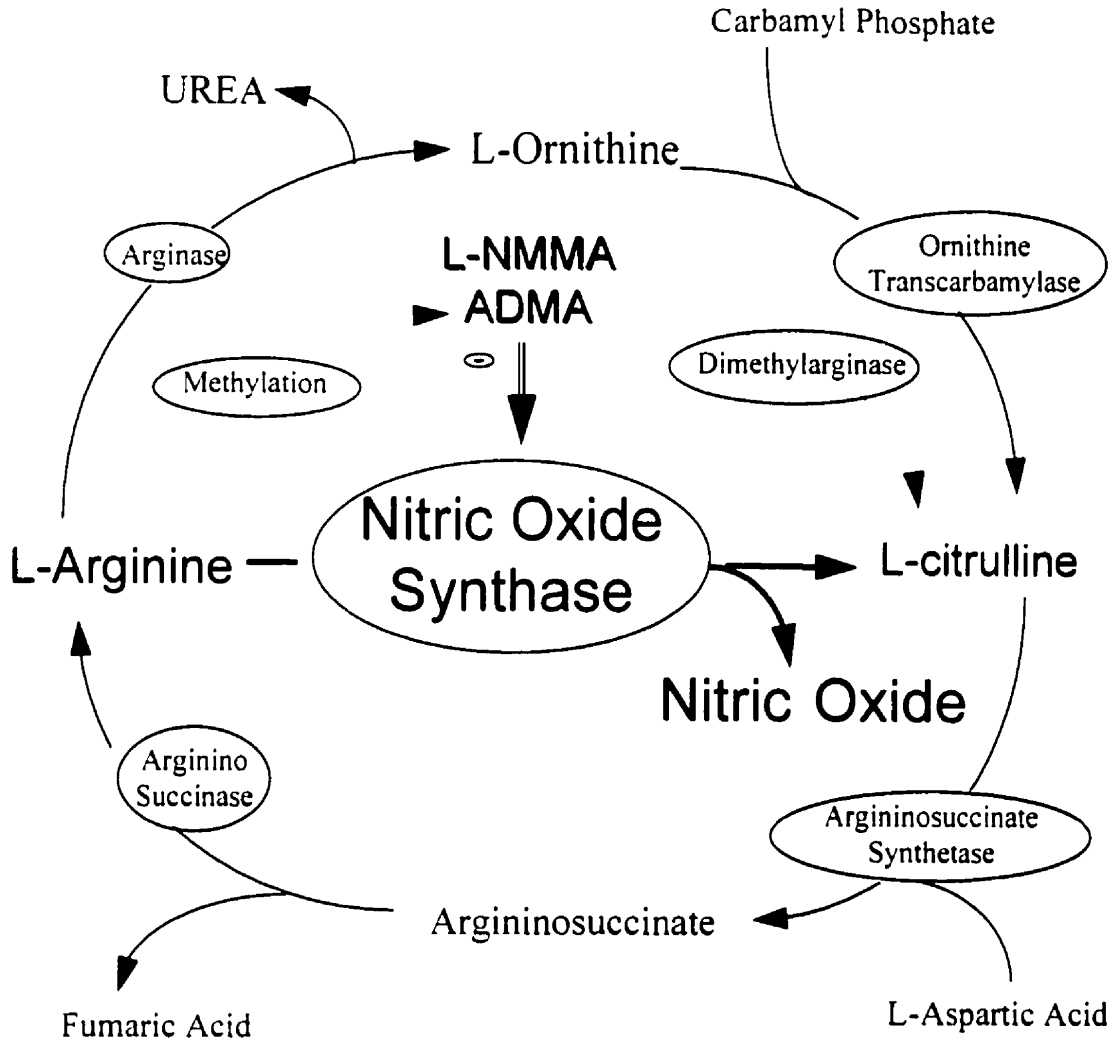


Figure 1: The biosynthetic and metabolic pathways of L-arginine including the synthesis of nitric oxide and its inhibition.

Tetrahydrobiopterin is an essential cofactor for the nitric oxide synthase. It increases synthase activity by serving as an electron donor for the hydroxylation of L-arginine (Tayeh & Marletta, 1989). Tetrahydrobiopterin also serves to stabilize the active dimeric state of the nitric oxide synthase (Klatt *et al.*, 1995; Nathan & Zie, 1994b; Venema *et al.*, 1997). iNOS has been shown to be dependent on tetrahydrobiopterin availability (Cho *et al.*, 1995; Gross & Levi, 1992). As well, tetrahydrobiopterin deficiency has been associated with the enzyme cNOS producing oxygen radicals in combination with nitric oxide (Cosentino & Katusic, 1995). Thus, supplementing with tetrahydrobiopterin may restore nitric oxide synthase activity and decrease oxygen radical formation which would effectively increase the biological activity of nitric oxide. Stroes *et al.* (1997) demonstrated that infusion of tetrahydrobiopterin in humans restored endothelial function in patients with hypercholesterolemia and abolished the rate limiting role of L-arginine (Stroes *et al.*, 1997), possibly due to the mechanisms mentioned above. Tetrahydrobiopterin is an important aspect of nitric oxide synthesis and endothelial dysfunction and one that needs further investigation.

1.3.1 Nitric Oxide Activity

Nitric oxide is released in response to a large variety of cell surface receptor agonists including adenine nucleotides, thrombin, bradykinin, acetylcholine, substance P, epinephrine, histamine, vasopressin, platelet-derived products, and shear stress (Feng & Hedner, 1990a; Furchgott & Zawadzki, 1980; Moncada *et al.*, 1991). Interactions among these multiple receptor pathways may be the most important mechanisms regulating the generation of nitric oxide (Michel *et al.*, 1993). In addition to its potent vasodilator

effects, nitric oxide is an inhibitor of platelet adhesion and aggregation (Radomski *et al.*, 1991).

The biological actions of nitric oxide differ from other major mediators in that it diffuses freely from its site of synthesis to act on adjacent tissues and that no enzymatic mechanism is required for its removal because of its free radical nature and short half-life (Conner & Grisham, 1995). Nitric oxide acts on vascular smooth muscle and platelets through the stimulation of soluble guanylate cyclase and elevation of intracellular cyclic guanosine monophosphate (GMP) (Rapoport & Murad, 1983). A cyclic GMP-dependent protein kinase (PKG) is then activated and through its phosphorylation actions stimulates the extrusion of calcium via the sarcolemmal and sarcoplasmic reticulum calcium/magnesium ATPase membrane pumps, resulting in decreased intracellular calcium concentrations (Goy, 1991; Lincoln, 1989). The activated PKG may also phosphorylate potassium channels in the cell membrane causing hyperpolarization which may inhibit voltage sensitive L-type calcium channels, resulting in decreased calcium entry and lower intracellular calcium concentrations (Walsh, 1994). The decrease in intracellular calcium concentration causes dissociation of calmodulin from and consequential inactivation of myosin light chain kinase allowing the dephosphorylation of myosin and resultant relaxation of the smooth muscle (Dusting, 1995; Nelson *et al.*, 1990; Thornbury *et al.*, 1991).

The biological half-life of nitric oxide is very short, a matter of seconds, due to the abundance of oxyhemoglobin which results in the conversion of nitric oxide to inactive nitrite and nitrate. Superoxide radicals can also participate in nitric oxide inactivation and

further reduce the biological half-life of nitric oxide (Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986). In arginine depleted cells, nitric oxide synthase catalyzes synthesis of both nitric oxide and superoxide which may result in increased levels of peroxynitrite, a free radical which can potentially cause tissue damage through lipid peroxidation, thiol oxidation, nitrosation, and DNA damage (Huie & Pakmaja, 1993; Xia *et al.*, 1996). Increased nitric oxide degradation may be associated with decreased nitric oxide activity in heart failure as has been demonstrated in hypercholesterolemia (Stroes *et al.*, 1997). Superoxide dismutase and analogues of the enzyme which destroy superoxide radicals have been shown to functionally increase the biological half-life of nitric oxide (Gryglewski *et al.*, 1986; Kasten *et al.*, 1995; Rubanyi & Vanhoutte, 1986). Therefore, treatments which would either decrease the production of superoxide radicals or decrease their concentrations may effectively increase the half life of nitric oxide and improve vasodilation in heart failure.

1.3.2 Systemic Effects of Nitric Oxide: Implications in Heart Failure

Evidence supporting a role for nitric oxide in the regulation of systemic vascular resistance was elucidated in experiments using L-arginine analogues which are inhibitors of nitric oxide synthase, namely N^G-N^G-dimethyl arginine or asymmetric dimethylarginine (ADMA) and N^G-monomethyl-L-arginine (L-NMMA), figure 1. Studies have shown that these compounds increase blood pressure and produce vasoconstriction which is accompanied by a significant decrease in the amount of nitric oxide generated (Calver *et al.*, 1993; Calver *et al.*, 1992; Vallance *et al.*, 1989; Vallance *et al.*, 1992a). These effects are reversible by L-arginine supplementation because of the

competitive nature of the nitric oxide synthase inhibition (Azuma *et al.*, 1995). Since L-NMMA is not a vasoconstrictor in its own right, but rather an inhibitor of the synthesis of an endogenous vasodilator, it has been suggested that there is an L-arginine, nitric oxide-dependent tone in the cardiovascular system which is important in the regulation of blood flow and blood pressure (Moncada *et al.*, 1991). If the decrease in endothelium-dependent relaxation in heart failure is the result of increased endogenous levels of ADMA it is possible that supplementation with L-arginine will improve this impairment through direct competition with these nitric oxide synthase antagonists.

The contribution of nitric oxide to vascular tone is not uniform throughout the vascular system. It has been suggested that nitric oxide activity is highest in arteries of large diameter that are subjected to the greatest mechanical forces of blood flow and shear stress, where nitric oxide is involved in the autoregulation of blood flow (Ignarro, 1989; Pepine *et al.*, 1997). As well, endothelium-dependent relaxation has been shown to be greater in arteries than in veins which suggests that arteries generate more nitric oxide than do veins (Luscher *et al.*, 1988). In support of such regional differences in nitric oxide production, the physiological role, expression, and activity of cNOS in capillary and venous endothelium differ from those of resistance arteries and arterioles possibly due to differences in local stimuli such as shear stress (Kelly *et al.*, 1996). If such regional differences in nitric oxide production are closely linked to blood flow and shear stress, it may suggest that stimulated endothelium-dependent release of nitric oxide is impaired in heart failure as a consequence of chronically reduced cardiac output and the resultant decrease in shear stress (Drexler *et al.*, 1994).

The data surrounding the levels and release of nitric oxide in heart failure are interesting. It has been suggested that there is a dissociation of stimulated and basal release of nitric oxide which may play a role in the pathophysiology of heart failure (Drexler *et al.*, 1994). Drexler states that the basal release of nitric oxide appears to be preserved or enhanced in advanced heart failure whereas the endothelium-dependent dilation stimulated by acetylcholine is blunted (Drexler *et al.*, 1992; Drexler *et al.*, 1994).

1.3.2.1 Basal Nitric Oxide Release

The opinions on the conditions under which the basal release of nitric oxide is altered are controversial. Basal release of nitric oxide plays an important role in modulating tissue perfusion in distal resistance vessels such as in the forearm (Drexler *et al.*, 1994). Teerlink demonstrated that early in heart failure, while there is yet no endothelial dysfunction to acetylcholine, there is impaired basal release of nitric oxide (Teerlink *et al.*, 1994). Ontkean *et al.* demonstrated that basal nitric oxide release is impaired in pulmonary artery segments of heart failure rats (Ontkean *et al.*, 1991). Drexler showed preserved or enhanced basal nitric oxide production in rats with advanced heart failure (Drexler *et al.*, 1994; Drexler & Lu, 1992), and Hildebrand *et al.* also suggested that basal release of nitric oxide is increased in heart failure (Hildebrand *et al.*, 1991). Thus, the status of basal nitric oxide release appears to vary both with time and the progression of the disease of heart failure as well as with the species used in the study. More work is needed to investigate the properties and role of basal nitric oxide release in heart failure.

iNOS is not considered to be normally expressed in healthy tissues as it is an inflammatory response stimulated by increased levels of circulating cytokines such as tumor necrosis factor (Geller *et al.*, 1993; Levine *et al.*, 1990). Researchers have shown that patients with heart failure express higher levels of iNOS in the ventricular myocardium (Habib *et al.*, 1996; Haywood *et al.*, 1996). iNOS activity may be enhanced by increased intracellular cAMP or activation of protein kinase C isoforms; this suggests that elevated intracardiac or systemic levels of catecholamines and angiotensin II, which are characteristic of heart failure could promote and sustain iNOS expression in this syndrome (Kelly *et al.*, 1996). The expression of iNOS by cardiac muscle in response to lipopolysaccharides and specific cytokines results in large amounts of nitric oxide being produced; the physiological and pathophysiological consequences of this are not fully elucidated but it may result in increased basal levels of nitric oxide and contribute in a compensatory manner to maintaining adequate tissue perfusion at rest.

1.3.2.2 Stimulated Nitric Oxide Release

Many labs, including our own, have found that endothelium-dependent dilation to acetylcholine is attenuated in rats with heart failure (Feng *et al.*, 1997; Teerlink *et al.*, 1993; Teerlink *et al.*, 1994). The vasodilator effects of acetylcholine and methacholine are blunted in the forearm circulation of patients with heart failure (Drexler *et al.*, 1992; Kubo *et al.*, 1991) and in the aorta and mesenteric arteries from rats with heart failure (Feng *et al.*, 1997; Feng *et al.*, 1996c). Recent studies demonstrated for the first time that decreased endothelial-dependent relaxation in heart failure is due at least in part to decreased cNOS activity (Feng *et al.*, 1997; Feng *et al.*, 1996b; Ontkean *et al.*, 1991). It

is not known what other mechanisms are involved in producing these changes in NOS activity, nor whether these changes and other cardiovascular alterations associated with heart failure can be modified through treatment with L-arginine.

1.3.3 Endothelial Dysfunction in Heart Failure

Chronic, severe heart failure could potentially induce endothelial dysfunction involving nitric oxide through several mechanisms. Recall that the levels of endothelin are elevated in heart failure (Cody *et al.*, 1992; Margulies *et al.*, 1990), and that endothelin is negatively regulated by nitric oxide (Rubanyi, 1993; Vane *et al.*, 1990). If endothelium-dependent release of nitric oxide is impaired in heart failure then there is reduced negative regulation of the vasoconstricting effects elicited by endothelin resulting in increased vasoconstriction that is characteristic of heart failure.

Alternatively, decreased release of nitric oxide may occur as a result of activation of the renin-angiotensin system and increased expression and activity of the vascular tissue angiotensin converting enzyme (ACE) which is identical to kininase II, the enzyme that degrades bradykinin (Luscher, 1993a). Since bradykinin is a powerful stimulator of nitric oxide release (Boglie *et al.*, 1991), decreased tissue levels of bradykinin would result in lower levels of nitric oxide and possibly impaired vasodilation.

It is also possible that heart failure exhibiting depressed cardiac output leads to reduced levels of shear stress and consequently diminished nitric oxide release (Drexler *et al.*, 1994). One may also consider that tumor necrosis factor (TNF) levels are increased in severe chronic heart failure (Levine *et al.*, 1990). TNF has been shown to decrease nitric oxide mRNA expression and to impair the stimulated release of nitric oxide and

therefore elevated plasma levels may cause endothelial dysfunction in patients with chronic heart failure (Drexler *et al.*, 1994).

In addition, the levels of endogenous nitric oxide synthase inhibitors, such as ADMA, may be elevated in heart failure as has been demonstrated in patients with hypercholesterolemia or renal failure (Bode-Boger *et al.*, 1996; Vallance *et al.*, 1992a). It is in this instance that a possible role for L-arginine treatment of heart failure is most clearly implicated.

1.4 L-arginine

Over a century ago arginine's role in normal physiology began to unfold when German scientists first recognized, identified, isolated, and named arginine as a component of proteins (Schulze & Steiger, 1886 reviewed in Reyes *et al.* 1994). In the 1930's studies on the synthesis of urea in liver slices incubated with arginine, citrulline, or ornithine led Krebs and Henseleit to postulate the existence of the urea cycle (Krebs & Henseleit, 1932) which established a key role of arginine in a major metabolic pathway, figure 1.

In addition to being involved in protein, urea, and creatinine synthesis, L-arginine is the physiological precursor to nitric oxide and has the potential to affect the release of hormones such as catecholamines, glucagon, growth hormone, insulin, prolactin, somatostatin, and corticosterone (Reyes *et al.*, 1994); all of which are capable of affecting a vast array of biological processes. Thus, L-arginine is involved in regulation pathways ranging from the urea cycle, muscle metabolism, immune system function, and vascular regulation to neurotransmission.

1.4.1 L-arginine Kinetics

Improved understanding of the intracellular pharmacokinetics of L-arginine and its role as a nutrient substrate in disease states is required before its full pharmacological effects can be explored. The amino acid L-arginine is generally classified as semi-essential (Rose, 1937). The sources of L-arginine are exogenous from dietary intake and endogenous via synthesis in the liver and kidney and release through muscle metabolism. In the growing animal, much of the L-arginine is used primarily for the synthesis of connective tissue proteins and other L-arginine-rich proteins. In the injured or wounded animal an increase in the amount of L-arginine utilized in the synthesis of reparative connective tissue could also be expected. It is only in such states of growth, wound healing, trauma, injury or sepsis, when L-arginine degradation and utilization is increased, that L-arginine becomes an essential amino acid (Castillo *et al.*, 1993b). Under such cases it is recommended that the dietary intake of L-arginine be increased.

It has been estimated that the average North American diet contains 5.4g of L-arginine daily which is provided primarily from meat sources (Visek, 1986). The LD_{50} (intraperitoneal [i.p.] after 24 hour starvation) for L-arginine-HCl in rats is 18mM/kg (3.8g/kg) (Gullino *et al.*, 1955), which places L-arginine in a class of compounds with very low toxicity for normally nourished animals. In some human studies, subjects ingested 30 grams of L-arginine daily for one week and showed no side effects besides mild gastrointestinal discomfort (Barbul *et al.*, 1981). L-Arginine infusions have been shown to lead to hypophosphatemia and hyperkalemia in both normal subjects and insulin-dependent diabetics (Massara *et al.*, 1980). Much of the accumulated evidence

suggests that high doses of L-arginine (30-60g/day) are well tolerated in humans (Barbul, 1986)

As with other amino acids, the intestinal absorption of L-arginine occurs in the jejunum where there is a specific transport system (Kim *et al.*, 1991; Wang *et al.*, 1991) known as system y^+, a sodium-independent carrier primarily for cationic amino acids (Christensen, 1984; Kim *et al.*, 1991; Wang *et al.*, 1991; White, 1985; White & Christensen, 1982). After its absorption by the brush-border membrane, L-arginine is extensively catabolized by the enterocyte (Blachier *et al.*, 1991a; Blachier *et al.*, 1991b). It has been reported that approximately 30-44% of the ingested arginine is absorbed (Castillo *et al.*, 1993a). The effect of altered dietary L-arginine on these kinetics are not known.

Tissues must actively take up L-arginine from the circulation. This transport may be a rate-limiting or regulatory step for subsequent metabolic events. Greene *et al.* demonstrated that at physiological circulating L-arginine concentrations, approximately 70% of arginine transport by pulmonary artery endothelial cells occurs via system y^+ and the rest through a sodium-dependent carrier named system $B^{0,+}$ (Greene *et al.*, 1993).

L-arginine uptake into smooth muscle cells has been suggested as the limiting factor for nitric oxide production by lipopolysaccharide-induced iNOS (Schott *et al.*, 1993). The activity of the L-arginine transporter system y^+ is stimulated by certain inflammatory cytokines in iNOS-expressing cells (Greene *et al.*, 1993; Pacitti *et al.*, 1992). Thus, L-arginine transport is stimulated by the same mediators, tumor necrosis factor and lipopolysaccharides, that induce iNOS to synthesize nitric oxide. It has also

been shown that inducers of iNOS, namely lipopolysaccharide and interferon-γ, co-induce activity and mRNA expression for argininosuccinate synthetase which is the rate limiting enzyme for the recycling of L-citrulline to L-arginine (Nussler *et al.*, 1994). These mechanisms may compensate for the increased utilization of L-arginine upon stimulation of iNOS. Citrulline recycling would contribute to nitric oxide production under conditions when extracellular L-arginine concentrations were insufficient.

The majority of endogenous L-arginine synthesis occurs in the liver and kidney through the transfer of an amino group from L-aspartic acid or L-glutamic acid to L-citrulline, figure 1. This reaction uses ATP and is mediated by the enzyme argininosuccinate synthase. It should be noted that the liver extracts far more L-arginine than it releases into the blood stream. The majority of L-arginine synthesized in the liver is utilized locally in the urea cycle (Rogers *et al.*, 1972; White & Christensen, 1982). In the kidney, circulating L-arginine is filtered in the glomerulus and almost entirely reabsorbed in the proximal tubule which is also the major site of L-arginine synthesis (Levillain *et al.*, 1991). L-arginine that is synthesized in the proximal convoluted tubule enters systemic circulation while most of the L-arginine that is synthesized in the proximal straight tubule is metabolized to urea (Featherson *et al.*, 1973). Maintaining normal plasma levels of L-arginine, therefore, depends mainly on dietary intake and synthesis in the kidney.

1.4.2 Physiological Effects of L-arginine

While this thesis investigates the effects of L-arginine as a substrate of nitric oxide, oral administration of L-arginine will likely affect all pathways in which L-arginine plays a role. L-Arginine has multiple and potent secretagogue activities on several endocrine glands including the pituitary, pancreas, and adrenal glands (Barbul, 1986). It has been shown that animals given L-arginine in their diet show an increase in weight gain, improved nitrogen balance, increased wound healing and collagen deposition, as well as improved immune function including enhanced thymic, lymphocyte, and natural killer cell activity (Barbul *et al.*, 1983; Brittenden *et al.*, 1994; Swanson, 1990). While the mechanism for L-arginine induced wound healing likely involves increased macrophage and neutrophil activity and vasodilation involving nitric oxide production, an appreciation of the other potential effects of L-arginine is required to fully explore the consequences of chronic oral L-arginine treatment.

Administration of L-arginine has been shown to cause increased pituitary growth hormone secretion (Franchimont *et al.*, 1979; Isidori *et al.*, 1981; Knopf *et al.*, 1965; Masuda *et al.*, 1990; Merimee *et al.*, 1965; Nakaki & Kato, 1994; Rakoff *et al.*, 1973). L-Arginine also affects the release of insulin and glucagon (Cherrington *et al.*, 1974; Floyd *et al.*, 1966; Nakaki & Kato, 1994; Palmer *et al.*, 1975b; Utsumi *et al.*, 1979), pituitary prolactin (Rakoff *et al.*, 1973), and adrenal catecholamines (Imms *et al.*, 1969).

L-arginine's stimulation of growth hormone release is worthy of note when one considers the potential effects of growth hormone on the heart, figure 2. It is believed that L-arginine stimulates growth hormone secretion through inhibition of somatostatin

release from the hypothalamus (Alba-Roth *et al.*, 1988; Masuda *et al.*, 1990), possibly involving muscarinic receptors (Delitala *et al.*, 1982). The severity of cardiac failure in dilated cardiomyopathy has been linked to decreased circulating growth hormone (Giustina *et al.*, 1996), therefore, growth hormone may be involved in regulatory responses to heart failure and the failure of this compensatory mechanism may result in the worsening of ventricular function.

Growth hormone has been shown to affect the heart in such a way as to allow the cardiac muscle to function more economically (Yang *et al.*, 1995). Short term administration of growth hormone results in an increase in myocardial contractility in normal humans, while chronic growth hormone treatment enhances cardiac output, increases glomerular filtration rate, and improves exercise capacity in growth hormone deficient adults (Yang *et al.*, 1995). Treatment with growth hormone in heart failure rats improves cardiac output and contractility while reducing left ventricular end-diastolic pressure and systemic vascular resistance (Yang *et al.*, 1995).

The expression of growth hormone's local effector, insulin-like growth factor I (IGF-I) is increased in hypertrophic myocardium (Duerr *et al.*, 1995) and has been shown to increase the size of cultured cardiomyocytes and increase the expression of myosin, actin, and troponin (Sacca & Fazio, 1996). IGF-I increases blood flow through a nitric oxide dependent mechanism (Fryburg, 1996), stimulating nitric oxide production in vessels and decreasing vascular contractility (Walsh *et al.*, 1996). Although IGF-I is produced locally there is a long lag time for these responses which may suggest that complex mechanisms are involved (Walsh *et al.*, 1996). In rats with myocardial

infarction, IGF-I induced additional myocyte growth and consequently restored cardiac function as did the growth hormone treatment mentioned above (Duerr *et al.*, 1995; Yang *et al.*, 1995). These results suggest that L-arginine through the release of growth hormone and IGF-I may improve cardiac function through both increasing myocardial contractility and decreasing peripheral vascular resistance in heart failure.

An additional L-arginine pathway that may contribute to improving cardiovascular performance in heart failure involves the conversion of L-arginine to agmatine by the enzyme arginine decarboxylase. Agmatine is an endogenous non-catecholamine α_2-agonist that acts on the nucleus tractus solitarius in the brainstem to decrease peripheral sympathetic stimulation (Nakaki & Kato, 1994), figure 2. As catecholamines are known to promote vasoconstriction, agmatine may participate in the hypotensive effects of L-arginine administration, however, more research is needed to elucidate this pathway.

Thus, L-arginine pathways that do not involve nitric oxide production may prove beneficial in the treatment of heart failure, but that does not preclude a role for L-arginine's effects elicited through nitric oxide production.

1.4.3 Effects of L-arginine and Nitric Oxide Production

It has been suggested that systemically administered L-arginine releases nitric oxide in man following the observation that L-arginine administration increased cardiac output, decreased peripheral resistance, and increased plasma concentrations of cGMP, L-citrulline, and urine nitrate and nitrite levels in normotensive humans (Hishikawa *et al.*, 1991; Hishikawa *et al.*, 1992). The effects elicited by L-arginine through the production of nitric oxide are stereo-specific because the inactive optical isomer, D-arginine, is not a

substrate for nitric oxide synthase and does not affect acetylcholine-induced vasodilation (Egashira *et al.*, 1996).

Endothelium-dependent relaxation is decreased in many disease states including atherosclerosis (Cooke & Tsao, 1992b; Cox *et al.*, 1989), hypertension (Panza *et al.*, 1993a; Panza *et al.*, 1993b; Panza *et al.*, 1990; Taddei *et al.*, 1996), myocardial ischemia and reperfusion (Weyrich *et al.*, 1992), hypercholesterolemia (Bode-Boger *et al.*, 1996; Celermajer *et al.*, 1992; Cooke *et al.*, 1992a; Drexler *et al.*, 1991), angina pectoris (Egashira *et al.*, 1996), and heart failure (Katz *et al.*, 1993; Kubo *et al.*, 1991; Thuillez *et al.*, 1995), however, relaxation in response to sodium nitroprusside, an endothelium-independent source of nitric oxide, is not affected. This suggests that the locus for impaired vasorelaxation is specific and located at the level of the endothelial cell, that is, prior to diffusion of the signal carried by nitric oxide. In these states, the acute administration of L-arginine, either orally or *in vitro*, normalizes this vascular dysfunction without affecting the response to nitroprusside which would indicate that the defect is not a consequence of receptor down-regulation (Cooke *et al.*, 1992a; Cooke & Tsao, 1992b; Drexler *et al.*, 1991; Egashira *et al.*, 1996). The administration of L-arginine has additional cardiovascular benefits such as reducing systolic and diastolic pressures when infused into healthy humans and patients with essential hypertension (Nakaki & Kato, 1994). L-arginine has also been shown to increase cardiac output and reduce pulmonary vascular resistance in the canine pacing model of heart failure (Ogilvie & Zborowska-Sluis, 1995), figure 2.

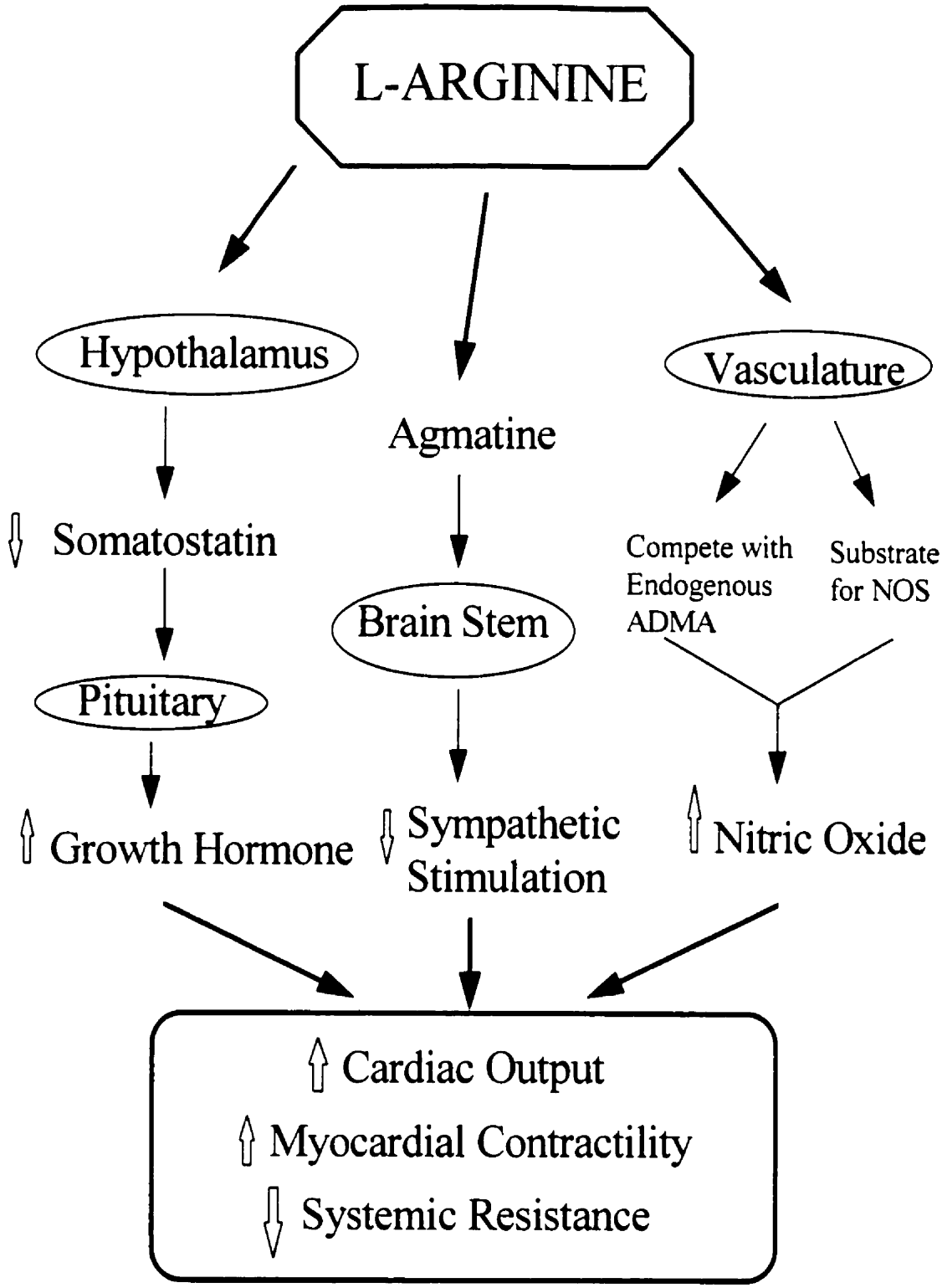


Figure 2: Possible L-arginine pathways for improving cardiac performance in heart failure.

Mechanisms such as depletion of L-arginine, impaired L-arginine transport into cells, blockade of recycling of L-citrulline to L-arginine are all possible pathways which may explain the decreased endothelium-dependent relaxation seen in heart failure and outline possible ways in which L-arginine treatment could ameliorate the condition of impaired nitric oxide production in heart failure. It is also possible that L-arginine administration produces cardioprotective effects through increased nitric oxide release by inhibiting neutrophil aggregation and stimulation, or by reducing the production of superoxide free radicals which are one of the most important inactivators of nitric oxide (Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986; Xia *et al.*, 1996).

While many studies indicate that exogenous administration of L-arginine can improve disease states where nitric oxide release or endothelium-dependent relaxation is impaired (Girerd *et al.*, 1990), others have demonstrated that sufficient amounts of endogenous L-arginine are available to saturate nitric oxide synthase and that the addition of exogenous L-arginine does not enhance endothelium-dependent relaxation in normal vessels (Gold *et al.*, 1989). This has been referred to as the L-arginine paradox (Forstermann *et al.*, 1994). Plasma L-arginine concentrations in humans are normally in the 80-110μM range (Sax *et al.*, 1988). The intracellular concentration of L-arginine is presumed to be about 100 μM (Nakaki & Kato, 1994). Since the K_m value of the constitutive NOS for L-arginine is 10 μM this enzyme is theoretically saturated (Nakaki & Kato, 1994). The apparent K_m of iNOS for L-arginine is 150 μM, approximately one order of magnitude larger than for the constitutive NOS (Bogle *et al.*, 1992a; Iyengar *et al.*, 1987). Thus, cellular nitric oxide production could be substrate-limited at physiologic

concentrations of L-arginine (Nussler *et al.*, 1994) but recall that the L-arginine transporter may be stimulated by the same agents that stimulate iNOS expression (Greene *et al.*, 1993; Pacitti *et al.*, 1992), thus, the L-arginine paradox endures.

Cellular nitric oxide production is determined by intracellular L-arginine concentration, nitric oxide synthase activity, availability of the various cofactors required for the nitric oxide synthase reaction, and cell responsiveness to stimuli. Thus, there are multiple potential sites at which nitric oxide production may be regulated and the actual regulated steps may vary for different cell types and may be altered in disease states. The study by Nussler *et al.* indicates that the recycling of citrulline to arginine may be an important regulatory mechanism for determining rates of cellular nitric oxide production *in vivo* only when L-arginine concentrations are insufficient to support maximal or near maximal rates of nitric oxide synthase (Nussler *et al.*, 1994). Stroes *et al.* suggest that the decreased affinity of nitric oxide synthase for L-arginine due to tetrahydrobiopterin deficiency may contribute to the L-arginine paradox in hypercholesterolemia (Stroes *et al.*, 1997). Further investigation is needed in this area in any state where L-arginine administration ameliorates conditions through unknown mechanisms.

Getting away from the L-arginine paradox, a mechanism through which L-arginine may improve endothelium-dependent relaxation involves oxygen radicals. It is well known that superoxide anion is involved in the breakdown of nitric oxide (Gryglewski *et al.*, 1986). Recall that in L-arginine depleted cells, nitric oxide synthase produces both nitric oxide and superoxide anion (Xia *et al.*, 1996). Thus, L-arginine

supplementation may both decrease the levels of oxygen radicals produced and increase nitric oxide production and its biologically active half-life.

Another possible explanation for the beneficial effects of exogenous L-arginine is the presence of endogenous nitric oxide synthase inhibitors, ADMA or L-NMMA (Nakajima *et al.*, 1971; Vallance *et al.*, 1992a), whose inhibition of nitric oxide synthesis may be out-competed by exogenous L-arginine (Vallance *et al.*, 1992b). Both ADMA and L-NMMA have been shown to accumulate in the plasma of patients with acute renal failure (Vallance *et al.*, 1992a), and ADMA was shown to be increased in hypercholesterolemic patient plasma (Bode-Boger *et al.*, 1996) and in heart failure rats (Feng *et al.*, 1997). Thus, inhibition of nitric oxide synthase by these compounds could explain, in part, the hypertension that occurs in these conditions and the impaired endothelium-dependent relaxation in heart failure. Administration of L-arginine thus has the potential of improving nitric oxide production through competition with ADMA and L-NMMA for the active site on nitric oxide synthase. Treatment with L-arginine effectively restores impaired endothelium-dependent relaxation in heart failure rats exhibiting elevated levels of ADMA (Azuma *et al.*, 1995), suggesting a role for L-arginine in treatment of these diseases. Increasing plasma L-arginine levels, while the elevated levels of ADMA and L-NMMA remain the same, results in elevation of the ratio of L-arginine to inhibitor above the normal level which may account partially for the restored nitric oxide formation seen in the above cases (Bode-Boger *et al.*, 1996).

Bogle *et al.* demonstrated that some nitric oxide synthase inhibitors, L-NMMA for example, also inhibit L-arginine uptake via the system y^+ unidirectional transporter

(Bogle *et al.*, 1992b). Close coupling of the L-arginine transporter and nitric oxide synthase could account for the regulation of nitric oxide release as well as for the ability of circulating L-arginine to reverse the inhibition of nitric oxide synthase by L-NMMA (Bogle *et al.*, 1992b). If L-arginine supplementation does improve the production of nitric oxide the consequence would be improved vasodilation, supporting a role for L-arginine as a type of vasodilator therapy.

Short-term, oral L-arginine treatment in humans with heart failure, 4-12g L-arginine per day for three weeks, was shown to decrease mean arterial pressure, and was accompanied by a decrease in circulating levels of endothelin. The patients exhibited increased exercise tolerance and general quality of life as measured by a questionaire, showed increased forearm blood flow during exercise, but no change was detected in basal nitric oxide synthase activity as evaluated by vasoconstrictor responses to L-NMMA (Rector *et al.*, 1996). Thus, oral L-arginine treatment has beneficial effects in heart failure in humans notably improving conditions during exercise.

The purpose of this thesis was to evaluate the effectiveness of chronic oral L-arginine as a treatment to improve the impaired endothelium-dependent vasorelaxation in heart failure and to examine any beneficial effects on overall hemodynamics. Since L-arginine is a substrate of nitric oxide synthase and nitric oxide release is attenuated in heart failure, it was investigated whether administration of exogenous L-arginine would increase nitric oxide production and improve endothelial function. The studies identified above support the belief that acute administration of L-arginine restores endothelial function stimulated by acetylcholine and thus improves disease states such as

hypertension and heart failure (Feng *et al.*, 1996b; Girerd *et al.*, 1990; Hirooka *et al.*, 1994). The present study investigates whether these effects are maintained during chronic L-arginine treatment. We hypothesized that chronic oral L-arginine treatment would restore endothelium-dependent relaxation and improve basal cardiac function and cardiac output in heart failure rats.

2. Materials and Methods

2.1 Animal Protocol

Animals used in this study were handled in accordance with the guidelines of the Animal Care Committee at the University of Western Ontario, Canada.

All experiments were conducted on male Sprague-Dawley rats weighing 210-250g. All animals were maintained on normal rat chow and given water *ad libitum* in a twelve hour light-dark cycle. Animals were caged individually after surgical operation.

2.1.1 Rationale for the Use of Current Heart Failure Model

Experimental coronary occlusion with consequent myocardial infarction has been produced for many years by surgical ligation of one or more coronary arteries. Heart failure due to coronary artery ligation in rats is a well-established animal model that has provided significant information about the pathophysiology of chronic heart failure and endothelial dysfunction (Drexler *et al.*, 1992; Drexler & Lu, 1992; Feng *et al.*, 1992; Hill & Singal, 1996; Pfeffer *et al.*, 1979; Pfeffer *et al.*, 1985; Sun *et al.*, 1995; Thuillez *et al.*, 1995; Yang *et al.*, 1995).

2.1.2 Coronary Artery Ligation Surgery

Rats were randomly selected to undergo coronary artery ligation or sham surgery using the techniques similar to those previously described (Pfeffer *et al.*, 1979). Rats were anaesthetized with sodium pentobarbital (50 mg/kg intraperitoneally [i.p]., MTC Pharmaceuticals, Cambridge, ON), then intubated and artificially ventilated with a respirator (SAR-830, CWE, Inc., Ardmore, PA.). Tidal volume was approximately 3 cc at 70 breaths per minute.

A left intercostal thoracotomy was performed. The chest was opened through the third or fourth intercostal space, ribs spread using a chest retractor, and the left side of the heart was exposed. In the Sprague-Dawley rat, the left coronary artery is the predominant supplier of blood to the left ventricle. The artery lies beneath the epicardium, buried in heart muscle but it can be seen in the intact beating heart as a tiny pink streak beneath the heart's surface. After opening the pericardium, the proximal left coronary artery was ligated between the pulmonary out-flow tract and the left atrium by positioning a suture of 6-0 silk thread (Ethicon, Inc., Somerville, New Jersey) around the artery together with a small bundle of heart muscle that is transfixed with it (Feng *et al.*, 1992; Feng *et al.*, 1990b). The lungs were thereafter hyper-inflated using positive end-expiratory pressure and the thorax closed with three intercostal sutures of 2-0 silk thread. The muscle and skin layers were then closed using 4-0 silk stitches. The usual time necessary to complete the procedure was thirty minutes to one hour.

Changes immediately following left coronary artery ligation included a sudden pallor of the myocardium which was often accompanied by cardiac arrhythmias, including ventricular tachycardia and fibrillation which subsided after twenty to thirty minutes. After coronary artery ligation approximately 30% of the rats die within several hours, those surviving this initial period have about 11% mortality within eight weeks. Sham-operated rats underwent the same surgery minus the coronary artery ligation. Following surgery, both groups of rats ate and drank normally and moved about in their cages without difficulty.

2.1.3 Post Mortem Examination

At the time of experimentation, approximately eight weeks post-surgery, a blood sample was usually taken from the rats prior to lethal injection of sodium pentobarbital. Subsequently, the heart and aorta were excised and internal organs examined for evidence of heart failure including pleural effusions and edema, or other abnormalities such as infection which may have affected the outcome. The heart was dissected free of adjacent tissues, the ventricles were separated from the atria, and the right ventricular free wall was dissected from the septum. Right and left ventricle and atrial sections were blotted dry and weighed. Left ventricle volume was then measured by the weight of water injected into the left ventricle under atmospheric pressure. All samples were stored at -80°C.

2.1.4 Measurement of Infarct Size

Left coronary artery occlusion in rats can readily provide left ventricular free wall infarctions of varying sizes. Within twenty-one days of the infarction, the necrotic myocardial tissue was completely replaced by connective tissue (Fishbein *et al.*, 1978). Ligated hearts show progression of pathologic changes including discolouration, necrosis, scar tissue, as well as left ventricular wall thinning and volume expansion (Pfeffer *et al.*, 1979). Heart failure in this model was caused primarily by decreased myocardial contractility due to decreased myocardial mass.

The infarct size resulting from coronary artery ligation was evaluated by cutting the left ventricles of ligated hearts into four transverse slices of equal thickness from apex to base. Photographs were taken of these slices and the infarcted area was measured by tracing the endocardial circumferences of the fibrotic and normal areas on the

photographs with a distance meter. Infarct size was expressed as a fraction of the total cross-sectional endocardial circumference of the left ventricle (Feng *et al.*, 1996c; Sun *et al.*, 1995).

Other methods of evaluating infarct size involve again cutting the left ventricle into four transverse slices from apex to base but then cutting five-micron thick sections, staining with Masson's trichrome stain and mounting. The endocardial and epicardial circumferences of the infarcted and non-infarcted circumference are then determined with a planimeter Digital Image Analyzer. The results of all four slices are summed separately, averaged, and expressed as a percentage for infarct size (Hill & Singal, 1996; Pfeffer *et al.*, 1979; Sakai *et al.*, 1996; Yang *et al.*, 1995). Alternatively, the left ventricle may be opened with an incision along the septum from base to apex and pressed flat so that the circumferences of the left ventricle and the region of infarction may be outlined on a clear plastic sheet to represent the endocardial and epicardial surfaces. Infarct size was then calculated as a percentage of left ventricle surface area as determined by weight of the traced sections of the plastic sheet (Chien *et al.*, 1988; Ontkean *et al.*, 1991). The method employed in the present study was deemed acceptable as it yields accurate measurements in a relatively inexpensive manner.

Previous studies have evaluated the relationship between size of the healed myocardial infarction and left ventricular performance three weeks after coronary artery ligation (Pfeffer *et al.*, 1979). They determined that rats with small (4-30%) myocardial infarctions have no discernible impairment in either baseline hemodynamics or peak indices of pumping and pressure-generating ability when compared to sham-operated,

non-infarcted rats. Rats with moderate (31-46%) infarctions have normal baseline hemodynamics but reduced peak flow indices and developed pressure. Rats with infarctions greater than 46% have congestive heart failure characterized by elevated left ventricular end-diastolic pressures, reduced rate of change of ventricular pressures (dP/dt), lower left ventricular systolic pressures, reduced cardiac output, and a minimal capacity to respond to pre-and afterload stresses. Thus, in this model of histologically healed myocardial infarction, the impairment of left ventricular function was directly related to the loss of myocardium (Pfeffer *et al.*, 1979). The range of post-infarction ventricular dysfunction observed in this model reflects the spectrum of ventricular dysfunction observed in man. The range of infarctions associated with depressed ventricular performance and the condition of heart failure are unique to the rat model because of the rat's ability to survive such large infarctions, unlike animals like the dog in which, an infarction greater than 30% results in fatal arrhythmias (Kumar *et al.*, 1970; Pfeffer *et al.*, 1979). Only rats with calculated infarct sizes greater than 30% of the left ventricle were included in the studies since this level of infarction is standardly used in the literature for representing typical signs of heart failure (Pfeffer *et al.*, 1979; Drexler *et al.*, 1992).

2.2.1 Rationale for Hemodynamic Study

The presence of heart failure was assessed, as previously described (Pfeffer *et al.*, 1979; Sakai *et al.*, 1996; Thuillez *et al.*, 1995), by evaluating left ventricular end-diastolic and systolic pressures, left ventricular pressure development, heart rate, and mean arterial pressures. It is of note that studies by Pfeffer were done only 21 days post-surgery and hemodynamic measurements were made during anesthesia. The present study was done

under a longer time frame and in conscious animals, experimental conditions which should have allowed for further progression and expression of the heart failure condition. Others have also demonstrated hemodynamic evidence of heart failure in this rat model after a period of thirty days (Thuillez *et al.*, 1995), six weeks (Yang *et al.*, 1995), eight weeks (Feng *et al.*, 1996c; Hill & Singal, 1996), ten weeks (Ontkean *et al.*, 1991), and sixteen weeks (Hill & Singal, 1996). We have conducted hemodynamic measurements on our particular model to confirm that a state of heart failure was induced in our model after eight weeks and that the present model adheres to previous criteria.

2.2.2 Hemodynamic Protocol

Eight weeks post-surgery, rats were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.) for catheter placements. A total of four vessels were cannulated in preparation for this study. All lines contained heparinized saline (0.9%) to prevent blockage by blood clotting. In addition, the lines were secured onto the backs of the rats to allow for free movement.

The tail artery was cannulated with polyethylene tubing (PE-50, Becton Dickinson & Company, Sparks, MD) for the purpose of measuring mean arterial blood pressure and heart rate. A second PE-50 catheter was inserted into the left ventricle through the right carotid artery for the measurement of left ventricle systolic and end-diastolic pressures as well as the maximal rate of pressure development (+dP/dt) and rate of relaxation (-dP/dt) of left ventricle. Pressures and heart rate measurements were taken using a Hewlett-Packard pressure transducer (model 1290A) connected to a pressure monitor and recorded on a Gould recorder (model 2400S).

Measurement of cardiac output (CO) required two separate cannulations. Firstly, a thermodilution probe (2.5F, Baxter, Mississauga, Ontario) was passed into the upper thoracic aorta via the right femoral artery so that the tip was at the aortic arch. The position of the probe was confirmed by visualization after completion of the study. Secondly, a PE-50 catheter was inserted into the superior vena cava via the right jugular vein for injection of heparinized saline (0.9% NaCl). Cardiac output measurements were obtained by injecting 0.2 cc of 20-22°C saline into the superior vena cava and detecting changes in aortic blood temperature by a cardiac output computer (American Edwards Laboratories, CA) via the thermodilution probe. Measurements were made in duplicate and normalized by dividing by the body weight of the individual rats. This normalized value of cardiac output is expressed as mL/min/100g body weight throughout the thesis. Right atrial pressure was not measured because catheter placement in the right atrium is often associated with significant bradycardia in conscious rats. Thus, we were unable to calculate peripheral vascular resistance. However, previous studies have measured peripheral vascular resistance in anesthetized rats and have shown the values to be significantly increased in heart failure (Drexler, 1986).

The thermodilution method of quantifying cardiac output is an application of the indicator dilution principle in which the indicator is cold saline. This is the most widely used invasive method for assessing cardiac output in man for the reasons that it is easy to perform and allows instantaneous determination of cardiac output (Branthwaite & Bradley, 1968; Ganz *et al.*, 1971; Gola *et al.*, 1996). It is also a popular technique for use in rats (Carbonell *et al.*, 1985; Yang *et al.*, 1995) and dogs (O'Murchu *et al.*, 1994).

Non-invasive methods of assessing cardiac output involve Doppler echocardiography. The most accurate and reproducible method by Doppler measures the left ventricular outflow tract, however, this method is not used extensively in clinical cardiology studies (Gola *et al.*, 1996), and was not available in the current study.

All hemodynamic measurements were made during the conscious state, two to three hours post-surgery, at which time the animals were alert and able to execute coordinated movements. Hemodynamic measurements were obtained when the rats were resting quietly. The present hemodynamic measurements taken three hours after surgery have partially recovered from the depressing effect of anaesthesia when the values are compared to measurements taken immediately post-surgery (own observations). Our experience indicates that extending the recovery time is detrimental to the rat's survival because of the intra-ventricular catheter which causes severe arrhythmias.

2.3.1 Rationale for Organ Bath Study

The *in vitro* organ bath is a long-established method of evaluating endothelium-dependent relaxation of vascular smooth muscle (O'Murchu *et al.*, 1994). Thoracic aortae were used in these studies because of their feasibility as well as their long-standing reputation and use in these studies (Cooke *et al.*, 1992a; Feng *et al.*, 1996c; Noll *et al.*, 1994; Ontkean *et al.*, 1991; Teerlink *et al.*, 1994; Thuillez *et al.*, 1995). Aortic endothelial dysfunction in the coronary artery ligated rat model of heart failure has been demonstrated as early as four weeks post-surgery (Thuillez *et al.*, 1995). The present studies investigate aortic endothelial function eight weeks post surgery.

Ontkean found that the abnormalities in nitric oxide production were more severe in the pulmonary arteries than in the thoracic aorta (Ontkean *et al.*, 1991). While these are both relatively large conduit arteries, the changes observed indicate that the aorta is a useful model for studying the pathophysiology of heart failure in the vasculature and for testing the effects of therapeutic modalities. It has also been demonstrated that small arteries are more affected with regards to endothelial dysfunction than larger arteries (Teerlink *et al.*, 1993; Thuillez *et al.*, 1995). Thus, if vascular dysfunction is observed in thoracic aortic ring segments it might imply that the pulmonary and smaller peripheral arteries would also display impaired endothelial function and perhaps to a higher degree, however, this hypothesis was not addressed in the current studies.

2.3.2 Organ Bath -- Ring Preparation

Eight weeks post-surgery rats were killed via lethal injection of sodium pentobarbital and the thoracic aorta immediately removed and placed in cold Krebs' bicarbonate solution. While in Krebs' solution the vessels were dissected free of fat and connective tissues, cut into transverse ring segments of 4 mm in length, and mounted in a 5 mL organ bath containing aerated Krebs' solution. Care was taken to avoid endothelial damage.

2.3.3 Organ Bath -- Apparatus

The vascular ring segments were placed around two horizontal stainless steel wires which were mounted on vertical holders. One holder was attached to a Grass FT03 Force-Displacement Transducer (Grass Instruments, Quincy, MA) connected to a Grass Model 79E Polygraph for the continuous recording of changes in vessel wall tension. The

other holder was fixed to a brass manipulator which was used to apply passive tension to the vessels.

The ring segments were bathed in Krebs' solution which was maintained at 37°C in a double-walled, temperature-controlled organ bath with internal volume of 5 mL. The Krebs' bicarbonate solution had the following composition (mM): NaCl, 118; $NaHCO_3$, 22; Glucose, 11; KCl, 4.7; $MgSO_4 \bullet 7H_2O$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5. Krebs' in the bath was aerated with 95% O_2 - 5% CO_2, maintaining a pH of 7.4. Vessels were mounted at a resting tension of one gram, and allowed to equilibrate for one hour under these conditions before the experiment was begun.

2.3.4 Organ Bath Protocol

To evaluate the functional state of the aortic rings, vessels were constricted with 100 mM KCl for a 10-15 minute period. This was repeated three to four times until a consistent maximal response to KCl was established.

Cumulative concentration-responses to acetylcholine and sodium nitroprusside were performed; acetylcholine was used to evaluate the endothelium-dependent relaxation of the vessel and sodium nitroprusside to evaluate the endothelium-independent relaxation of the vessel. Since the dilator response to both acetylcholine and nitroprusside involves activation of the guanylate cyclase system, a generalized defect within the guanylate cyclase system can be ruled out as a possible explanation for endothelial dysfunction if a response occurs to nitroprusside but not to acetylcholine.

Vessel rings were preconstricted with 10^{-5} M prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, dinoprost tromethamine, 5 mg/mL, The Upjohn Company of Canada, Don Mills, Ontario)

(O'Murchu *et al.*, 1994; Ontkean *et al.*, 1991) and challenged with increasing amounts of either acetylcholine (iodide, Sigma A-7000) from 10^{-9} to 10^{-5} M (Noll *et al.*, 1994; Teerlink *et al.*, 1994), or sodium nitroprusside (Sigma S-0501) from 10^{-10} to 10^{-6} M (Thuillez *et al.*, 1995). These ranges of concentrations produced full concentration-response curves for their respective drugs. The response to each dose added was allowed to reach a stable plateau before the next dose was added, approximately two to three minutes. Responses were expressed as a percentage of the maximum constriction to $PGF_{2\alpha}$ for each tissue. Between individual concentration-response curves, vessels were washed repeatedly with fresh aerated Krebs' solution and allowed to return to baseline tension.

2.4.1 Rationale for Nitrite Assay

Extracellular fluids such as plasma are continuously being circulated to all parts of the body. Rapid transport and subsequent mixing between plasma and the extracellular fluid via diffusion through the capillary endothelial cells allows all cells in the body to be exposed to essentially the same extracellular environment. Thus, the presence of nitrate and nitrite in the extracellular fluid is a good indicator of those metabolic processes that occur at the cellular and tissue level (Grisham *et al.*, 1995).

Nitric oxide itself is difficult to quantitate because it is generally produced in small amounts and has a half-life of less than six seconds (Granger *et al.*, 1995; Iyengar *et al.*, 1987). The major route of nitric oxide decomposition *in vivo* is the reaction with oxyhemoglobin in the red blood cells to form nitrate and methemoglobin (Beckman *et al.*, 1990). Another possible route is through formation of peroxynitrite by the reaction of

nitric oxide with superoxide (Beckman *et al.*, 1990). Peroxynitrite can react directly with organic molecules such as sulphydryls resulting in nitrite and nitrate being formed (Koppenol *et al.*, 1992). The resulting nitrite proceeds to react with oxyhemoglobin once it enters the vascular system and as a result only small amounts are present in plasma. The reaction of nitrite with oxyhemoglobin results in stoichiometric formation of nitrate and methemoglobin. The majority of nitric oxide synthesized from L-arginine is detected in plasma, serum, and urine as nitrate; however, nitrite that enters body fluids without traversing a vascular space may not be completely oxidized to nitrate (Granger *et al.*, 1995). Thus, determination of plasma levels of both nitrate and nitrite is a useful method of quantifying basal systemic nitric oxide production since the only known endogenous source of nitrate and nitrite is the L-arginine-nitric oxide pathway. It should be noted that plasma nitrate levels reflect not only endogenous nitric oxide production but also total nitrate ingestion from the diet, and the minor contribution made by bacterial metabolism of amino compounds in the gut (Grisham *et al.*, 1995).

Our protocol employs the Griess reaction for indirect determination of nitric oxide and is similar to that used by Grisham and others (Grisham *et al.*, 1995; Kichuk *et al.*, 1996; Kumura *et al.*, 1994; Schmidt, 1995; Schmidt *et al.*, 1992; Tracey *et al.*, 1995; Vodovotz *et al.*, 1996).

The Griess reaction, developed in 1879 (reviewed in Grisham *et al.*, 1995), is based on the formation of a chromophore during the reaction of nitrite with sulfanilamide and heterocyclic amines such as N-(1-napthyl)ethylenediamine (NED) under acidic conditions. During this reaction, acidified nitrite undergoes a diazotization reaction with

sulfanilamide to form a diazonium salt which then couples to NED to form a red chromophore with a characteristic absorption spectrum. Griess reagents may also react with nitrosothiols and nitrosoamines, however, nitrate does not undergo the diazotization reaction and therefore must first be chemically reduced to nitrite in order to react with the Griess reagents (Granger *et al.*, 1995).

The nitrate reduction in the following experiments was performed using bacterial nitrate reductase (Grisham *et al.*, 1995; Tracey *et al.*, 1995) but the reaction may also be done using cadmium metal (Green *et al.*, 1982; Kumura *et al.*, 1994). Other indirect methods of measuring nitric oxide include bioassays for cGMP which are fairly nonspecific and other assays for nitrate and nitrite such as gas chromatography-mass spectrometry (Tesch *et al.*, 1976), chemiluminescence (Archer, 1993), and high-performance liquid chromatography (Romero *et al.*, 1989). Most methods available require expensive equipment. Mass spectrometry is the most sensitive technique but it is not suitable for routine analyses (Leone *et al.*, 1995).

2.4.2 Nitrate Assay Protocol

Blood samples were collected from rats eight weeks post-surgery. Rats were anaesthetized with sodium pentobarbital (50 mg/kg i.p.) and the right carotid artery was cannulated with a PE-50 catheter for blood collection. Blood samples were collected in heparinized tubes, centrifuged for 15 minutes at 10,000 g, and plasma collected and stored at -80°C for future use. A 10 mM stock solution of sodium nitrate ($NaNO_3$; Sigma S-5506) standard was stored in 100 µL aliquots at -70°C for up to two months. This stock was diluted to yield a standard curve ranging from 5 to 50 µM.

To begin, 100 μL of sample, standard, or water blank was added to a red polypropylene Eppendorf tube containing 200μL H_2O, 25 μL 1M Hepes buffer (pH 7.4, Sigma H-3375), 25 μL 0.1 mM flavin adenine dinucleotide disodium salt (FAD, Sigma F-6625), 50 μL 1mM nicotinamide adenine dinucleotide phosphate (NADPH, Sigma N-7505), and 100 μL 1U/mL *E.coli* nitrate reductase (Boehringer Mannheim 981-249) and incubated at 37°C for three hours. Next, 5 μL 1500U/mL lactic dehydrogenase (LDH from bovine muscle, Sigma L-1378) and 50 μL 100 mM pyruvic acid (sodium salt, Sigma P-2256) were added and the mixture was incubated for 10 minutes at 37°C.

Since the plasma samples contained heparin, which may precipitate upon addition of the Griess reagent and interfere with spectrophotometric reading (Grisham *et al.*, 1995), samples were applied with 50 μL 10 mg/mL protamine sulphate (from Salmon; Sigma P-4020) to remove heparin. Samples were vortexed, incubated for 5 minutes at room temperature, and centrifuged for 10 minutes at 10,000 rpm to remove the precipitate. 500 μL of the supernatant was transferred to glass culture tubes and 1 mL premixed Griess reagent added. The Griess reagent consists of 0.2% NED in 5% phosphoric acid combined with an equal volume of 2% sulfanilamide in 5% phosphoric acid. After 10 minutes incubation at room temperature, the absorbance at 543 nm was read. All samples were done in duplicate and the average taken.

2.5.1 Rationale for L-arginine Treatment Dose

Previous studies administering L-arginine to rats in drinking water have used a variety of doses: 0.63 mmol/kg/day (Matsuoka *et al.*, 1996); 2.25% L-arginine HCl in drinking water, approximately 1.2g/day (Cooke *et al.*, 1992a); 0.05g free base L-

arginine/kg/day (Lubec *et al.*, 1996); 1.25g/L in drinking water, approximately 0.2g/kg/day (Resta & Walker, 1994). We administered two different concentrations of L-arginine to two separate populations of rats in order to observe any dose-dependent effects.

In order to establish the dose of L-arginine being administered to the rats chronically, randomly selected sham and heart failure rats were given a stock mixture of either 12.5g/L or 50g/L L-arginine (Sigma A-5006) as their drinking water and the consumption of said mixture was monitored.

2.5.2 L-Arginine Treatment

The amount of water consumed by both non-treated and L-arginine-treated rat groups was measured by weighing the water bottle and contents. These drinking water measurements were taken twice weekly and the average daily consumption was estimated by dividing the measured change in water present in the water bottle by the number of days since last measurement. A similar technique was used by Lubec (Lubec *et al.*, 1996). Water consumption was measured to ensure that the drinking habits of the different treatment groups were not significantly different and to establish the dose of L-arginine being administered over the course of the study. The average dose of L-arginine received by the two treatment groups was calculated by multiplying the estimated daily water consumption by the L-arginine stock concentration and dividing by the average rat body weight.

2.5.3 Measurement of Plasma L-arginine Levels

Blood samples were collected from rats eight weeks post-surgery. Rats were anaesthetized with sodium pentobarbital (50 mg/kg i.p.) and the right carotid artery was cannulated with a PE-50 catheter for blood collection. Blood samples were collected in heparinized tubes, centrifuged for 15 minutes at 10,000 g, and plasma collected and stored at -80°C for future use. Plasma samples were sent to the Children's Psychiatric Research Institute, London, Ontario, for detection of L-arginine by ion exchange chromatography in an automated amino acid analyzer using the ninhydrin detection system. Samples of 0.2mL heparinized plasma were deproteinized by addition of 0.3mL sulfosalicylic acid solution, and centrifuged at 16000 rpm. The supernatant was frozen and sent to the institute where it was filtered through a 0.22 μM filter prior to being applied to the analyzer.

2.6 Statistical Analysis

Data were expressed as the mean ± standard error of the mean (SEM). EC_{50} values were calculated with Graphpad Prism via non-linear regression. All statistical comparisons were performed with a commercially available package for PC computers (Microsoft EXCEL). One way analysis of variance analyses (ANOVA) were performed on data to detect significance between the multiple groups. These analyses, considered significant with $P<0.05$, were followed by two-tailed Fischer's Least Significant Difference procedure to detect significance between individual groups. In comparing

only two groups, such as in table I, only Students' t-test was employed. Again, differences were considered significant at the level of $P<0.05$.

Inclusion criteria for the entire study include an infarct size of greater than 30% of the left ventricle.

Additional exclusion criteria for the organ bath studies included tissues which failed to yield a constriction response to potassium chloride, had an unstable or shifting baseline, or which failed to dilate to 50% of the maximum constriction in response to sodium nitroprusside indicating severe vessel damage as a result of tissue handling. Tissue that exhibited very pronounced intrinsic oscillations after being exposed to $PGF_{2\alpha}$ or acetylcholine and from whose tracing a credible measurement could not be made were also excluded.

Exclusion criteria for the hemodynamic study included rats in whom the thermal dilution probe position was found to be below the thoracic aorta upon visual confirmation at autopsy. As well, it should be noted that some measurements were unobtainable as a result of a line being pulled loose or blocked by a coagulated blood even though measures were taken to avoid such instances. The criteria mentioned here account for any discrepancies in sample size numbers within groups.

3. Results

3.1 Characterization of the Rat Heart Failure Model

Rats that underwent the coronary artery ligation surgery were not readily discernible from sham rats by their general appearance. There were no outwardly visible signs of peripheral edema, respiratory distress, nor failure to groom, and there was no significant difference in the body weights of these two groups. Differences were, however, detected upon hemodynamic analysis. Table I lists the various characteristics of heart failure and sham rats.

Figure 3 includes photographs representing the second section of typical ligated and sham left ventricles which were sliced transversely from apex to base. It was from such photographs that the infarct size was calculated for all ligated rats. Scar tissue, ventricular wall thinning in the infarcted area, volume expansion, as well as hypertrophy of the non-infarcted ventricular muscle characteristic of heart failure were all obvious in comparing the infarcted photograph to that of sham. There was no significant difference in the infarct sizes of the rat population used in the hemodynamic compared to organ bath studies, 42.4±2.1% and 38.1±1.3%, respectively ($P=0.09$).

The heart weights standardized by body weight of heart failure rats were significantly increased compared to sham ($P<0.01$), this result was uniform throughout the heart as it was seen in atria, and both the right and left ventricles, table I. Similarly, the left ventricular volume of heart failure rats was also significantly increased compared to sham ($P<0.05$).

Hemodynamic parameters are also listed in Table I. Heart failure rats displayed decreased cardiac index compared to sham ($P<0.05$). As well, the ligated rats exhibited

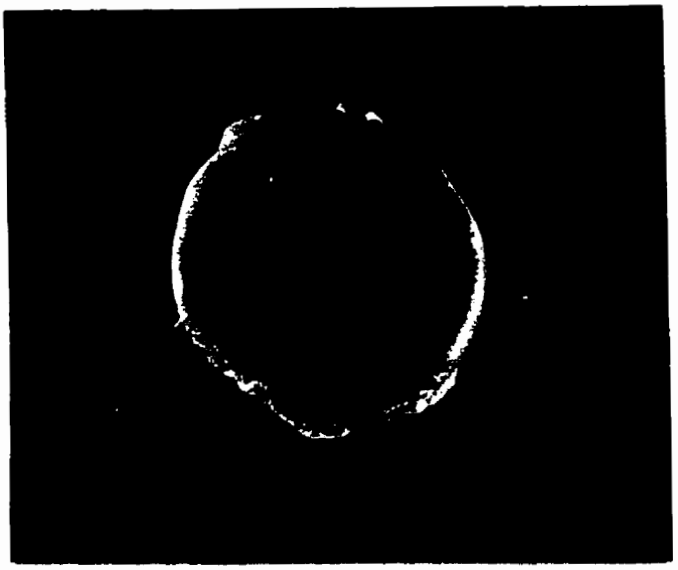
elevated left ventricular end-diastolic pressures, depressed systolic left ventricular pressures, and reduced rates of pressure development and relaxation (+dP/dt and -dP/dt, respectively) ($P<0.01$). There was no significant change in heart rate nor in mean arterial pressures.

TABLE I: Physical and hemodynamic characteristics of sham compared to heart failure rats 8 weeks following surgery.

	Sham	Heart Failure
N	11	11
WEIGHTS		
BODY (g)	509±13	500±13
HEART/BODY (mg/g)	2.5±0.1	3.1±0.2*
ATRIA/BODY (mg/g)	0.21±0.01	0.44±0.08*
RV/BODY (mg/g)	0.46±0.01	0.74±0.09*
LV/BODY (mg/g)	1.80±0.04	1.92±0.05$^{\#}$
LV VOLUME (mL)	0.109±0.010	0.291±0.05*
CI (mL/min/100g body)	21.2±1.0	17.7±1.2*
LVEDP (mmHg)	4.2±1	12.1±2.2**
SLVP (mmHg)	145±3	124±4**
+ dP/dt (mmHg/sec)	2935±66	2296±119**
- dP/dt (mmHg/sec)	3014±121	2366±144**
MAP (mmHg)	114±5	107±4
HEART RATE (bpm)	407±13	400±15

Values expressed as Mean±SEM. *P<0.05, **P<0.01 vs. Sham, $^{\#}$P=0.05 vs. Sham.

(a)



Scale: 1cm

(b)

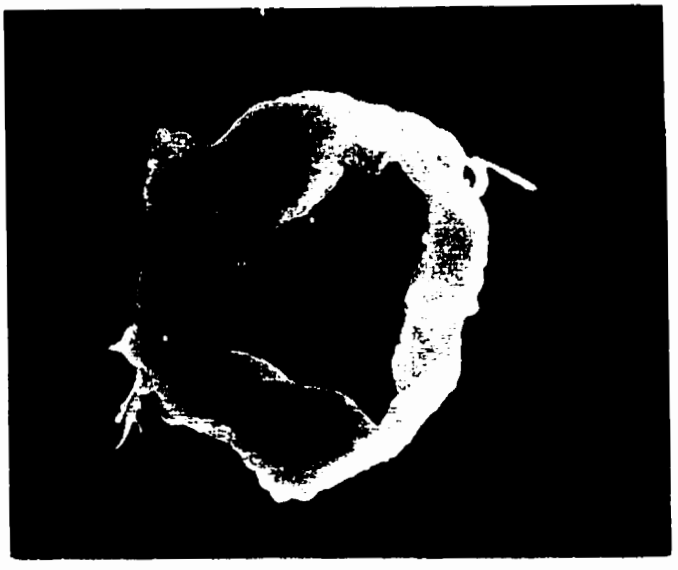


Figure 3: Left ventricles were sliced transversely from apex to base in order to evaluate the infarct size. Above are representative photographs of the second section of typical sham (a) and ligated (b) left ventricles. The infarcted heart displays scarring, volume expansion, and hypertrophy. Only rats with infarcts greater than 30% of the left ventricle were included in the studies as heart failure rats.

3.2 Characterizing the Chronic Oral L-arginine Treatment

Figure 4 illustrates the trend in water consumption for all six rat groups, untreated sham and heart failure, low dose L-arginine treated sham and heart failure, and high dose L-arginine treated sham and heart failure rats. In the first week post-surgery water consumption was significantly lower compared to following weeks ($P<0.01$). Water consumption increased over the following weeks and plateaued at approximately 50 mL of water per day after the third week. The average low dose of L-arginine received by the rats in the final week was calculated as 1.36 ± 0.04 g/kg/day. The average high dose of L-arginine was calculated as 5.09 ± 0.15 g/kg/day. Figure 5 shows that the low dose L-arginine treated sham and heart failure rats received similar doses of L-arginine ($P=0.18$), as did the high dose L-arginine treated sham and heart failure rats ($P=0.09$).

In order to confirm that the L-arginine consumed was being absorbed by the rats, plasma concentrations of L-arginine were measured via ion exchange chromatography, figure 6. Heart failure rats exhibit a trend towards lower plasma L-arginine levels compared to sham. Low dose L-arginine treatment in heart failure rats appears to have restored plasma levels to that of untreated sham rats while the high dose L-arginine treated heart failure rats had significantly higher plasma arginine levels than untreated sham rats ($P<0.05$). High dose treated sham rats, however, displayed significantly higher plasma L-arginine levels than untreated sham and heart failure rats but were not different from high dose L-arginine treated heart failure rats ($P<0.05$, $P<0.05$, and P=n.s., respectively). There appears to be a dose-dependent increase in plasma L-arginine levels.

Plasma nitrate and nitrite levels were measured as an indication of basal nitric oxide production. Figure 7 shows that there was no significant difference between either untreated sham and heart failure rats, nor between untreated and L-arginine treated groups.

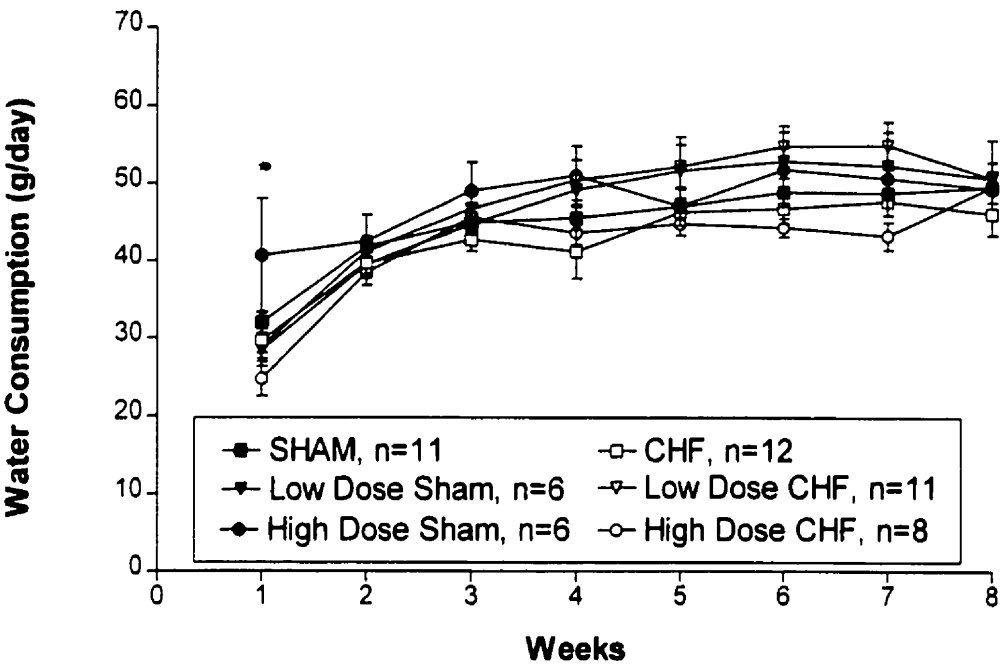


Figure 4: Rats were treated with either a high or low dose of L-arginine, or water placebo for 8 weeks following coronary artery ligation or sham surgery. The amount of water consumed was measured by weighing the water bottle and contents twice weekly. The average daily consumption (g/day) was estimated for each week. The water consumption in the first week post-surgery was significantly lower than consumption in the following weeks (* $P<0.01$).

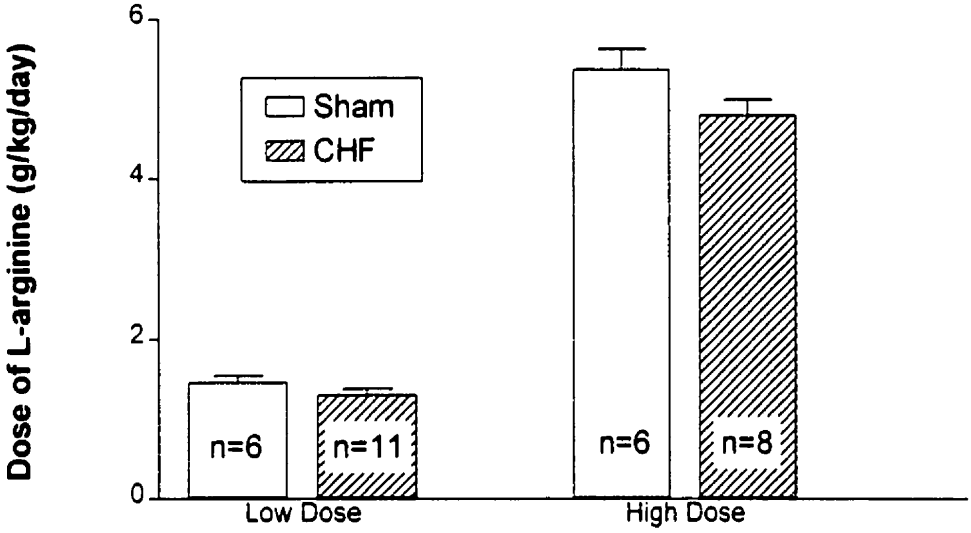


Figure 5: The average dose of L-arginine consumed by the low and high dose treated rats was calculated by multiplying the estimated daily water consumption by the L-arginine stock concentrations, 12.5g/L and 50g/L respectively, and dividing by body weight. There was no significant difference within treatment groups. P=0.2 and 0.09, respectively for low and high doses.

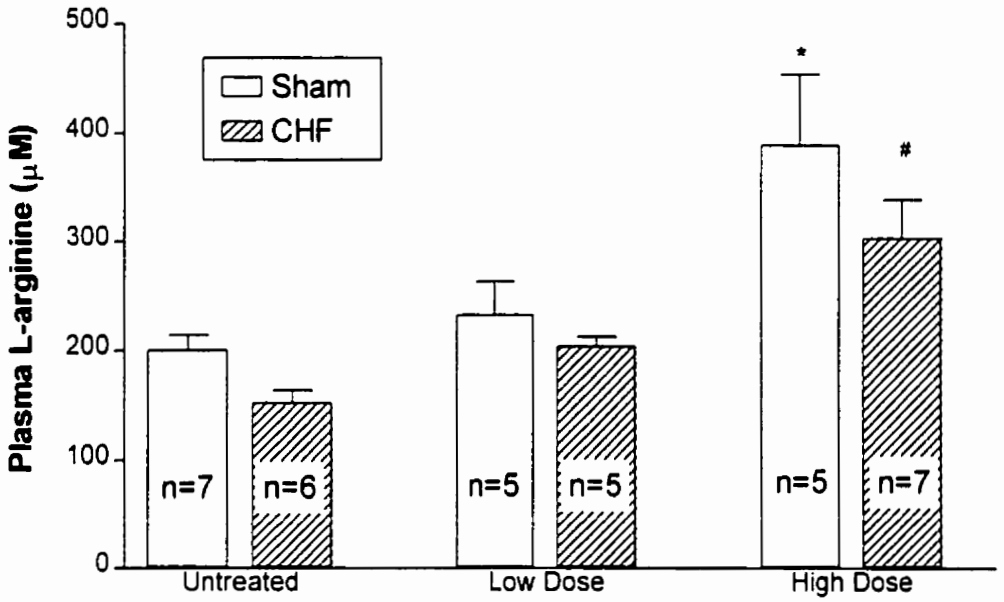


Figure 6 : The plasma L-arginine concentrations were measured by ion exchange chromatography and are expressed as mmol/L. There appears to be a trend towards decreased plasma arginine concentrations in untreated CHF vs. Sham, and an L-arginine treatment-dependent increase in plasma concentrations. *P<0.05 vs. Untreated and Low Dose L-Arginine treated Sham and CHF; # P<0.05 vs. Untreated and Low Dose L-Arginine treated CHF and Untreated Sham.

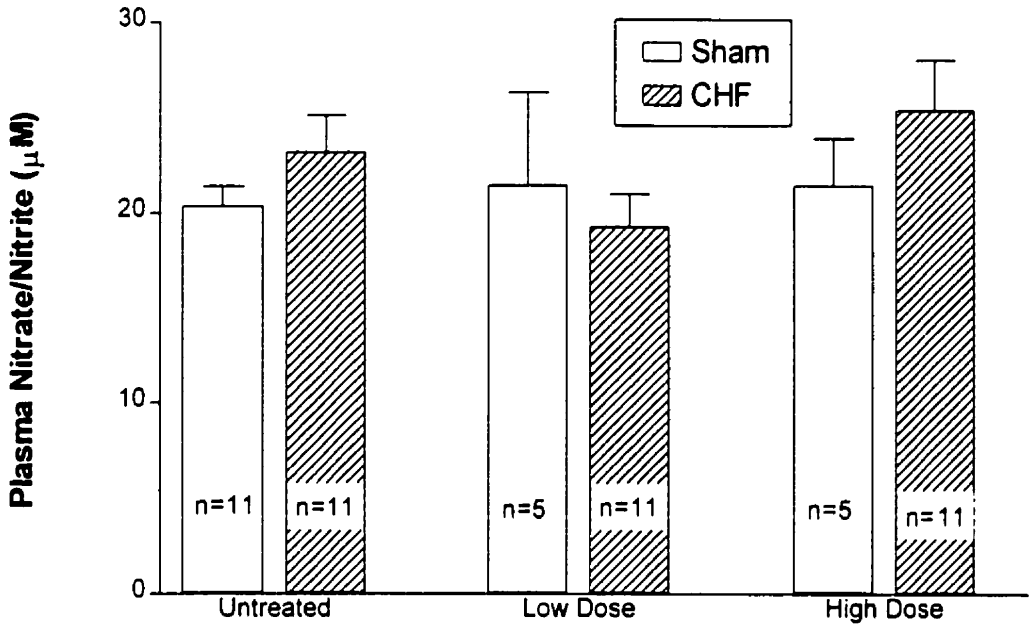


Figure 7: Plasma levels of nitrate and nitrite were assayed using the Griess reaction for indirect determination of basal nitric oxide levels in plasma. No significant difference between the plasma nitrate/nitrite levels of untreated or L-arginine treated sham or heart failure rats was detected. ANOVA P=0.47

3.3 The Effects of L-Arginine Treatment on Cardiac Function in Sham Rats

Sham rats were given L-arginine treatment to serve as controls for the treated heart failure rats. There was no sign of infarction in these rats and neither low nor high dose L-arginine treatment group was visibly discernible from untreated sham. All rats ate and drank normally, moved about freely in the cage, and groomed normally.

Table II lists the characteristics of these three sham groups. There was no difference between the body weights of untreated and low and high dose L-arginine treated sham rats. There was no significant difference between any of the three sham groups in heart weights normalized for body weights and this was seen consistently throughout all sections of the heart. There was no significant difference in the left ventricular volumes between untreated and L-arginine treated sham rats.

Hemodynamic analyses, also presented in Table II, showed no significant difference in the cardiac outputs of untreated and L-arginine treated sham groups, nor was there any difference in left ventricular end-diastolic pressure, systolic left ventricular pressure, or in rates of developed pressure and relaxation.

TABLE II: Physical and hemodynamic characteristics of untreated and L-arginine treated sham rats after 8 weeks of treatment post-surgery.

	SHAM	LOW DOSE SHAM	HIGH DOSE SHAM
N	11	5	5
WEIGHTS			
BODY (g)	509±13	493±19	452±8
HEART/BODY (mg/g)	2.5±0.1	2.6±0.1	2.5±0.1
ATRIA/BODY (mg/g)	0.21±0.01	0.22±0.02	0.22±0.03
RV/BODY (mg/g)	0.46±0.01	0.49±0.02	0.47±0.01
LV/BODY (mg/g)	1.80±0.04	1.91±0.10	1.86±0.04
LV VOLUME (mL)	0.109±0.01	0.094±0.01	0.089±0.01
CI (mL/min/100g body)	21.2±1.0	18.5±1.5	20.8±1.3
LVEDP (mmHg)	4.2±1	4.8±1.6	4.6±1.7
SLVP (mmHg)	145±3	138±5	145±5
+ dP/dt (mmHg/sec)	2935±66	2951±95	2952±168
- dP/dt (mmHg/sec)	3013±121	2931±155	3068±171
MAP (mmHg)	114±5	109±8	115±8
HEART RATE (bpm)	407±13	403±23	406±15

Values expressed as Mean ± SEM.

3.4 The Effects of L-Arginine Treatment on Cardiac Function in Heart Failure Rats

Figure 8 demonstrates that there was no significant difference between the infarct sizes produced among untreated and L-arginine treated heart failure groups (P=0.4), while sham rats had no myocardial infarction.

There was no significant difference in the body weights of untreated and low or high dose L-arginine treated heart failure rats, figure 9. The heart weights, normalized for body weight, were not significantly different between the untreated and L-arginine treated heart failure groups, but they were significantly increased compared to sham rats ($P<0.05$), figure 10. This characteristic was uniform throughout the heart as it was evident in the atria, right and left ventricles, figure 11 a, b and 12 a. Thus, no effect of the L-arginine treatment was seen.

Left ventricular volume of both untreated and L-arginine treated heart failure rats was significantly increased compared to untreated sham rats ($P<0.05$), figure 12b. However, there was no difference between untreated and L-arginine treated heart failure rats.

Cardiac output in untreated heart failure was decreased compared to sham ($P<0.05$). Low dose L-arginine treatment did not improve cardiac output in heart failure compared to untreated, however, high dose L-arginine treated heart failure rats had cardiac outputs that were not significantly different from either untreated sham or heart failure, see figure 13.

Left ventricular end-diastolic and systolic pressures, figures 14 and 15, were significantly different from sham for all three heart failure groups ($P<0.05$) while there

was no difference between untreated and L-arginine treated heart failure rats. Similarly, the heart failure rats displayed decreased rates of pressure development and relaxation ($\pm dP/dt$) compared to sham ($P<0.05$) but no differences were seen between untreated and L-arginine treated heart failure rats, figure 16 a and b.

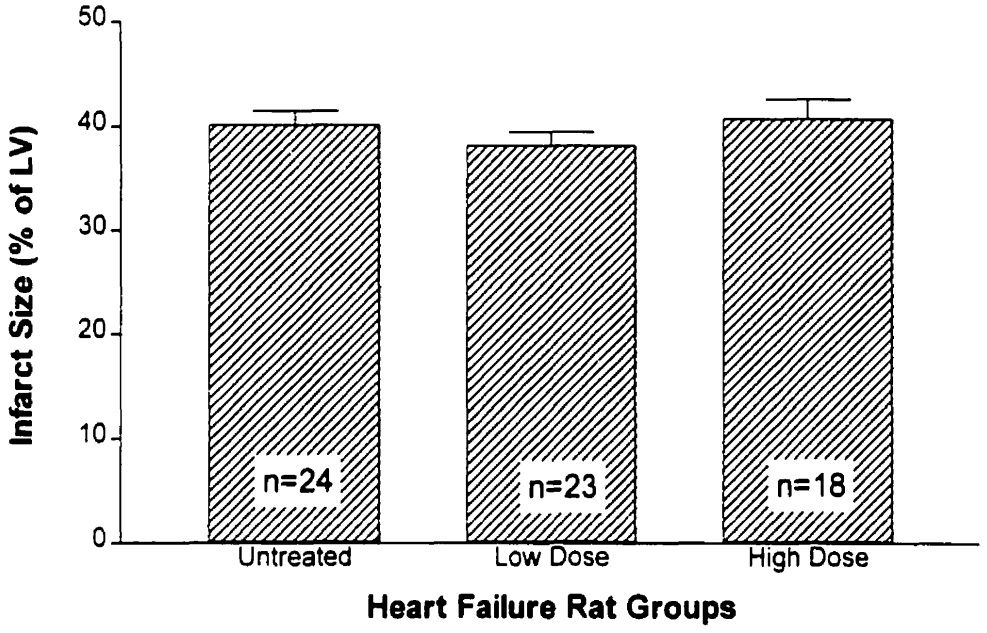


Figure 8: Comparison of infarct sizes induced by coronary artery ligation in untreated, low dose, and high dose L-arginine treated heart failure rats. Infarct size was evaluated by tracing the circumferences of fibrotic and normal cross-sectional areas in a ligated heart. Infarct size is expressed as a fraction of the total cross-sectional endocardial circumference of the left ventricle. Only rats with infarct size greater than 30% of the left ventricle were included in the studies. No significant difference between groups was observed. ANOVA P=0.4.

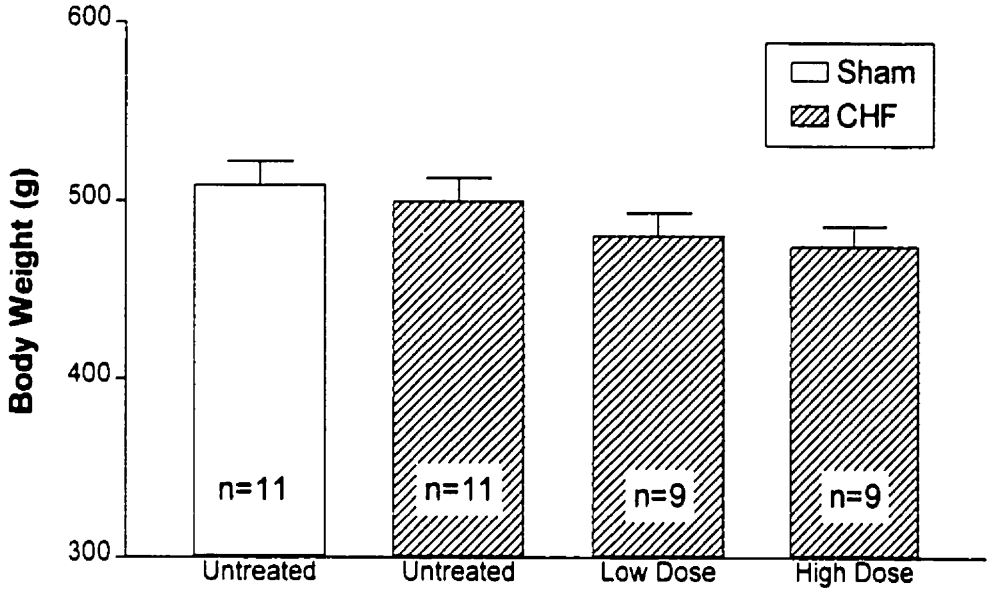


Figure 9: Comparison of body weights of rats 8 weeks following coronary artery ligation or sham surgery. There was no significant difference between the body weights of untreated sham, untreated heart failure, low or high dose L-arginine treated heart failure rats. ANOVA P=0.1.

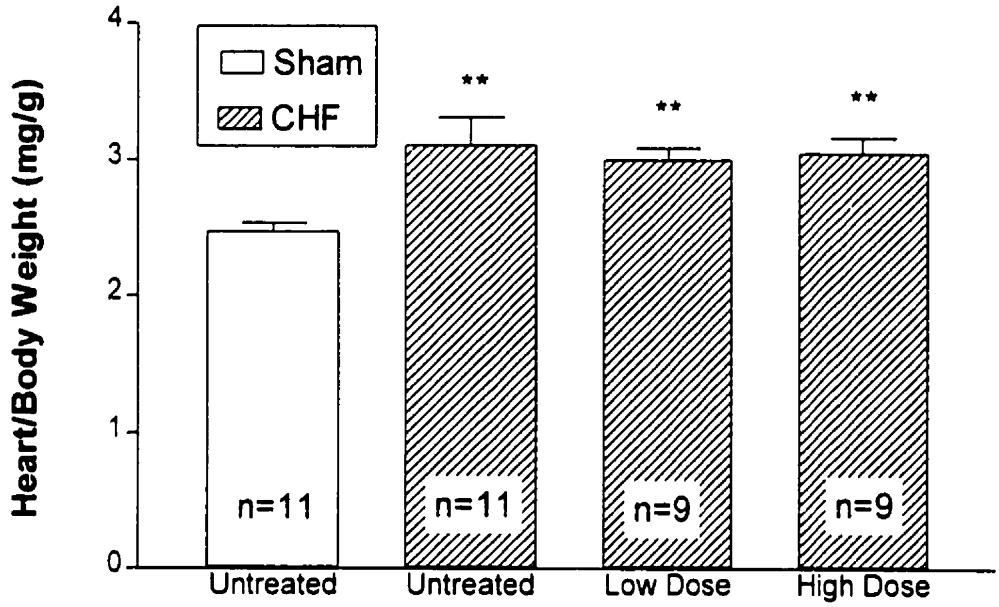


Figure 10: Comparison of heart weights of rats 8 weeks following coronary artery ligation or sham surgery. Values are expressed as heart weight (mg) normalized for individual rat body weights (g). All heart failure groups, both untreated and L-arginine treated, had heart weights greater than untreated sham rats. ** $P<0.01$ vs. untreated sham.

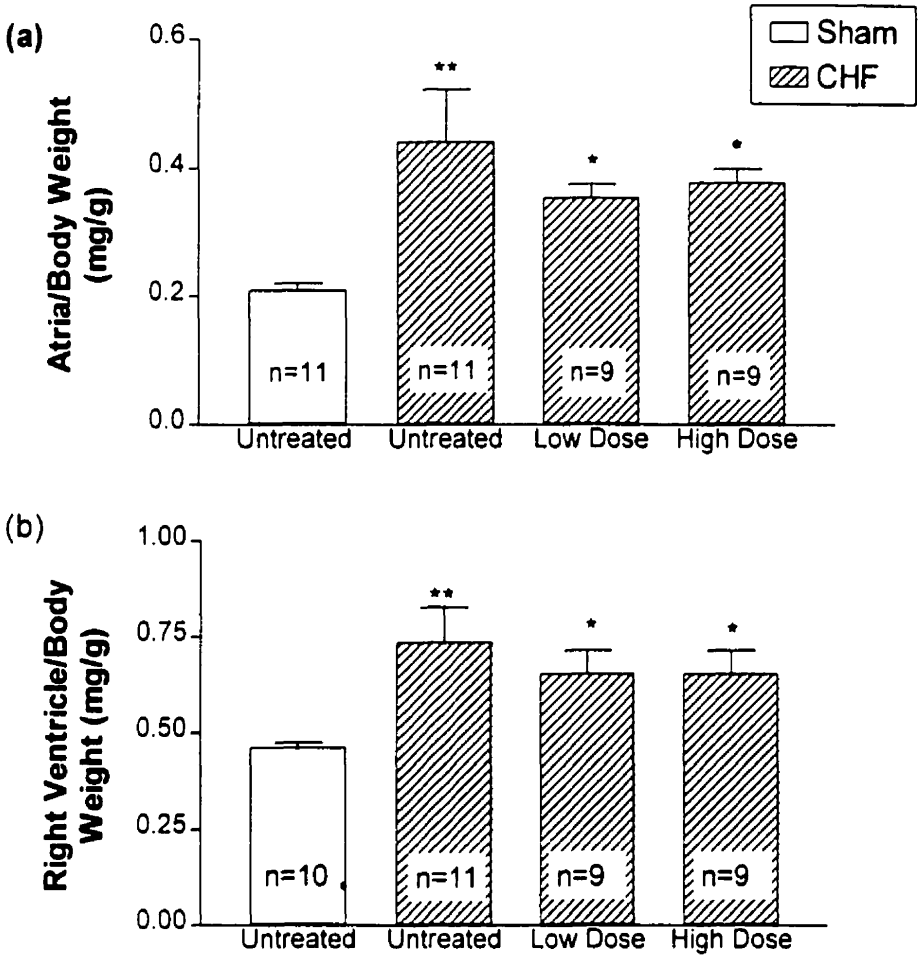


Figure 11: Eight weeks following coronary artery ligation or sham surgery, hearts were excised, atria were removed from the ventricles, and the right ventricle free wall was dissected from the septum. Tissue was blotted dry and weighed. Increased weight was seen consistently in the heart sections of heart failure rats compared to sham rats. There was no difference between the weights of untreated and low or high dose L-arginine treated heart failure rats. *P<0.05 **P<0.01 vs. untreated sham.

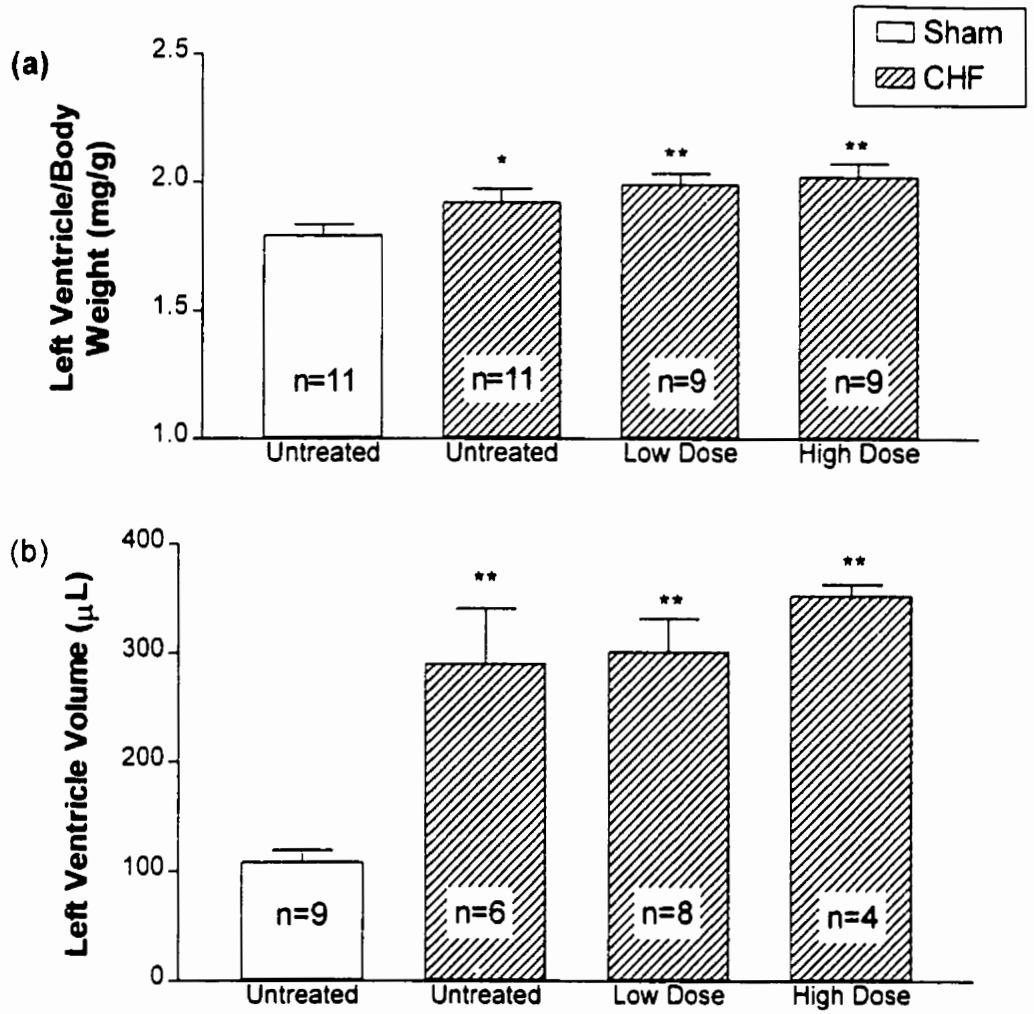


Figure 12: Comparison of left ventricle weights (a) and volumes (b) of rats 8 weeks following coronary artery ligation or sham surgery. Hearts were excised and the left ventricle was isolated. Volume was measured by weighing the ventricle both before and after fluid injection under atmospheric pressure, volume is presented in mL (b). The dry ventricle weight was normalized for whole body weight and is presented as mg ventricle/ g body weight (a). The untreated and L-arginine treated heart failure rats exhibited both significantly increased left ventricular weights and volumes compared to sham.
* $P<0.05$ ** $P<0.01$ vs. untreated sham.

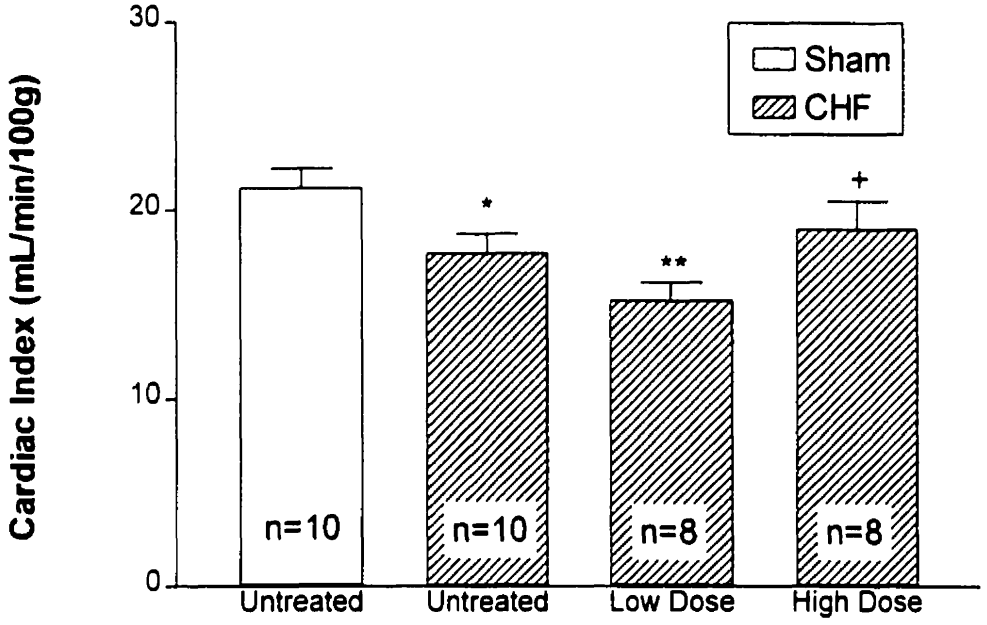


Figure 13: Cardiac output was measured by thermodilution method and normalized for individual rat body weights to yield cardiac index which is expressed as mL/min/100g body weight. The cardiac output was significantly depressed in both untreated and low dose L-arginine treated rats compared to untreated sham, *P<0.05 **P<0.01. High dose L-arginine treated heart failure rats had cardiac output not signficantly different from either untreated sham or heart failure, but was significantly increased compared to low dose L-arginine treatment, + P<0.05.

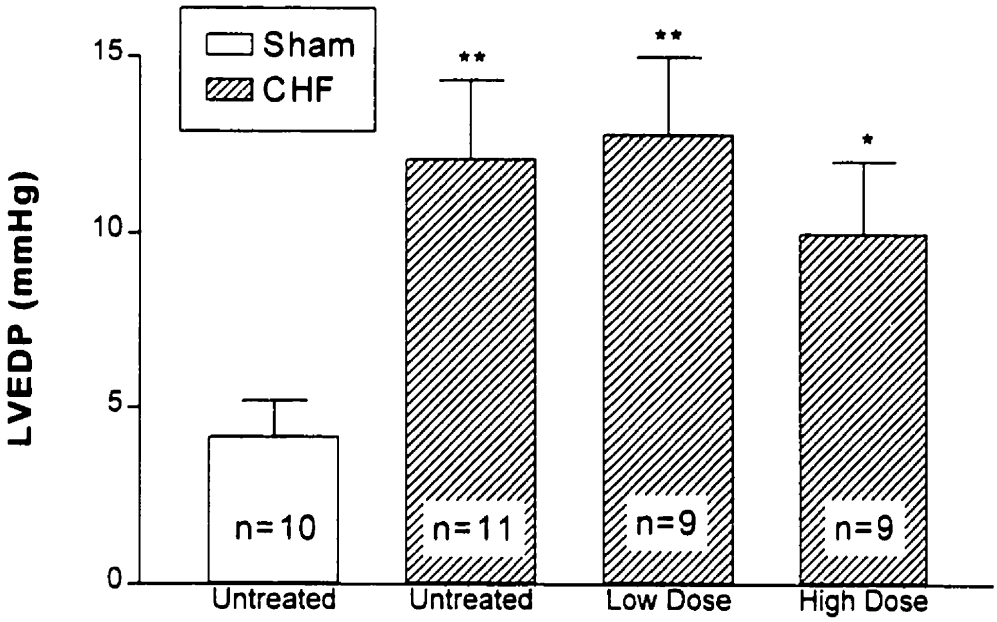


Figure 14: The right carotid artery was cannulated and the catheter advanced into the left ventricle for measurment of left ventricular end-diastolic pressures (LVEDP) using a Hewlett-Packard pressure transducer. LVEDP was elevated in all heart failure groups, both untreated and L-arginine treated. There was no significant difference within the heart failure groups. * P<0.05, ** P<0.01 vs. untreated sham.

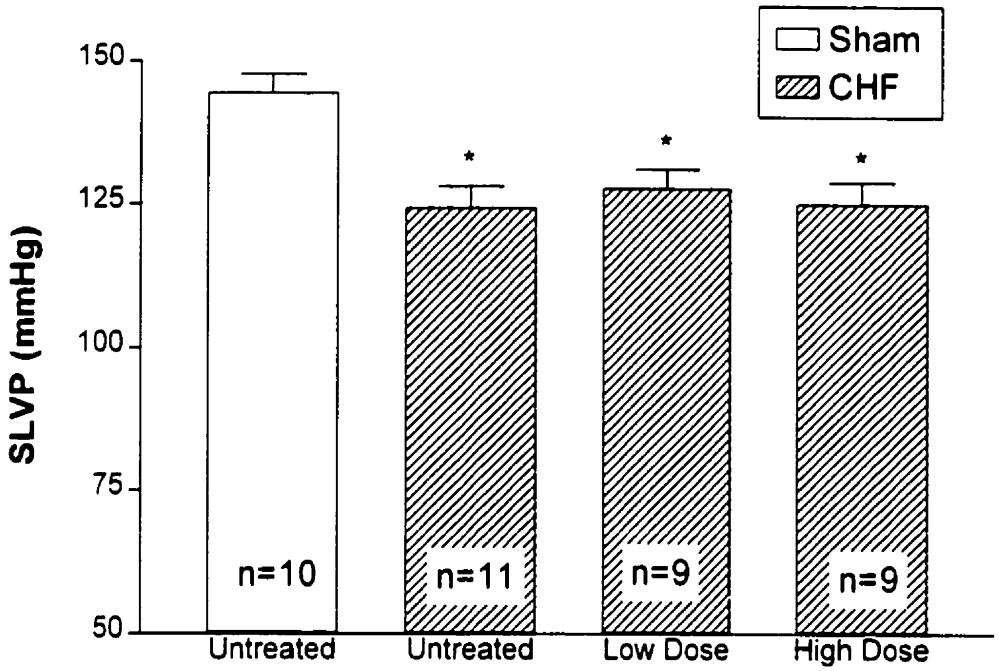


Figure 15: The right carotid artery was cannulated and the catheter was advanced into the left ventricle for measurement of left ventricular systolic pressure (SLVP) using a Hewlett- Packard pressure transducer. SLVP was depressed in all heart failure groups. There was no significant difference between untreated and L-arginine treated heart failure groups. * P<0.05 vs. untreated sham.

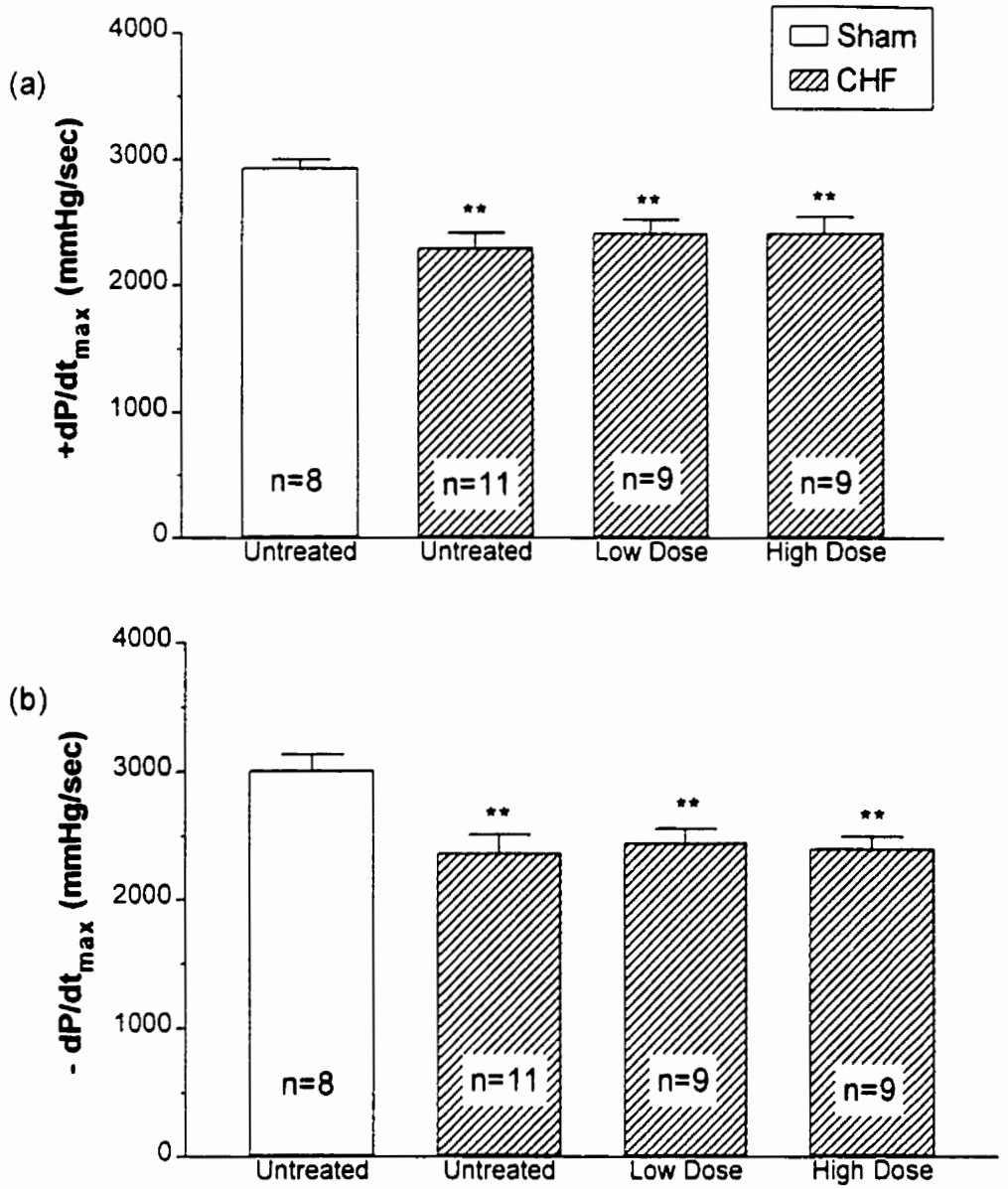


Figure 16: The right carotid artery was cannulated and the catheter was advanced into the left ventricle for pressure measurement using a Hewlett-Packard pressure transducer. The maximal rate of pressure development (+dP/dt, (a)) and the maximal rate of relaxation (-dP/dt, (b)) were significantly depressed in all heart failure groups compared to sham. There was no difference in these values between untreated and L-arginine treated heart failure groups. **P<0.01 vs. untreated sham.

3.5 The Effects of L-Arginine Treatment on Endothelium-Dependent Relaxation

Organ bath experiments were conducted to evaluate the endothelium-dependent and -independent relaxation of thoracic aortae. Figure 17a shows the right shift in endothelium-dependent relaxation induced by acetylcholine of untreated heart failure rats compared to untreated sham rats. The EC_{50} value of the untreated heart failure group was higher than that of sham ($P<0.05$), table III. There was no significant difference between untreated sham and heart failure in the endothelium-independent relaxations to sodium nitroprusside, figure 17b.

There was no significant difference between the untreated and L-arginine treated sham groups in either endothelium-dependent or endothelium-independent relaxations, figures 18a and b.

Figure 19a illustrates the left-shift in the endothelium-dependent relaxation to acetylcholine of low dose L-arginine treated heart failure rats compared to untreated heart failure. This significant shift in the EC_{50} values of the dose response curve to acetylcholine demonstrated a restoration of endothelium-dependent relaxation to the level of untreated sham rats. The EC_{50} values of low dose L-arginine treated heart failure rats were similar to those of untreated sham, table III, but were significantly decreased from untreated heart failure, $P<0.05$. There was no significant difference between the endothelium-independent relaxations to nitroprusside of the low dose L-arginine treated and untreated heart failure rats, figure 19b.

The endothelium-dependent relaxation to acetylcholine in high dose L-arginine treated heart failure rats was not significantly different from that of untreated heart failure

rats, figure 20a. As well, there was no difference between the endothelium-independent relaxation to nitroprusside between these two groups, see figure 20b.

TABLE III: Comparison of the EC_{50} values for untreated and L-arginine treated heart failure rats in relaxation responses to acetylcholine and sodium nitroprusside.

	ACETYLCHOLINE (Log[M])	NITROPRUSSIDE (Log[M])
SHAM		
Untreated	-7.54±0.12	-8.47±0.12
Low Dose L-Arg	-7.74±0.06	-8.28±0.15
High Dose L-Arg	-7.66±0.18	-8.20±0.32
HEART FAILURE		
Untreated	-7.11±0.09*	-8.53±0.12
Low Dose L-Arg	-7.43±0.12	-8.48±0.16
High Dose L-Arg	-7.17±0.09*#	-8.39±0.19

Values expressed as Mean±SEM.
* P<0.05 vs. Untreated Sham. # P<0.05 vs. High Dose L-Arg Sham.

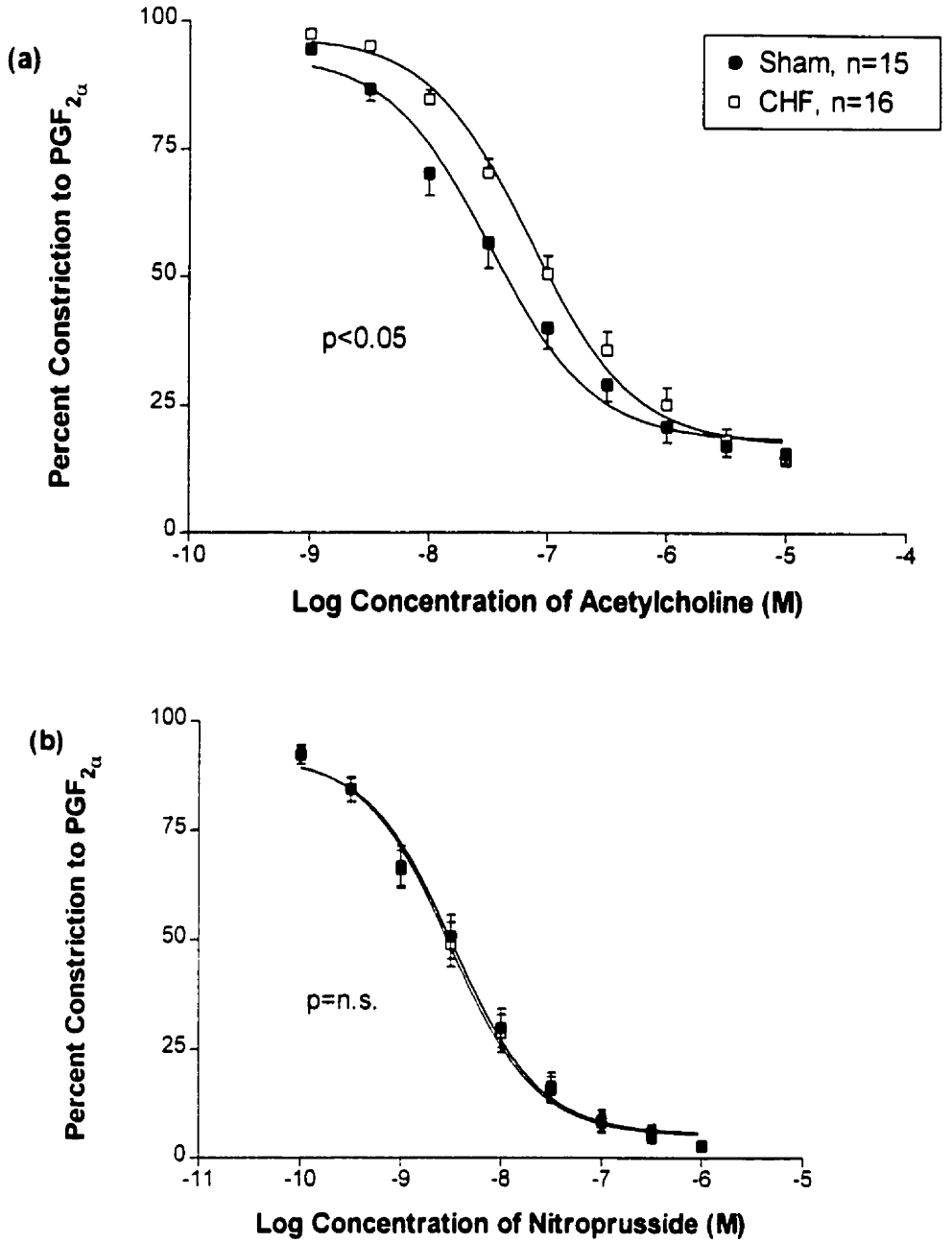


Figure 17: Cumulative concentration-response curves to acetylcholine (a) and sodium nitroprusside (b) were done on thoracic aortic ring segments. Results are expressed as percent of the maximal constriction to $PGF_{2\alpha}$. The endothelium-dependent relaxation response to acetylcholine (a) of heart failure rats was significantly right-shifted compared to sham, $P<0.05$. There was no significant difference in the endothelium- independent relaxations to nitroprusside of untreated sham and heart failure rats, $P=0.7$.

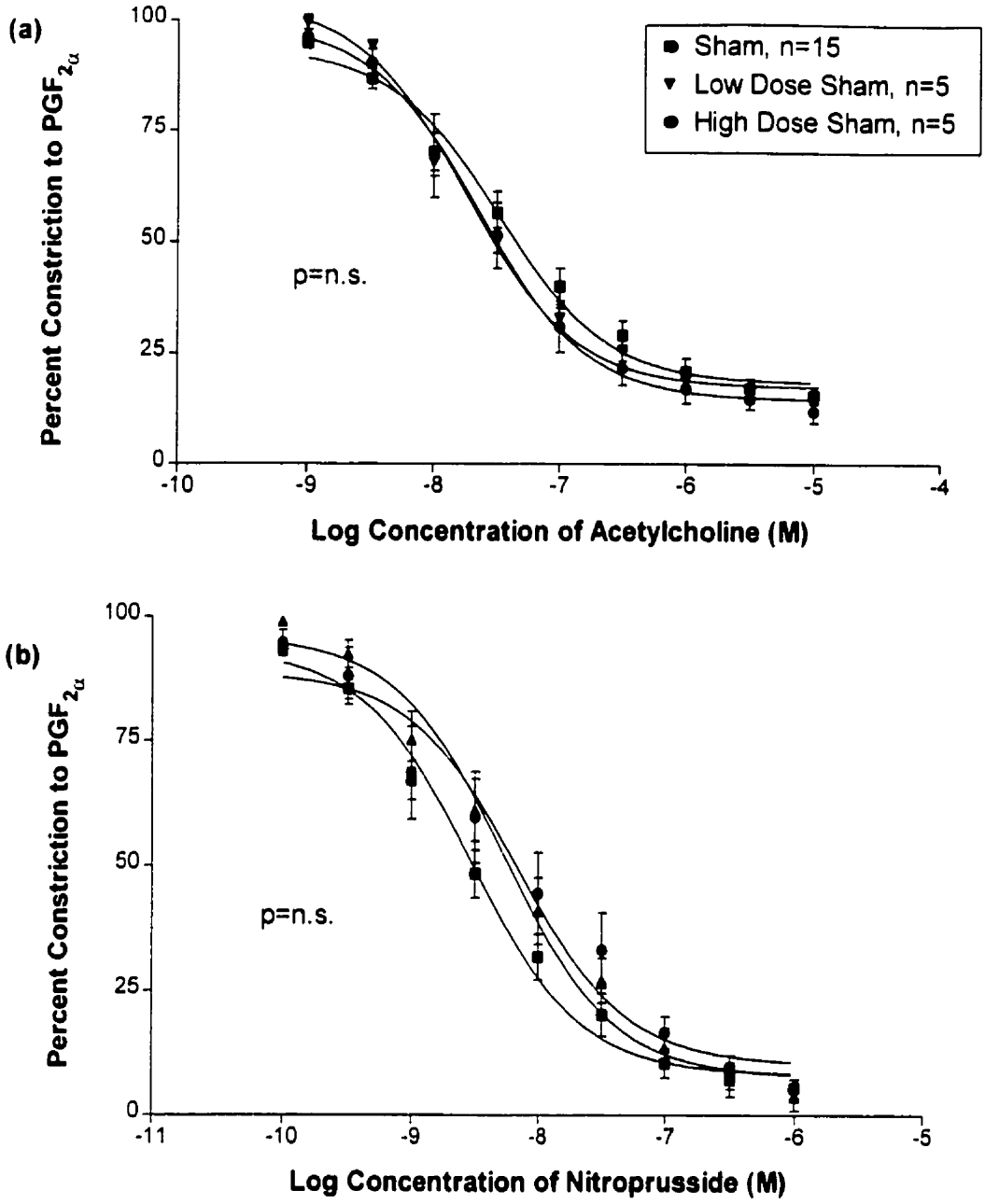


Figure 18: Cumulative concentration-response curves to acetylcholine (a) and sodium nitroprusside (b) were done on thoracic aortic ring segments. Results are expressed as a percent of the maximal constriction to $PGF_{2\alpha}$. There was no significant difference in either endothelium-dependent (a) or endothelium-independent (b) relaxations between untreated and low or high dose L-arginine treated sham rats. ANOVA P=0.5 and 0.5, respectively.

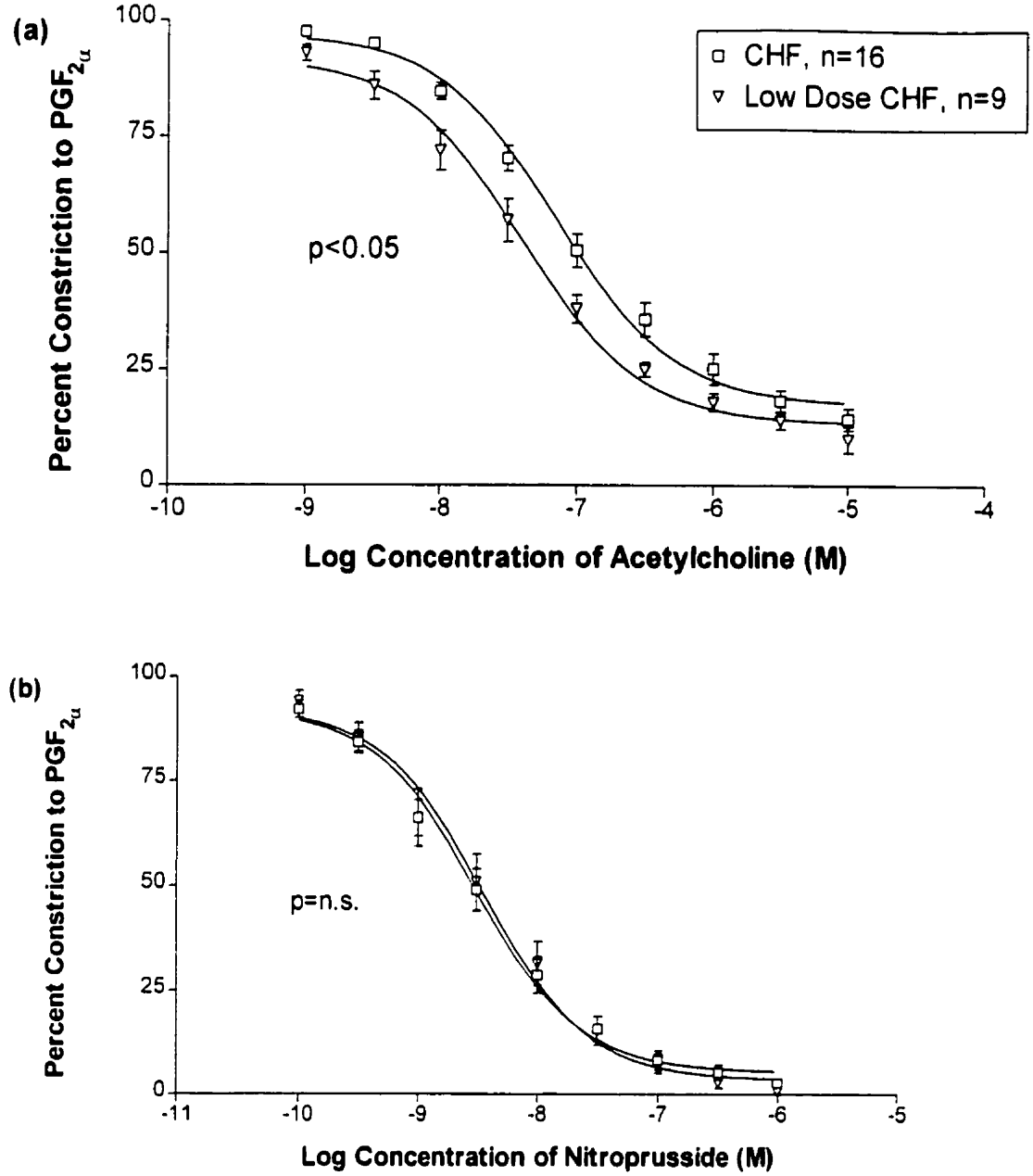


Figure 19: Cumulative concentration-response curves to acetylcholine (a) and sodium nitroprusside (b) were done on thoracic aortic ring segments preconstricted with $PGF_{2\alpha}$. Results are expressed as a percent of the maximal constriction. The endothelium-dependent relaxation response to acetylcholine (a) of heart failure rats treated with low dose L-arginine was significantly improved as shown by a left-shift in the curve compared to untreated heart failure rats, $P<0.05$. There was no significant difference in the endothelium- independent relaxations to nitroprusside (b) of untreated and low dose L-arginine treated heart failure rats, $P=0.8$.

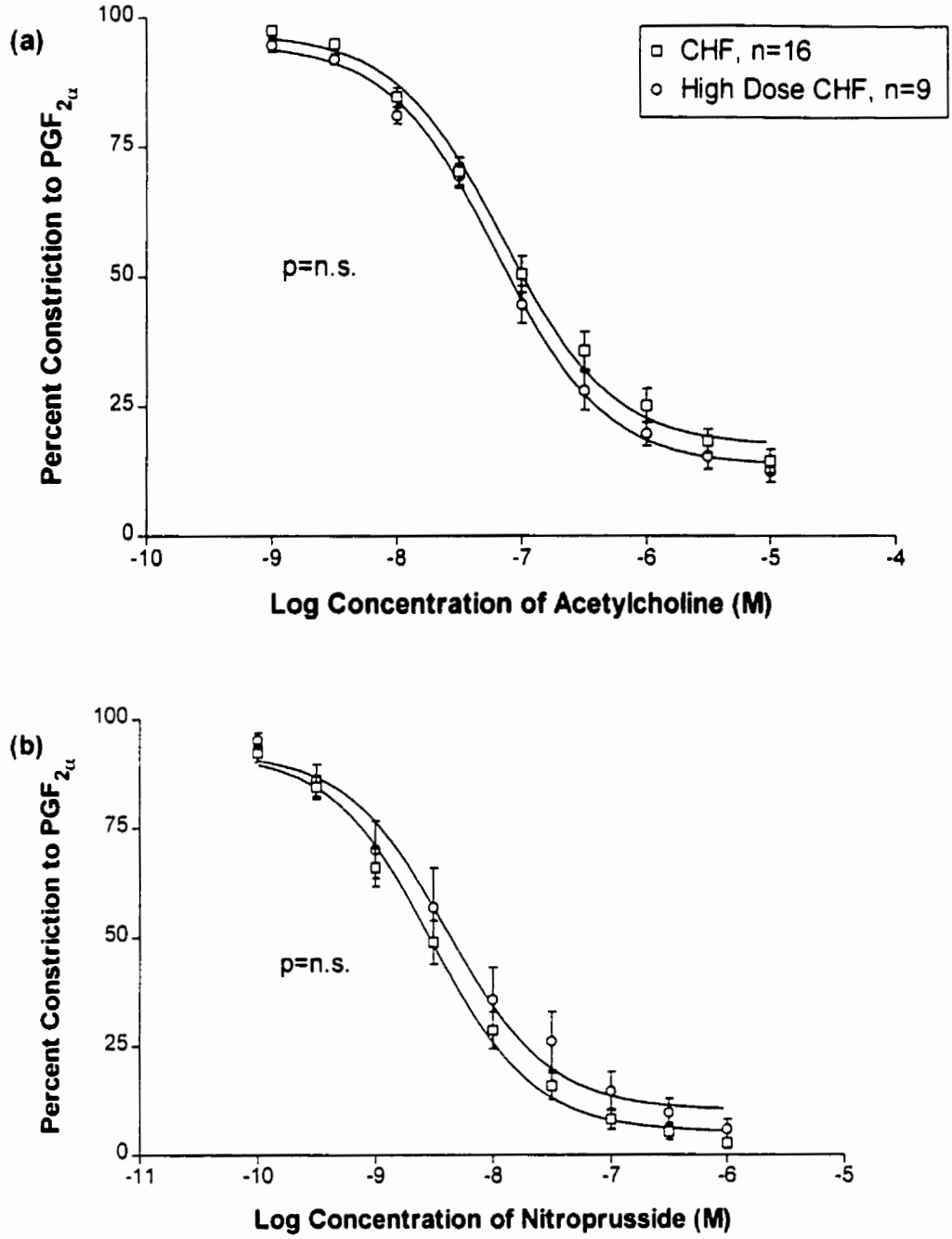


Figure 20: Cumulative concentration-response curves to acetylcholine (a) and sodium nitroprusside (b) were done on thoracic aortic ring segments preconstricted with $PGF_{2\alpha}$. Results are expressed as a percent of the maximal constriction. There was no significant difference in endothelium-dependent (a) or endothelium-independent (b) relaxations between untreated and high dose L-arginine treated heart failure rats. ANOVA P=0.6 & 0.5, respectively.

4. Discussion

4.1 Establishing the Heart Failure Model

Initially, studies were carried out in order to confirm that this well established model of heart failure could be reproduced in my hands and, therefore, that the results obtained following treatment with L-arginine were attributable to the treatment's effects on the condition of heart failure. In this rat model of heart failure the etiology involved induction of myocardial infarction, thus, the observation of an infarcted area on the left ventricle of the ligated hearts was the most obvious parameter used to discriminate between rats with heart failure and those without. Evidence of an infarcted area with necrosis, scar tissue, and alterations in ventricular wall thickness and volume expansion indicated that the syndrome of heart failure was present.

As mentioned, there was no difference seen in the body weights of sham and heart failure rats. This result is in agreement with previous studies that also found no significant difference between the body weights of sham and heart failure rats (Ontkean *et al.*, 1991; Pfeffer *et al.*, 1979). There are, however, other reports of significant decreases in body weights in heart failure rats (Sakai *et al.*, 1996; Thuillez *et al.*, 1995), but these studies were done in alternate strains of rats at various ages and weights which may explain the difference.

As a consequence of myocardial injury, the heart dilates to expand its volume and hypertrophies in an attempt to increase both stroke volume, strength of contraction, and cooperatively improve cardiac output. As discussed earlier, cardiac dilatation and hypertrophy attempt to maintain cardiac output, however, in heart failure the injured heart is incapable of increasing cardiac output past a critical point and the Frank-Starling

mechanism fails. The photographs of the ligated heart clearly illustrate what is also demonstrated by the heart weight and left ventricular volume measurements, that is, that there was hypertrophy of non-infarcted area and increased ventricular volume in heart failure rats. The marked hypertrophy of the right ventricle may have been indicative of elevated pulmonary pressure which was manifested as increased left ventricular end-diastolic pressure in heart failure rats. The combination of injury and dilatation of the left ventricle also contributed to the decreased contractile force generated and the elevated end-diastolic and decreased systolic pressures which directly reflected the condition of heart failure.

The present model demonstrated such typical traits of heart failure as depressed cardiac output, decreased left ventricular systolic pressure and rates of pressure development, impaired endothelium-dependent relaxation, and elevated left ventricular end-diastolic pressures. These hemodynamic parameters indicated that, indeed, the heart was unable to compensate for the myocardial injury and was not able to pump effectively. Although mean arterial pressure was not significantly different between groups there was a slight trend towards decreased mean arterial pressure in heart failure. Studies by others generally noted decreased mean arterial pressures and reduced cardiac output accompanied by no change in peripheral resistance in rats with heart failure (Pfeffer *et al.*, 1979; Yang *et al.*, 1995). Considering all the evidence provided, it appears that this rat model represents the pathophysiological state of heart failure.

4.2 L-Arginine Availability in Heart Failure

Plasma levels of L-arginine exhibit a strong trend towards being lower in untreated heart failure rats compared to sham rats. Preliminary results in our lab (Feng *et al.*, unpublished observation) have shown that aortic tissue levels of L-arginine were also decreased in heart failure rats. This may suggest that there is decreased L-arginine availability to the nitric oxide synthase enzyme in heart failure. It is possible that the observed trend in plasma levels of L-arginine may reflect a larger alteration in intracellular levels of L-arginine, particularly if L-arginine is actively sequestered in parts of the cell requiring arginine, such as in the vicinity of nitric oxide synthases. Although the cause of the decreased L-arginine concentrations in heart failure rats is not known, mechanisms such as impaired L-arginine transport from the jejunum or into cells, blockade of L-arginine recycling from L-citrulline, or L-arginine depletion by use in other pathways or by excessive iNOS stimulation are suggested as possible explanations for this observation. Therefore, L-arginine supplementation may increase L-arginine availability and subsequently increase nitric oxide production by nitric oxide synthase and improve the impaired endothelium-dependent relaxation in heart failure.

The plasma concentrations of L-arginine may be affected by alterations in extracellular fluid volume in the body. Under conditions where extracellular volume was increased due to, for example, sodium and water retention which may occur in heart failure (DiBona *et al.*, 1988), the measured concentration of L-arginine would be diluted and, therefore, not yield an accurate picture of L-arginine levels in heart failure relative to sham rats.

Future studies should investigate the aspect of L-arginine availability and whether transport is inhibited or whether L-arginine is being utilized in another pathway such as increased urea production or increased production of endogenous ADMA which could have additional consequences with respect to the impaired endothelium-dependent relaxation in heart failure.

4.3 L-arginine Treatment, Dosage, and Plasma Levels

In addition to establishing the heart failure model, it was also necessary to determine the characteristics of the L-arginine treatment. The lower water consumption in the first week of the treatment was likely a consequence of the rats undergoing an extensive surgical procedure. The increased water consumption of later weeks corresponded with the rats recovery from the surgery and their growth as indicated by increased body weight.

The plasma concentrations of L-arginine corresponded well with the dosing regimen of untreated, low and high dose L-arginine groups, that is, there was a dose-dependent increase in plasma L-arginine levels in both sham and heart failure rats in the present study. The elevated plasma L-arginine concentration of the treated groups demonstrated that oral L-arginine was being absorbed and distributed in the plasma and thus had physiological relevance. Since the low dose L-arginine treatment appeared to have restored plasma L-arginine levels to that of sham rats, the normalized plasma L-arginine levels may account for any beneficial effects observed in the heart failure rats. As well, the extremely high plasma levels achieved by the high dose L-arginine treatment may account for any difference between the effects observed in the high and low dose L-

arginine treatment groups. In the present study, basal level of plasma L-arginine in untreated sham rats was about 200μM which agrees with the values reported by other investigators (Pieper & Peltier, 1995).

Future studies should look at the cellular distribution of L-arginine and whether plasma concentrations are reflective of intracellular levels of L-arginine and whether this source of L-arginine is distributed into the metabolically active pool of arginine.

4.4 Plasma Nitrate Levels

No difference was seen in the plasma levels of nitrate and nitrite in heart failure rats, nor was there any difference seen with the L-arginine treatments. While it is possible that restriction of nitrates in the diet may improve the sensitivity of the assay, in most studies the rats were not fasted prior to obtaining blood samples but were simply fed standard food as in the present study. Such studies were able to detect significant alterations in plasma nitrate and nitrite levels (Kumura *et al.*, 1994; Matsuoka *et al.*, 1996). Kumura *et al.* fed the rats with standard diet and found plasma nitrate and nitrite concentrations of approximately 33μM. The values in the present study ranged between 20 to 25μM. This slight difference in values may be accounted for by different chow fed to the rats or the difference in age or strain.

A study in humans looked at the effect of acute L-arginine supplementation on cardiovascular performance in patients with hypercholesterolemia and found no significant improvement (Wennmalm *et al.*, 1995). It is interesting that they also found no alteration in plasma nitrate concentration upon supplementation with L-arginine which they interpreted as indicating that the formation of nitric oxide was not markedly

increased in the patients studied (Wennmalm *et al.*, 1995). While this interpretation could also be applied to the present study, it would then be difficult to explain the observed improvement in endothelium-dependent relaxation.

Although the major source of nitrates and nitrites endogenously is the L-arginine, nitric oxide pathway, it has been discussed that the diet may contribute as much as 60% to the plasma levels (Grisham *et al.*, 1995). Thus, under conditions where subjects are maintained on a defined diet and there is no abnormality in renal clearance of nitrate and nitrite, the accumulation of nitrate and nitrite is proportional to the total activity of nitric oxide synthases per unit time (Granger *et al.*, 1995). It has been shown that glomerular filtration rates are not different between sham and heart failure rats (Feng *et al.*, 1997) (Hostetter *et al.*, 1983), thus, the nitrate and nitrite concentrations presented here should reflect the basal activity of nitric oxide synthases. In the present study it was not feasible to obtain plasma nitrate levels that more closely reflected endogenously produced nitric oxide by putting the rats on a nitrate reduced diet or fasting the animals.

If one assumes that rats drank in response to or concurrent with eating one could suppose that the rats ate similar amounts since the drinking measurements were found to be uniform. Since the rats ate the same food the dietary contribution to plasma levels should be uniform across the groups. Assuming, then, that diet uniformly contributes a significant amount to these levels, the remaining percentage of plasma nitrate and nitrite would be attributable to basal nitric oxide production and these values may reflect a difference in basal levels not detectable unless isolated from the exogenous pool. Therefore, it is possible that the nitrate levels reflecting the true basal levels of nitric

oxide release may be buried in the total levels measured in the present study due to a substantial amount of nitrate coming from the diet. Future studies should further investigate the relative contribution of diet to plasma nitrate and nitrite concentrations.

In addition, as when considering plasma L-arginine concentrations, alterations in extracellular fluid volume in the body, may affect the measurement of nitrate and nitrite. Heart failure rats may have increased fluid retention and substances in the blood would be diluted. In this case the plasma concentrations on nitrate and nitrite would not yield an accurate picture of the basal nitric oxide release in heart failure compared to sham rats.

Another limitation of this method for determining basal nitric oxide levels was that it was not possible to discern whether the nitrate and nitrite measured were derived from nitric oxide which had been able to elicit a biological effect prior to being oxidized. It should be recognized that oxygen radical inactivation may play a role in decreasing the biological activity of nitric oxide (Bode-Boger *et al.*, 1996).

4.5 Effects of L-Arginine on Cardiac Function in Sham Rats

Since no infarct was induced in the sham groups, it was not expected that there would be a significant difference between any of the parameters that characterize the heart failure model unless there was a non-specific effect of the L-arginine treatment. No differences were observed between the sham groups in any parameters, including endothelium-dependent and endothelium-independent relaxations.

The supplementation of exogenous L-arginine was unlikely to produce any effects in the sham rats because this model represents normal physiology. Since under normal physiological conditions the constitutive nitric oxide synthase is theoretically saturated

and in sham rats there is no reason to expect altered L-arginine availability, there would be no void which exogenous L-arginine could fill, instead the excess L-arginine is likely taken up by the liver and shunted through the urea cycle (Rogers *et al.*, 1972; White & Christensen, 1982). In addition, the possibility that L-arginine may be exerting effects through its secretagogue actions on other organs has not been ruled out.

4.6 Effects of L-Arginine on Cardiac Function in Heart Failure Rats

Although L-arginine has been shown to have cardioprotective effects in several models of myocardial ischemia and reperfusion (Amrani *et al.*, 1995; Carrier *et al.*, 1996; Weyrich *et al.*, 1992), a few studies have shown L-arginine treatment to be of no benefit or even to be detrimental (Takeuchi *et al.*, 1995). In the present study no cardioprotective effects of L-arginine treatment were reported using the model of coronary artery ligation induced heart failure.

Ogilvie *et al.* infused L-arginine into a dog model of heart failure resulting in improved cardiac output and decreased pulmonary resistance but no change in other hemodynamic parameters (Ogilvie & Zborowska-Sluis, 1995). The present study demonstrated no significant differences between L-arginine treated and untreated heart failure rats in any morphological or hemodynamic criteria used to characterize the disease state of heart failure. This includes the measurement of cardiac output for the high dose L-arginine treated heart failure rats which was not significantly different from either untreated sham or heart failure groups. Thus, it would appear that chronic oral L-arginine treatment fails to improve basal state hemodynamics or reduce infarct size or cardiac remodelling in heart failure rats. Recall that the half-life of nitric oxide may be

substantially decreased by elevated levels of oxygen free radicals (Gryglewski *et al.*, 1986). Thus, the supplementation of L-arginine alone may not be sufficient to overcome the negative influence increased oxidative stress that are present in heart failure (Hill & Singal, 1996).

It is worthy to note that there was no change in mean arterial pressure when L-arginine was administered chronically (Matsuoka *et al.*, 1996), although acute administration of L-arginine has been shown to reduce blood pressure in hypertensive humans and animals (Chen & Sanders, 1991; Nakaki *et al.*, 1990). It was suggested that compensatory mechanisms take over during the chronic phase of L-arginine administration (Matsuoka *et al.*, 1996), this may explain some of the results in the present study.

A study administering a bolus L-arginine intravenously in coronary artery ligation model of heart failure two months post-surgery observed a decrease in arterial pressure as well as a negative inotropic effect in response to L-arginine (Feng *et al.*, 1996c). Woo *et al.* administered a chronic oral L-arginine dose of 1g/kg/day for 35 days post-surgery (Woo *et al.*, 1996). They observed decreased hypertrophy of the left ventricle as well as preservation of left ventricular function in the form of positive inotropy in a Langendorff preparation. These results were not observed in our *in vivo* measurements. This may also be a consequence of the different time course of the experiments. It is possible that these beneficial effects of L-arginine treatment were present earlier in the disease state but the mechanism was overwhelmed by the time we measured cardiac function, possibly

through down-regulation of L-arginine transport from the gastrointestinal tract or into cells, or through a shift in L-arginine metabolism to other pathways.

4.7 Effects of L-Arginine on Endothelium-Dependent Relaxation

Abnormalities in vasomotor tone are well known components of chronic heart failure (Ontkean *et al.*, 1991). Both enhanced vasoconstriction at rest and impaired responsiveness to vasodilator stimuli such as exercise and ischemia have been described previously (Wilson *et al.*, 1986; Zelis & Flaim, 1982; Zelis *et al.*, 1968). In the present study, the *in vitro* data confirmed that endothelium-dependent vasodilation to acetylcholine was impaired in heart failure rats. The EC_{50} of acetylcholine was significantly increased in heart failure but not the maximum response. This could imply that competitive inhibition of the nitric oxide synthase is involved in impaired endothelial function in heart failure.

Endothelium-dependent relaxations are significantly inhibited by exogenous ADMA applied *in vitro* (Azuma *et al.*, 1995). This inhibition is dose-dependent in that a dose of 10^{-5}M ADMA shifts only the EC_{50} but that higher doses of ADMA shift both the EC_{50} as well as inhibiting the maximum response (Feng *et al.*, 1997). In the present study, the impaired endothelium-dependent relaxation in heart failure expresses traits resembling those of 10^{-5}M ADMA inhibition.

Low dose L-arginine treatment restored the impaired endothelial function in heart failure as shown by the reduction of the acetylcholine EC_{50} value to the level of untreated sham rats. Since there was no difference in the endothelium-independent relaxation stimulated by nitroprusside, the positive effect seen in the low dose L-arginine treated rats

was specific to improving endothelium-dependent relaxation and the production of nitric oxide.

The improvement of endothelium-dependent relaxation by the low dose L-arginine treatment may have been a consequence of the treatment restoring plasma L-arginine levels of heart failure rats to the physiological level of sham rats. The resulting increased L-arginine plasma concentration in heart failure may have multiple effects which may include providing adequate substrate for nitric oxide synthase, balancing the effects of endogenous vasoconstrictors such as endothelin through increased nitric oxide production, reducing the production of the nitric oxide inactivating oxygen radicals, and competing with endogenous nitric oxide synthase inhibitors such as ADMA. Future studies should address these issues in order to elucidate the mechanisms by which L-arginine is producing its beneficial effects in this condition.

While the low dose L-arginine treatment did not appear to improve vasoconstriction as detected through improved cardiac output or altered mean arterial pressure, it did improve endothelium-dependent relaxation in the aorta *in vitro*. This may represent improvement in the vasodilatory response to stimuli, which would not be seen in measuring basal hemodynamic parameters, but may be observable under conditions of stress, such as exercise. This would be an interesting aspect to investigate in future studies.

It was curious that the rats treated with high dose L-arginine failed to exhibit the same restorative effect on endothelium-dependent relaxation, however, the plasma concentration of L-arginine was significantly higher than the physiological levels of sham

rats and this may have had a negative feedback effect on either L-arginine transporters or on one or many of the enzymes which metabolize L-arginine. It is possible that high levels of L-arginine may inhibit either its own uptake into the body or cell or may stimulate the production of other regulatory factors, such as ADMA (MacAllister *et al.*, 1994a), to counteract the vasorelaxing effects of nitric oxide. Arginine has been shown to be partly metabolized to form dimethylarginines in cultured endothelial cells (MacAllister *et al.*, 1994a), and in humans (Bode-Boger *et al.*, 1996), however, the results are not yet clear in rabbits (Bode-Boger *et al.*, 1996). While the concentrations of dimethylarginines within cells are not known, their levels are concentrated intracellularly (MacAllister *et al.*, 1994a), therefore, the small increase observed in plasma ADMA concentrations in the study by Bode-Boger *et al.* (1996) was suggested to reflect a higher increase in the vicinity of nitric oxide synthase intracellularly. Thus, it is possible that under conditions of excess L-arginine, additional dimethylarginine may be formed, resulting in increased inhibition of nitric oxide synthesis and decreased L-arginine concentrations due to increased metabolism. Alternatively, the high dose of L-arginine may be working through other mechanisms mentioned previously such as stimulation of growth hormone and IGF. This may account for the apparent effect on cardiac output in that it was not significantly different from sham since growth hormone has been shown to improve cardiac output (Yang *et al.*, 1995).

The organ bath studies presented were limited to the stimulated, endothelium-dependent vasodilation and additional studies using blockers of nitric oxide synthase are

required in order to provide data concerning the basal nitric oxide release and its contributions to basal arterial tone.

4.8 Summary and Conclusions

The studies presented in this thesis demonstrate that a low dose of L-arginine given chronically to heart failure rats may restore plasma L-arginine levels and improve endothelium-dependent vasorelaxation to the level of sham rats, however, it fails to significantly alter basal cardiac performance. High dose treatment of heart failure rats, on the other hand, produces plasma L-arginine concentrations in excess of those of sham rats and fails to improve either endothelial function or cardiac performance.

The lack of effect of the chronic oral L-arginine treatment on basal hemodynamic parameters reveals no obvious benefit of such a treatment, however, the improved endothelial function elicited by the low dose L-arginine treatment indicates a positive effect that may have consequences not elucidated by the current studies. A possible cardioprotective and hemodynamically beneficial effect of L-arginine treatment should not yet be ruled out, but rather, the present studies should stimulate further research to investigate potential effects in later stage heart failure and under exercise conditions where endothelial-dependent relaxation is stimulated. The possibility of long-term effects of L-arginine supplementation on the condition of heart failure, particularly with regards to its effect on mortality from the disease should also be investigated.

Heart failure is a disease state that involves multiple systems and it is often necessary to combine therapies in order to more effectively combat the many deleterious factors. In light of this and the fact that we have shown a beneficial effect of low dose

chronic oral L-arginine treatment on endothelium-dependent relaxation in heart failure, it is possible that this positive aspect may be incorporated into other treatment protocols in which potentiation and overall improvement in cardiac performance might be seen. Such combination therapy might involve the use of antioxidants, diuretics, or other vasodilators such as the ACE inhibitors. In any case, the potential for chronic oral L-arginine as a treatment of heart failure should not yet be dismissed.

REFERENCES

Alba-Roth, J., Muller, O.A., Schopohl, J. & von Werder, K. (1988). Arginine stimulates growth hormone secretion by suppressing endogenous somatostatin secretion. *Journal of Clinical Endocrinology and Metabolism*, **67,** 1186-1189.

Amrani, M., Schyns, C.J., Chester, A.H., Yacoub, M.H. & Jayakumar, J. (1995). L-arginine reverses low coronary reflow and enhances post-ischemic recovery of cardiac mechanical function. *Cardiovascular Research*, **30,** 200-204.

Archer, S. (1993). Measurement of nitric oxide in biological model. *FASEB Journal*, **7,** 349-360.

Azuma, H., Sato, J., Hamasaki, H., Sugimoto, A., Isotani, E. & Obayashi, S. (1995). Accumulation of endogenous inhibitors for nitric oxide synthesis and decreased content of L-arginine in regenerated endothelial cells. *British Journal of Pharmacology*, **115,** 1001-4.

Barbul, A. (1986). Arginine: biochemistry, physiology, and therapeutic implications. *Journal of Parenteral and Enteral Nutrition*, **10,** 227-238.

Barbul, A., Rettura, G., Levenson, S.M. & Seifter, E. (1983). Wound healing and thymotropic effects of arginine: a pituitary mechanism of action. *The American Journal of Clinical Nutrition*, **37,** 786-794.

Barbul, A., Sisto, D. & Wasserkrug, H.L. (1981). Arginine stimulates lymphocyte immune responses in healthy humans. *Surgery*, **90,** 244-251.

Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A. & Freeman, B.A. (1990). Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proceedings of the National Academy of Science. U.S.A.*, **87,** 1620-1624.

Blachier, F., Darcy-Vrillon, B., Sener, A., Duee, P.H. & Malaisse, W.J. (1991a). Arginine metabolism in rat enterocytes. *Biochimica et Biophysica Acta*, **1092,** 304-310.

Blachier, F., M'Rabet-Touil, H., Darcy-Vrillon, B., Posho, L. & Duee, P.H. (1991b). Stimulation by D-glucose of the direct conversion of arginine to citrulline in enterocytes isolated from pig jejunum. *Biochemical and Biophysical Research Communications*, **177,** 1171-1177.

Bode-Boger, S.M., Boger, R.H., Kienke, S., Junker, W. & Frolich, J.C. (1996). Elevated L-arginine/dimethylarginine ratio contributes to enhanced systemic NO production by dietary L-arginine in hypercholesterolemic rabbits. *Biochemical and Biophysical Research Communications*, **219,** 598-603.

production by dietary L-arginine in hypercholesterolemic rabbits. *Biochemical and Biophysical Research Communications*, **219,** 598-603.

Bogle, R.G., Baydoun, A.R., Pearson, J.D., Moncada, S. & Mann, G.E. (1992a). L-arginine transport is increased in macrophages generating nitric oxide. *Biochemical Journal*, **284,** 15-8.

Bogle, R.G., Moncada, S., Pearson, J.D. & Mann, G.E. (1992b). Identification of inhibitors of nitric oxide synthase that do not interact with the endothelial cell L-arginine transporter. *British Journal of Pharmacology*, **105,** 768-770.

Boglie, R.G., Coade, S.B., Moncada, S., Pearson, J.D. & Mann, G.E. (1991). Bradykinin and ATP stimulate L-arginine uptake and nitric oxide release in vascular endothelial cells. *Biochemical and Biophysical Research Communications*, **180,** 926-932.

Branthwaite, M.A. & Bradley, R.D. (1968). Measurement of cardiac output by thermal dilution in man. *Journal of Applied Physiology.*, **24,** 434-438.

Bredt, D.S. & Snyder, S.H. (1994). Nitric oxide: a physiologic messenger molecule. *Annual Reviews in Biochemistry*, **63,** 175-195.

Brittenden, J., Heys, S.D., Ross, J., Park, K.G.M. & Eremin, O. (1994). Nutritional pharmacology: effects of L-arginine on host defences, response to trauma and tumour growth. *Clinical Science*, **86,** 123-132.

Brophy, J.M. (1992). Epidemiology of congestive heart failure: Canadian data from 1970-1989. *Canadian Journal of Cardiology*, **8,** 495-498.

Busse, M., Calibaba, J., Girard, D., Hoffman, M., Hutchins, L., LeBlanc, A., McDougall, S., Mireault, J. & Weitzman, B. (1994). *The Canadian Red Cross Society: First aid the vital link.* Toronto: Mosby Lifeline.

Calver, A., Collier, J., Leone, A., Moncada, S. & Vallance, P. (1993). Effect of local intra-arterial asymmetric dimethylarginine (ADMA) on the forearm arteriolar bed of healthy volunteers. *Journal of Human Hypertension*, **7,** 193-4.

Calver, A., Collier, J., Moncada, S. & Vallance, P. (1992). Effect of local intra-arterial N^G-monomethyl-L arginine in patients with hypertension: the nitric oxide dilator mechanism appears abnormal. *Journal of Hypertension*, **10,** 1025-1031.

Carbonell, L.F., Salom, M.G., Salazar, F.L., Garcia-Estan, J., Ubeda, M. & Quesada, T. (1985). Normal hemodynamic parameters in conscious Wistar rats. *Ref Esp Fisiol*, **41,** 437-442.

Carrier, M., Khalil, A., Tourigny, A., Eng, Solymoss, B.C. & Pelletier, L.C. (1996). Effect of L-arginine on metabolic recovery of the ischemic myocardium. *Annals of Thoracic Surgery*, **61,** 1651-1657.

Castillo, L., Chapman, T.E., Sanchez, M., Yu, Y.M., Burke, J.F., Ajami, A., Vogt, J. & Young, V.R. (1993a). Plasma arginine and citrulline kinetics in adults given adequate and arginine-free diets. *Proceedings of the National Academy of Science. USA.*, **90,** 7749-7753.

Castillo, L., Chapman, T.E., Yu, Y.M., Ajami, A., Burke, J.F. & Young, V.R. (1993b). Dietary arginine uptake by the splanchnic region in adult humans. *American Journal of Physiology*, **265,** E532-E539.

Celermajer, D.S., Sorensen, K.E. & Gooch, V.M. (1992). Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *Lancet*, **340,** 1111-1115.

Chen, P.Y. & Sanders, P.W. (1991). L-arginine abrogates salt-sensitive hypertension in Dahl/Rapp rats. *Journal of Clinical Investigation*, **88,** 1559-1567.

Cherrington, A.D., Kawamori, R. & Pek, S. (1974). Arginine infusion in dogs: Model for the roles of insulin and glucagon in regulating glucose turnover and free fatty acid levels. *Diabetes*, **23,** 805-815.

Chien, Y.W., Barbee, R.W., MacPhee, A.A., Frohlich, E.D. & Trippodo, N.C. (1988). Increased ANF secretion after volume expansion is preserved in rats with heart failure. *American Journal of Physiology*, **23,** R185-R191.

Cho, H.J., Martin, E., Zie, Q. & Nathan, C. (1995). Inducible nitric oxide synthase: identification of amino acid residues essential for dimerization and binding of tetrahydrobiopterin. *Proceedings of the National Academy of Science. USA*, **92,** 11514-11518.

Christensen, H.N. (1984). Organic ion transport during seven decades. The amino acids. *Biochimica et Biophysica Acta*, **779,** 255-269.

Cody, R.J., Haas, G.J., Binkley; P.F., Capers, Q. & Kelley, R. (1992). Plasma endothelin correlates with the extent of pulmonary hypertension in patients with chronic congestive heart failure. *Circulation*, **85,** 504-509.

Conner, E.M. & Grisham, M.B. (1995). Nitric Oxide: Biochemistry, physiology, and pathophysiology. *Methods: A Companion to Methods in Enzymology*, **7,** 3-13.

Cooke, J.P., Singer, A.H., Tsao, P., Zera, P., Rowan, R.A. & Billingham, M.E. (1992a). Antiatherogenic effects of L-arginine in the hypercholesterolemic rabbit. *Journal of Clinical Investigations*, **90,** 1168-1172.

Cooke, J.P. & Tsao, P. (1992b). Cellular mechanisms of atherogenesis and the effects of nitric oxide. *Current Opinions in Cardiology*, **7**.

Cooper, G. (1987). Cardiocyte adaptation to chronically altered load. *Annual Reviews of Physiology*, **49,** 501-518.

Cooper, T., Pinakah, T. & Richardson, A. (1963). The use of the thermal dilution principal for measurement of cardiac output in the rat. *Medical Electronics and Biological Engineering*, **1,** 61-65.

Cosentino, F. & Katusic, Z. (1995). Tetrahydrobiopterin and dysfunction of endothelial nitric oxide synthase in coronary arteries. *Circulation*, **91,** 139-144.

Cox, D.A., Vita, J.A. & Treasure, C.B. (1989). Atherosclerosis impairs flow-mediated dilation of coronary arteries in humans. *Circulation*, **80,** 458-465.

Dargie, H.J., McAlpine, H.M. & Morton, J.J. (1987). Neuroendocrine activation in acute myocardial infarction. *Journal of Cardiovascular Pharmacology*, **9,** S21-S24.

Delitala, G., Frulio, T., Pacifico, A. & Maioli, M. (1982). Participation of cholinergic muscarinic receptors in glucagon- and arginine-mediated growth hormone secretion in man. *Journal of Clinical Endocrinology and Metabolism*, **55,** 1231-1233.

DeMarco, T., Chatterjee, K., Rouleau, J.L. & Parmley, W.W. (1988). Abnormal coronary hemodynamics and myocardial energetics in patients with chronic heart failure caused by ischemic heart disease and dilated cardiomyopathy. *American Heart Journal*, **115,** 809-815.

DiBona, G.F., Herman, P.J. & Sawin, L.I. (1988). Neuronal control of renal function in edema forming states. *American Journal of Physiology*, **254,** R1017-R1024.

Drexler (1986). Regional vascular adjustments during recovery from myocardial infarction in rats. *Journal of the American College of Cardiology*, **8,** 34-42.

Drexler, H., Hayoz, D., Munzel, T., Hornig, B., Just, H., Brunner, H.R. & Zelis, R. (1992a). Endothelial function in chronic congestive heart failure. *American Journal of Cardiology*, **69,** 1596-601.

Drexler, H., Hayoz, D., Munzel, T., Just, H., Zelis, R. & Brunner, H.R. (1994). Endothelial dysfunction in chronic heart failure. Experimental and clinical studies. [Review]. *Arzneimittel-Forschung*, **44,** 455-8.

Drexler, H. & Lu, W. (1992b). Endothelial dysfunction of hindquarter resistance vessels in experimental heart failure. *American Journal of Physiology*, **262,** H1640-H1645.

Drexler, H., Zeiher, A.M., Meinzer, K. & Just, H. (1991). Correction of endothelial dysfunction in coronary microcirculation of hypercholesterolaemic patients by L-arginine. *Lancet*, **338,** 1546-1550.

Duerr, R.L., Huang, S., Miraliakbar, H.R., Clark, R., Chien, K.R. & Ross, J.J. (1995). Insulin-like growth factor 1 enhances ventricular hypertrophy and function during the onset of experimental cardiac failure. *Journal of Clinical Investigation*, **95,** 619-627.

Dusting, G.J. (1995). Nitric oxide in cardiovascular disorders. *Journal of Vascular Research*, **32,** 143-161.

Dzau, V.J., Colucci, W.S., Hollenberg, N.K. & Williams, G.H. (1981). Relation of the renin-angiotensin-aldosterone system to clinical state in congestive heart failure. *Circulation*, **63,** 645-651.

Egashira, K., Hirooka, Y., Kuga, T., Mohri, M. & Takeshita, A. (1996). Effects of L-arginine supplementation on endothelium-dependent coronary vasodilation in patients with angina pectoris and normal coronary arteriograms. *Circulation*, **94,** 130-134.

Featherson, W.R., Rogers, Q.R. & Freedland, R.A. (1973). Relative importance of kidney and liver in synthesis of arginine by the rat. *American Journal of Physiology*, **224,** 127-129.

Feng, Q.P., Carlsson, S., Thoren, P. & Hedner, T. (1992). Effects of clonidine on renal sympathetic nerve activity, natriuresis and diuresis in chronic congestive heart failure rats. *Journal of Pharmacology and Experimental Therapeutics*, **261,** 1129-35.

Feng, Q.P. & Hedner, T. (1990a). Endothelium-derived relaxing factor (EDRF) and nitric oxide (NO). I & II. Physiology, pharmacology and pathophysiological implications. [Review]. *Clinical Physiology*, **10,** 407-26, 503-26.

Feng, Q.P., Hedner, T., Hedner, J. & Pettersson, A. (1990b). Blunted renal response to atrial natriuretic peptide in congestive heart failure rats is reversed by the alpha 2-

adrenergic agonist clonidine. *Journal of Cardiovascular Pharmacology*, **16,** 776-82.

Feng, Q.P., Lu, X., Fortin, A.J., Pettersson, A., Hedner, T., Kline, R.L. & Arnold, J.M.O. (1997). Elevation of an endogenous inhibitor of nitric oxide synthase in experimental congestive heart failure. *Journal of the American College of Cardiology*, **29,** 422A.

Feng, Q.P., Lu, X.R., Scott, J., McCormack, D.G. & Arnold, J.M.O. (1996a). Calcium dependent nitric oxide synthase activity is decreased while calcium independent nitric oxide synthase activity is increased in aorta of rats with congestive heart failure. *Journal of American College of Cardiology*, **27 (2 Suppl A),** 113A.

Feng, Q.P., Lu, X.R., Scott, J., McCormack, D.G. & Arnold, J.M.O. (1996b). Vascular and myocardial nitric oxide synthase activity in rats with ischemic congestive heart failure. *Cardiovascular Research*, **31,** 577-584.

Fishbein, M.C., Maclean, D. & Maroko, P.R. (1978). Experimental myocardial infarction in the rat. *American Journal of Pathology*, **90,** 57-70.

Floyd, J.C.J., Fajans, S.S. & Conn, J.W. (1966). Stimulation of insulin secretion by amino acids. *Journal of Clinical Investigations*, **45,** 1487-1502.

Forstermann, U., Close, E.I., Pollock, J.S., Nakane, M., Schwarz, P., Gath, I. & Kleinert, H. (1994). Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions. *Hypertension*, **23,** 1121-1131.

Franchimont, P., Luyckx, A. & Campistron, G. (1979). Effets de l'aspartate d'arginine sur la secretion de somatrotopine, insuline, et glucagon chez le rat. *Therapie*, **34,** 641-647.

Francis, G.S., Goldsmiths, R., Levine, T.B., Olivari, M.R. & Cohn, J.N. (1984). The neurohumoral axis in congestive heart failure. *Annals of Internal Medicine*, **101,** 370-377.

Fryburg, D.A. (1996). NG-monomethyl-L-arginine inhibits the blood flow but not the insulin-like response of forearm muscle to IGF-I. possible role of nitric oxide in muscle protein synthesis. *Journal of Clinical Investigation*, **97,** 1319-1328.

Furchgott, R.F. (1981). The requirement for endothelial cells in the relaxation of arteries by acetylcholine and some other vasodilators. *Trends in Pharmacological Science*, **2,** 173-176.

Furchgott, R.F. & Zawadzki, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288,** 373-376.

Ganz, W., Donoso, R., Marcus, H.S., Forrester, J.S. & Swan, H.J. (1971). A new technique for measurement of cardiac output by thermodilution in man. *The American Journal of Cardiology*, **27,** 392-396.

Geller, D., Lowenstein, C., Shapiro, R., Nussler, A.K., Di Silviio, M., Wang, S.C., Nakayama, D.K., Simmons, R.L., Snyder, S.H. & Billiar, T.R. (1993). Molecular cloning and expression of inducible nitric oxide synthase from human hepatocytes. *Proceedings of the National Academy of Science. USA.*, **90,** 3491-3495.

Girerd, X.J., Hirsch, A.T., Cooke, J.P., Dzau, V.J. & Creager, M.A. (1990). L-Arginine augments endothelium-dependent vasodilation in cholesterol-fed rabbits. *Circulation Research*, **67,** 1301-1308.

Giustina, A., Lorusso, R., Borghetti, V., Bugari, G., Misitano, V. & Alfieri, O. (1996). Impaired spontaneous growth hormone secretion in severe dilated cardiomyopathy. *American Heart Journal*, **131,** 620-622.

Goetz, K.L., Wang, B.C., Nadewed, J.B., Zhu, J.L. & Leadley, R.J.J. (1988). Cardiovascular, renal, and endocrine responses to intravenous endothelin in the anaesthetized dog. *American Journal of Physiology*, **255,** R1064-R1068.

Gola, A., Pozzoli, M., Capomolla, S., Traversi, E., Sanarico, M., Cobelli, F. & Tavazzi, L. (1996). Comparison of doppler echocardiography with thermodilution for assessing cardiac output in advanced congestive heart failure. *The American Journal of Cardiology*, **78,** 708-712.

Gold, M.E., Bush, P.A. & Ignarro, L.J. (1989). Depletion of arterial L-arginine causes reversible tolerance to endothelium-dependent relaxation. *Biochemical and Biophysical Research Communications*, **164,** 714-721.

Goy, M.F. (1991). cGMP: The wayward child of the cyclic nucleotide family. *Trends in Neuroscience*, **14,** 293-299.

Granger, D.L., Taintor, R., R., Boockvar, K.S. & Hibbs, J.B.J. (1995). Determination of Nitrate and Nitrite in Biological Samples Using Bacterial Nitrate Reductase Coupled with the Greiss Reaction. *Methods: A Companion to Methods in Enzymology*, **7,** 78-83.

Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S. & Tannenbaum, S.R. (1982). Analysis of nitrite, nitrate, and [^{15}N] in biological fluids. *Analytical Biochemistry*, **125,** 131-138.

Greene, B., Pacitti, A.J. & Souba, W.W. (1993). Characterization of L-arginine transport by pulmonary artery endothelial cells. *American Journal of Physiology*, **264,** L351-356.

Grisham, M.G., Johnson, G.G., Gautreaux, D.M. & Berg, R.D. (1995). Measurement of nitrate and nitrite in extracellular fluids: A window to systemic nitric oxide metabolism. *Methods*, **7,** 84-90.

Gross, S.S. & Levi, R. (1992). Tetrahydrobiopterin synthesis. An absolute requirement for cytokine-induced nitric oxide generation by vascular smooth muscle. *Journal of Biological Chemistry*, **36,** 25722-25729.

Group, T.D.I. (1996). Rationale, design, implementation, and baseline characteristics of patients in the DIG trial: A large, simple, long-term trial to evaluate the effect of digitalis on mortality in heart failure. *Controlled Clinical Trials*, **17,** 77-97.

Gryglewski, R.J., Palmer, R.M.J. & Moncada, S. (1986). Superoxide anion is involved in the breakdown of endothelium-derived relaxing factor. *Nature (London)*, **320,** 454-456.

Gullino, P., Winitz, M., Birnbaum, S.M., Cornfield, J., Otey, M.C. & Greenstein, J.P. (1955). The toxicity of essential amino acid mixtures with special reference to the protective effect of L-arginine. *Archives of Biochemistry and Biophysics*, **58,** 255-257.

Haas, G.J. & Leier, C.V. (1994). Vasodilators. In *Congestive Heart Failure: Pathophysiology, Diagnosis, and Comprehensive Approach to Management*. ed. Hosenpud, J.D. & Greenberg, B.H. pp. 400-453. New York: Springer-Verlag.

Habib, F.M., Springall, D.R., Davies, G.J., Oakley, C.M., Yacoub, M., H. & Polak, J.M. (1996). Tumor necrosis factor and inducible nitric oxide synthase in dilated cardiomyopathy. *Lancet*, **347,** 1151-1155.

Hahn, A.W., Resink, T.J., Scott-Burden, T., Powell, J., Dohi, Y. & Buhler, F.R. (1990). Stimulation of endothelin mRNA and secretion in rat vascular smooth muscle cells: a novel autocrine function. *Cell Regulation*, **1,** 649-659.

Haywood, G.A., Tsao, P.S., von der Leyen, H.E., Mann, M.J., Keeling, P.J., Trinidade, P.T., Lewis, N.P., Byrne, C.D., Rickenbacher, P.R., Bishopric, N.H., Cooke, J.P., McKenna, W.J. & Fowler, M.B. (1996). Expression of inducible nitric oxide synthase in human heart failure. *Circulation*, **93,** 1087-1094.

Hildebrand, F.L.J., Perrella, M.A. & Burnett, J.D.J. (1991). The role of endothelium-derived relaxing factor in experimental congestive heart failure. *Journal of American College of Cardiology*, **17.**

Hill, M.F. & Singal, P.K. (1996). Antioxidant and oxidative stress changes during heart failure subsequent to myocardial infarction in rats. *American Journal of Pathology*, **148,** 291-300.

Hirooka, Y., Imaizumi, T., Tagawa, T., Shiramoto, M., Endo, T., Ando, S. & Takeshita, A. (1994). Effects of L-arginine on impaired acetylcholine-induced and ischemic vasodilation of the forearm in patients with heart failure. *Circulation*, **90,** 658-68.

Hishikawa, K., Nakaki, T., Suzuki, H., Saruta, T. & Kato, R. (1991). L-arginine-induced hypotension [letter] [see comments]. *Lancet*, **337,** 683-4.

Hishikawa, K., Nakaki, T., Tsuda, M., Esumi, H., Ohshima, H., Suzuki, H., Saruta, T. & Kato, R. (1992). Effect of systemic L-arginine administration on hemodynamics and nitric oxide release in man. *Japan Heart Journal*, **33,** 41-48.

Hostetter, T.H., Pfeffer, J.M., Pfeffer, M.A., Dworkin, L.D., Braunwald, E. & Brenner, B.M. (1983). Cardiorenal hemodynamics and sodium excretion in rats with myocardial infarction. *American Journal of Physiology*, **245,** H98-H102.

Huie, R.E. & Pakmaja, S. (1993). The reaction of nitric oxide with superoxide. *Free Radical Research Communications*, **18,** 195-199.

Ignarro, L.F., Burns, R.E., Buga, G.M. & Wood, K.S. (1987). Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical. *Circulation Research*, **61,** 866-879.

Ignarro, L.J. (1989). Endothelium-derived nitric oxide: actions and properties. *FASEB Journal*, **3,** 31-36.

Imms, F.J., London, D.R. & Neame, R.L.B. (1969). The secretion of catecholamines from the adrenal gland following arginine infusion in the rat. *Journal of Physiology (London)*, **200,** 55P-56P.

Isidori, A., LoMonaco, A. & Cappa, M. (1981). Study of growth hormone release in man after oral administration of amino acids. *Current Medical Research Opinion*, **7,** 475-481.

Ito, H., Hirata, Y., Hiroe, M., Tsujino, M., Adachi, S., Takamoto, T., Nitta, M., Tanaguchi, K. & Marumo, F. (1991). Endothelin-1 induces hypertrophy with enhanced expression of muscle-specific genes in cultured neonatal rat cardiomyocytes. *Circulation Research*, **69,** 209-215.

Iyengar, R., Stuehr, D.J. & Marletta, M.A. (1987). Macrophage synthesis of nitrite, nitrate, and N-nitrosamines: precursors and role of the respiratory burst. *Proceedings of the National Academy of Science USA*, **84,** 6369-6373.

Kaiser, L., Spickard, R.C. & Olivier, N.B. (1989). Heart failure depresses endothelium-dependent responses in canine femoral artery. *American Journal of Physiology*, **256,** H962-H967.

Kannel, W.B., Ho, K. & Thom, T. (1994). Changing epidemilogical features of cardiac failure. *British Heart Journal*, **72,** S3-S9.

Kasten, T.P., Settle, S.L., Misko, T.P., Riley, D.P., Weiss, R.H., Currie, M.G. & Nickols, G.A. (1995). Potentiation of nitric oxide-mediated vascular relaxation by SC52608, a superoxide dismutase mimic. *Publication of the Society for Experimental Biology and Medicine*, **208,** 170-177.

Katz, S.D., Schwarz, M., Yuen, J. & LeJemtel, T.H. (1993). Impaired acetylcholine-mediated vasodilation in patients with congestive heart failure: Role of endothelium-derived vasodilating and vasoconstricting factors. *Circulation*, **88,** 55-61.

Kelly, R.A., Balligand, J.L. & Smith, T.W. (1996). Nitric oxide and cardiac function. *Circulation Research*, **79,** 363-380.

Kichuk, M.R., Seyedi, N., Zhang, X., Marboe, C.C., Michler, R.E., Addonizio, L.J., Kaley, G., Nasjletti, A. & Hintze, T.H. (1996). Regulation of nitric oxide production in human coronary microvessels and the contribution of local kinin formation. *Circulation*, **94,** 44-51.

Kim, J.W., Closs, E.I., Albritton, L.M. & Cunningham, J.M. (1991). Transport of cationic amino acids by the mouse ecotropic retrovirus receptor [see comments]. *Nature*, **352,** 725-8.

Klatt, P., Schmidt, K., Lehner, D., Glatter, O., Bachinger, H.P. & Mayer, B. (1995). Structural analysis of porcine brain nitric oxide synthase reveals a role for tetrahydrobiopterin and L-arginine in the formation of an SDS-resistant dimer. *European Molecular Biology Organization Journal*, **14,** 3687-3695.

Knopf, R.F., Conn, J.W., Fajans, S.S., Floyd, J.C., Guntsche, E.M. & Rull, J.A. (1965). Plasma growth hormone response to intravenous administration of amino acids. *Preliminary Conclusions*, **25,** 1140-1144.

Koppenol, W.H., Moreno, J.J., Pyror, W.A., Ischiropoulous, H. & Beckman, J.S. (1992). Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. *Chemical Research and Toxicology*, **5,** 834-842.

Krebs, H.A. & Henseleit, H. (1932). Untersuchungen uber die harnstoffbildung in tierkorper. *Hoppe-Seyler's Z Physiol Chem*, **210,** 33-66.

Kubo, S.H., Rector, T.S., Bank, A.J., Williams, R.E. & Heifetz, S.M. (1991). Endothelium-dependent vasodilation is attenuated in patients with heart failure. *Circulation*, **84,** 1589-1596.

Kumar, R., Hood, W.B.J., Joison, J., Norman, J.C. & Abelman, W.H. (1970). Experimental myocardial infarction. II. Acute depression and subsequent recovery of left ventricular function: Serial measurements in intact conscious dogs. *Journal of Clinical Investigations*, **49,** 55-62.

Kumura, E., Kosaka, H., Shiga, T., Yoshiine, T. & Hayakawa, T. (1994). Elevation of plasma nitric oxide end products during focal cerebral ischemia and reperfusion in the rat. *Journal of Cerebral Blood Flow and Metabolism*, **14,** 487-491.

Leone, A.M., Rhodes, P., Furst, V. & Moncada, S. (1995). Techniques for the measurement of nitric oxide. In *Signal Transduction Protocols*. ed. Kendall, D.A. & Hill, S.J. pp. 285-299. Totowa, New Jersey: Humana Press.

Levillain, O., Hus-Citharel, A., Morel, F. & Bankir, L. (1991). Localization of arginine synthesis along rat nephron. *American Journal of Physiology*, **259,** F916-F923.

Levine, B., Kalman, J., Mayer, L., Fillit, H.M. & Packer, M. (1990). Elevated circulating levels of tumor necrosis factor in severe chronic heart failure. *New England Journal of Medicine*, **323,** 236-241.

Lincoln, T.M. (1989). Cyclic GMP and mechanisms of vasodilatation. *Pharmacological Therapeutics*, **41,** 479-502.

Little, R.C. & Little, W.C. (1984). Cardiac preload, afterload, and heart failure. In *Congestive heart failure: update on diagnosis and treatment.* ed. Crevey, B.J. & Church, J. pp. 1-8. Ann Arbor, Michigan: American Medical Association.

Lubec, B., Hoeger, H., Kremser, K., Amann, G., Koller, D.Y. & Gialamas, J. (1996). Decreased tumor incidence and increased survival by one year oral low dose arginine supplementation in the mouse. *Life Sciences*, **58,** 2317-2325.

Luscher, T.F. (1993). Angiotensin, ACE-inhibitors and endothelial control of vasomotor tone. *Basic Research in Cardiology*, **88,** 15-24.

Luscher, T.F., Diederich, D. & Siebenmann, R. (1988). Difference between endothelium-dependent relaxation in arterial and in venous coronary bypass grafts. *New England Journal of Medicine*, **319,** 462-467.

MacAllister, R.J., Fickling, S.A., Whitley, G.S. & Vallance, P. (1994). Metabolism of methylarginines by human vasculature; implications for the regulation of nitric oxide synthesis. *British Journal of Pharmacology*, **112,** 43-48.

Mann, D.L. & Roberts, R. (1995). The molecular basis for hypertrophic growth of the adult heart. In *Heart Failure*. ed. McCall, D. & Rahimtoola, S.H. pp. 67-89. New York: Chapman and Hall.

Margulies, K.B., Hildebrand, F.L.J., Lerman, A., Perrella, M.A. & Burnett, J.C.J. (1990). Increased endothelin in experimental heart failure. *Circulation*, **82,** 226-2230.

Massara, F., Martelli, S. & Cagliero, E. (1980). The hypophosphatemic and hyperkalemic effect of arginine in man. *Journal of Endocrinology Investigations*, **3,** 177-180.

Masuda, A., Shibasaki, T., Hotta, M., Yamauchi, N., Ling, N., Demura, H. & Shizume, K. (1990). Insulin-induced hypoglycemia, L-dopa, and arginine stimulate growth hormone secretion through different mechanisms in man. *Regulatory Peptides*, **31,** 53-64.

Matsuoka, H., Nakata, M., Kohno, K., Koga, Y., Nomura, G., Toshima, H. & Imaizumi, T. (1996). Chronic L-arginine administration attenuates cardiac hypertrophy in spontaneously hypertensive rats. *Hypertension*, **27,** 14-18.

Mayer, B., Schmidt, K., Humbert, P. & Bohme, E. (1989). Synthesis of endothelium-derived relaxing factor: A cytosolic enzyme in porcine aortic endothelial cells Ca^{2+}-dependently converts L-arginine into an activator of soluble guanylyl cyclase. *Biochemical and Biophysical Research Communications*, **164,** 678-685.

McDonald, K.M., Francis, G.S. & Matthews, J. (1993). Long-term oral nitrate therapy prevents chronic ventricular remodelling in the dog. *Journal of American College of Cardiology*, **21,** 514-522.

McGiff, J.C., Crawshaw, K., Terragne, N.A., Malik, K.U. & Lonigro, A.J. (1972). Differential effect of noradrenaline and renal nerve stimulation on vascular resistance in the dog kidney and the release of a prostaglandin E-like substance. *Clinical Science*, **42,** 223-233.

Merimee, T.T., Lillicrap, D.A. & Rabinowitz, D. (1965). Effect of arginine on serum-levels of human growth hormone. *Lancet*, **2,** 668-670.

Michel, T., Kramer, B.K. & Kelly, R.A. (1993). Endothelial cell-derived vasoactive and inotropic paracrine factors. In *Heart Failure: Basic science and clinical aspects.*

ed. Gwathmey, J.K., Briggs, G.M. & Allen, P.D. pp. 519-540. New York: Marcel Dekker, Inc.

Moncada, S. & Higgs, A. (1993). The L-arginine-nitric oxide pathway. [Review]. *New England Journal of Medicine*, **329,** 2002-12.

Moncada, S., Korbut, R., Bunting, S. & Vane, J. (1978). Prostacyclin is a circulating hormone. *Nature*, **273,** 767-768.

Moncada, S., Palmer, R.M. & Higgs, E.A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. [Review]. *Pharmacological Reviews*, **43,** 109-42.

Nakajima, T., Matsuoka, Y. & Kakimoto, Y. (1971). Isolation and identification of NG-monomethyl, NGNG dimethyl- and NGN"Gdimethylargininen from the hydrosylate of proteins of bovine brain. *Biochimica et Biophysica Acta*, **230,** 212-222.

Nakaki, T., Hishikawa, K., Suzuki, H., Saruta, T. & Kato, R. (1990). L-arginine-induced hypotension [letter] [see comments]. *Lancet*, **336,** 696.

Nakaki, T. & Kato, R. (1994). Beneficial circulatory effect of L-arginine. [Review]. *Japanese Journal of Pharmacology*, **66,** 167-71.

Nathan, C. & Zie, Q. (1994). Nitric oxide synthases: roles, tolls and controls. *Cell*, **78,** 915-918.

Nelson, M.T., Patlak, J.B., Worley, J.F. & Standen, N.M. (1990). Calcium channels, potassium channels, and voltage dependence of arterial smooth muscle tone. *American Journal of Physiology*, **259,** C3-C18.

Noll, G., Tschudi, M.R., Novosel, D. & Luscher, T.F. (1994). Activity of the L-arginine/nitric oxide pathway and endothelin-1 in experimental heart failure. *Journal of Cardiovascular Pharmacology*, **23,** 916-921.

Nussler, A.K., Billiar, T.R., Liu, Z.Z. & Morris, S.M.J. (1994). Coinduction of nitric oxide synthase and argininosuccinate synthetase in a murine macrophage cell line. *Journal of Biological Chemistry*, **269,** 1257-1261.

Ogilvie, R.I. & Zborowska-Sluis, D. (1995). Acute effect of L-arginine on hemodynamics and vascular capacitance in the canine pacing model of heart failure. *Journal of Cardiovascular Pharmacology*, **26,** 407-413.

O'Murchu, B., Miller, V.M., Perrella, M.A. & Burnett, J.C., Jr. (1994). Increased production of nitric oxide in coronary arteries during congestive heart failure. *Journal of Clinical Investigation*, **93,** 165-71.

Ontkean, M., Gay, R. & Breenberg, B. (1991). Diminished endothelium-derived relaxing factor activity in an experimental model of chronic heart failure. *Circulation Research*, **69,** 1088-1096.

Pacitti, A.J., Copeland, E.M. & Souba, W.W. (1992). Stimulation of hepatocyte system y+-mediated L-arginine transport by an inflammatory agent. *Surgery*, **112,** 403-411.

Packer, M. (1992). Pathophysiology of chronic heart failure. *The Lancet*, **340,** 88-92.

Palmer, J.P., Walter, R.M. & Ensick, J.W. (1975). Arginine-stimulated acute phase of insulin and glucagon secretion: I. In normal man. *Diabetes*, **24,** 735-740.

Palmer, R.M.J., Ashton, D.S. & Moncada, S. (1988a). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, **333,** 664-666.

Palmer, R.M.J., Ferridge, A.G. & Moncada, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327,** 524-526.

Palmer, R.M.J. & Moncada, S. (1989). A novel citrulline-forming enzyme implicated in the formation of nitric oxide by vascular endothelial cells. *Biochemical and Biophysical Research Communications*, **158,** 348-352.

Palmer, R.M.J., Rees, D.D., Ashton, D.S. & Moncada, S. (1988b). L-Arginine is the physiological precursor for the formation of nitric oxide in the endothelium-dependent relaxation. *Biochemical and Biophysical Research Communications*, **153,** 1251-1256.

Panza, J.A., Casino, P.R., Badar, D.M. & Quyyumi, A.A. (1993a). Effect of increased availability of endothelium-derived nitric oxide precursor on endothelium-dependent vascular relaxation in normal subjects and in patients with essential hypertension [see comments]. *Circulation*, **87,** 1475-81.

Panza, J.A., Casino, P.R., Kilcoyne, C.M. & Quyyumi, A.A. (1993b). Role of endothelium-derived nitric oxide in the abnormal endothelium-dependent vascular relaxation of patients with essential hypertension. *Circulation*, **87,** 1468-74.

Panza, J.A., Quyyumi, A.A., Brush, J.E.J. & Epsteine, S.E. (1990). Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *New England Journal of Medicine*, **323,** 22-27.

Pardis, I.P., Kotler, M.N. & Ren, J.F. (1984). Development and regression of left ventricular hypertrophy. *Journal of American College of Cardiology*, **3**.

Pepine, C.J., Drexler, H. & Dzau, V.J. (1997). *Endothelial function in cardiovascular health and disease.* New York: Landmark Programs, Inc.

Pfeffer, M.A., Pfeffer, J.M., Fishbein, M.C., Fletcher, P.J., Spadaro, J., Kloner, R.A. & Braunwald, E. (1979). Myocardial infarct size and ventricular function in rats. *Circulation Research*, **44,** 503-512.

Pfeffer, M.A., Pfeffer, J.M., Steinberg, C. & Finn, P. (1985). Survival after an experimental myocardial infarction: beneficial effects of long-term therapy with captopril. *Circulation*, **72,** 406-412.

Pieper, G.M. & Peltier, B.M. (1995). Amelioration by L-arginine of a dysfunctional arginine/nitric oxide pathway in diabetic endothelium. *Journal of Cardiovascular Pharmacology*, **25,** 397-403.

Radomski, M.W., Palmer, R.M.J. & Moncada, S. (1991). Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet*, **2,** 1057-1058.

Rakoff, J.S., Siler, T.M., Sinha, Y.N. & Yen, S.S.C. (1973). Prolactin and growth hormone release in response to sequential stimulation by arginine and TRF. *Journal of Clinical Endocrinology and Metabolism*, **37,** 61-644.

Rapoport, R.M. & Murad, F. (1983). Agonist-induced endothelium-dependent relaxation in the rat thoracic aorta may be mediated through cGMP. *Circulation Research*, **52,** 352-357.

Rector, T.S., Bank, A.J., Mullen, K.A., Tschumperlin, L.K., Sih, R., Pillai, K. & Kubo, S.H. (1996). Randomized, double-blind, placebo-controlled study of supplemental oral L-arginine in patients with heart failure. *Circulation*, **93,** 2135-2141.

Resta, T.C. & Walker, B.R. (1994). Orally administered L-arginine does not alter right ventricular hypertrophy in chronically hypoxic rats. *American Journal of Physiology*, **266,** R559-63.

Reyes, A.A., Karl, I.E. & Klahr, S. (1994). Role of arginine in health and in renal disease. *American Journal of Physiology*, **36,** F331-F346.

Rogers, Q.R., Freedland, R.A. & Symmons, R.A. (1972). In vivo synthesis and utilisation of arginine in the rat. *American Journal of Physiology*, **223,** 236-240.

Romero, J.M., Lara, C. & Guerrero, M.G. (1989). Determination of intracellular nitrate. *Biochemical Journal*, **259,** 545-548.

Rose, W.C. (1937). The nutritive significance of the amino acids ad certain related compounds. *Science*, **86,** 298-300.

Rubanyi, G.M. (1991). Reversal of hypercholesterolemia-induced endothelial dysfunction by L-arginine. *Circulation*, **83,** 391-401.

Rubanyi, G.M. (1993). The role of endothelium in cardiovascular homeostasis and diseases. *Journal of Cardiovascular Pharmacology*, **22,** S1-S14.

Rubanyi, G.M. & Vanhoutte, P.M. (1986). Superoxide anions and hypoxia inactivate endothelium-derived relaxing factor. *American Journal of Physiology*, **250,** H822-H827.

Sacca, L. & Fazio, S. (1996). Cardiac performance: growth hormone enters the race. *Nature Medicine*, **2,** 29-30.

Sakai, S., Miyauchi, T., Sakurai, T., Kasuya, Y., Ihara, M., Yamaguchi, I., Goto, K. & Sugishita, Y. (1996). Endogenous endothelin-1 participates in the maintenance of cardiac function in rats with congestive heart failure. Marked increase in endothelin-1 production in the failing heart. *Circulation*, **93,** 1214-1222.

Sandler, G. & Fry, J. (1990). *Heart Failure*. London: Clinical Press Limited.

Sax, H.C., Hasselgren, P.O., Talamini, M.A., Edwards, L.L. & Fischer, J.F. (1988). Methods for studying protein synthesis and degradation in liver and skeletal muscle. *Journal of Surgical Research*, **45,** 50-55.

Schmidt, H.H.H.W. (1995). Determination of nitric oxide via measurement of nitrite and nitrate in culture media. *Biochemica*, **2,** 22.

Schmidt, H.H.H.W., Warner, T.D., Nakane, M., Forstermann, U. & Murad, F. (1992). Regulation and subcellular location of nitrogen oxide synthases in RAW264.7 macrophages. *Molecular Pharmacology*, **41,** 615-624.

Schocken, D.D., Arrieta, M.I., Leaverton, P.E. & Ross, E.A. (1992). Prevalence and mortality rate of congestive heart failure in the United States. *Journal of the American College of Cardiology*, **20,** 301-306.

Schott, C.A., Vetrovsky, P. & Stoclet, J.-C. (1993). Cationic amino acids inhibit the effects of L-arginine in rat aorta exposed to lipopolysaccharide. *European Journal of Pharmacology*, **236,** 155-157.

Schulze. E. & Steiger, E. (1886). Ueber das arginin. *Z.Physiol. Chem.*, **11,** 43-65.

Smith, V.E. & White, W.B. (1986). Improved left ventricular filling accompanies reduced ventricular mass during therapy of essential hypertension. *Journal of American College of Cardiology*, **8,** 1449-1454.

Stroes, E., Kastelein, J., Cosentino, F., Erkelens, W., Wever, R., Koomans, H., Luscher, T. & Rabelink, T. (1997). Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. *Journal of Clinical Investigation*, **99,** 41-46.

Sun, X.Y., Feng, Q.P., Zhao, X., Edvinsson, L. & Hedner, T. (1995). Cardiovascular and renal effects of alpha-trinositol in ischemic heart failure rats. *Life Sciences*, **57,** 1197-211.

Swanson, D.R. (1990). Somatomedin C and arginine: implicit connections between mutually isolated literatures. *Perspectives in Biology and Medicine*, **33,** 157-186.

Taddei, S., Virdis, A., Mattei, P., Ghiadoni, L., Sudano, I. & Salvetti, A. (1996). Defective L-arginine-nitric oxide pathway in offspring of essential hypertensive patients. *Circulation*, **94,** 1298-1303.

Takeuchi, K., Glynn, P., McGowan, F.X., Simplaceanu, E., Danh, H.C. & del Nido, P.J. (1995). Direct detrimental effects of L-arginine upon ischemia-reperfusion injury to myocardium. *Journal of Molecular and Cellular Cardiology*, **27,** 1405-1414.

Tayeh, M.A. & Marletta, M.A. (1989). Macrophage oxidation of L-arginine to nitric oxide, nitrite, and nitrate. Tetrahydrobiopterin is required as a cofactor. *Journal of Biological Chemistry*, **264,** 19654-19658.

Teerlink, J.R., Clozel, M. & Fischli, W. (1993). Temporal evolution of endothelial dysfunction in a rat model of chronic heart failure. *Journal of the American College of Cardiology*, **22,** 615-620.

Teerlink, J.R., Gray, G.A., Clozel, M. & Clozel, J.P. (1994). Increased vascular responsiveness to norepinephrine in rats with heart failure is endothelium dependent. Dissociation of basal and stimulated nitric oxide release. *Circulation*, **89,** 393-401.

Tesch, J.W., Rehg, W.R. & Sievers, R.E. (1976). Microdetermination of nitrates and nitrites in saliva, blood, water and suspended particulates in air by gas chromatography. *Journal of Chromatography*, **126,** 743-755.

Thornbury, K.D., Wark, S.M., Dalziel, H.H., Carl, A., Westfall, D.P. & Sanders, K.M. (1991). Nitric oxide and nitrosocysteine mimic nonadrenergic, noncholinergic

hyperpolarization in canine proximal colon. *American Journal of Physiology*, **261,** G553-G557.

Thuillez, C., Mulder, P., Elfertak, L., Blaysat, G., Compagnon, P., Henry, J.P., Richard, V., Scalbert, E. & Desche, P. (1995). Prevention of endothelial dysfunction in small and large arteries in a model of chronic heart failure. Effect of angiotensin converting enzyme inhibition. *American Journal of Hypertension*, **8,** 7S-12S.

Tracey, W.R., Tse, J. & Carter, G. (1995). Lipopolysaccharide-induced changes in plasma nitrite and nitrate concentrations in rats and mice: Pharmacological evaluation of nitric oxide synthase inhibitors. *The Journal of Pharmacology and Experimental Therapeutics*, **272,** 1011-1015.

Utsumi, M., Makimura, H. & Ishihara, K. (1979). Determination of immunoreactive somatostatin in rat plasma and responses to arginine, glucose, and glucagon infusion. *Diabetologia*, **17,** 319-323.

Vallance, P., Collier, J. & Moncada, S. (1989). Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. *Lancet*, **ii,** 997-1000.

Vallance, P., Leone, A., Calver, A., Collier, J. & Moncada, S. (1992a). Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. *Lancet*, **339,** 572-5.

Vallance, P., Leone, A., Calver, A., Collier, J. & Moncada, S. (1992b). Endogenous dimethylarginine as an inhibitor of nitric oxide synthesis. *Journal of Cardiovascular Pharmacology*, **20,** S60-S62.

Vane, J.R., Anggard, E.E. & Botting, R.M. (1990). Regulatory functions of the vascular endothelium. *New England Journal of Medicine*, **323,** 27-36.

Venema, R.C., Ju, H., Zou, R., Ryan, J.W. & Venema, V.J. (1997). Subunit interactions of endothelial nitric oxide synthase. *Journal of Biological Chemistry*, **272,** 1276-1282.

Visek, W.J. (1986). Arginine needs, physiological state and usual diets. A reevaluation. *American Institute of Nutrition*, **116,** 36-44.

Vodovotz, Y., Geiser, A.G., Chesler, L., Letterio, J.J., Campbell, A., Lucia, M.S., Sporn, M.B. & Roberts, A.B. (1996). Spontaneously increased production of nitric oxide and aberrant expression of the inducible nitric oxide synthase in vivo in the transforming growth factor β1 null mouse. *Journal of Experimental Medicine*, **183,** 2337-2342.

Walsh, M.F., Barazi, M., Pete, G., Muniyappa, R., Dunbar, J.C. & Sowers, J.R. (1996). Insulin-like growth factor I diminishes in vivo and in vitro vascular contractility: role of vascular nitric oxide. *Endocrinology*, **137,** 1798-1803.

Walsh, M.P. (1994). Regulation of vascular smooth muscle tone. *Canadian Journal of Physiology and Pharmacology*, **72,** 919-936.

Wang, H., Kavanauhg, M.P., North, R.A. & Kabat, D. (1991). Cell-surface receptor for ecotropic murine retroviruses is a basic amino-acid transporter. *Nature*, **352,** 729-731.

Wennmalm, A., Granstrom, E.F., Edlund, A. & Wiklund, O. (1995). Acute supplementation with the nitric oxide precursor L-arginine does not improve cardiovascular performance in patients with hypercholesterolemia. *Atherosclerosis*, **118,** 223-231.

Weyrich, A.S., Ma, X.L. & Lefer, A.M. (1992). The role of L-arginine in ameliorating reperfusion injury after myocardial ischemia in the cat. *Circulation*, **86,** 279-288.

White, M.F. (1985). The transport of cationic amino acids across the plasma membrane of mammalian cells. *Biochimica et Biophysica Acta*, **822,** 355-374.

White, M.F. & Christensen, H.N. (1982). Cationic amino acid transport into cultured animal cells. II. Transport system barely perceptible in ordinary hepatocytes, but active in hepatoma cell lines. *Journal of Biological Chemistry*, **258,** 8028-8038.

Wilson, J.R., Wiener, D.H., Fink, L.I. & Ferraro, N. (1986). Vasodilatory behavior of skeletal muscle arterioles in patients with nonedematous chronic heart failure. *Circulation*, **74,** 775-779.

Wong-Dusting, J.D., La, M. & Rand, M.J. (1991). Endothelin-1 enhances vasoconstrictor responses to sympathetic nerve stimulation and noradrenaline in the rabbit ear artery. *Clinical and Experimental Pharmacology and Physiology*, **18,** 131-136.

Woo, A., Stewart, D., Dawood, F., Wen, W.H., Aitken, K., McLaughlin, P.R. & Liu, P. (1996). The effect of nitric oxide modulation on left ventricular function and remodelling following large myocardial infarction. *Canadian Journal of Cardiology*, **12,** 128E.

Xia, Y., Dawson, V.L., Dawson, T.M., Snyder, S.H. & Zweier, J.L. (1996). Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. *Proceedings of the National Academy of Science. USA*, **93,** 6770-6774.

Yanagisawa, M., Kurihara, S., Kimura, S., Tomobe, S.Y., Kombayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. & Masaki, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*, **332,** 411-415.

Yang, R., Bunting, S., Gillett, N., Clark, R. & Jin, H. (1995). Growth hormone improves cardiac performance in experimental heart failure. *Circulation*, **92,** 262-267.

Zelis, R. & Flaim, S.F. (1982). Alterations in vasomotor tone in congestive heart failure. *Progress in Cardiovascular Disease*, **24,** 437-459.

Zelis, R., Mason, D.T. & Braunwald, E. (1968). A comparison of the effects of vasodilator stimuli on peripheral resistance vessels in normal subjects and in patients with congestive heart failure. *The Journal of Clinical Investigation*, **47,** 960-970.

Zusman, R.M. & Keiser, H.R. (1977). Prostaglandin biosynthesis by rabbit renomedullary interstitial cells in culture: stimulation by angiotensin II, bradykinin, and arginine vasopressin. *Journal of Clinical Investigation*, **60,** 215-223.

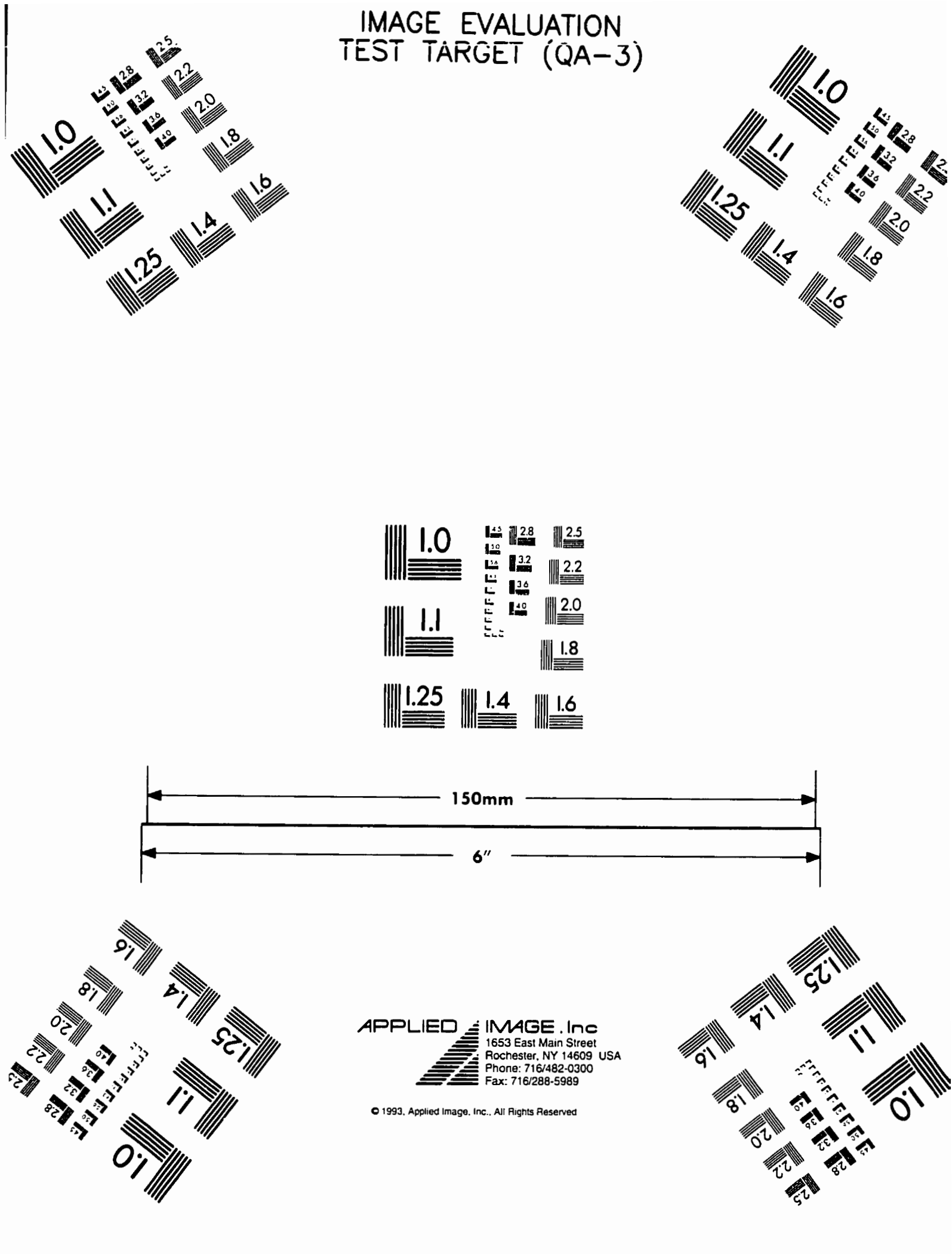


IMAGE EVALUATION
TEST TARGET (QA-3)
1.0
1.1
1.25
1.4
1.6
2.8
2.5
3.2
2.2
3.6
2.0
4.0
1.8
150mm
6"
APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989
© 1993, Applied Image, Inc., All Rights Reserved