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Re-expression of the Cardiac Fetal Gene Program in L-NAME Hypertension

by

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In partial fulfillment of the requirements for the degree of

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Abstract

Cardiac hypertrophy is an important adaptive response to increased hemodynamic overload but also represents the most powerful risk factor for cardiovascular morbidity and mortality. This myocardial response to work overload is associated with a complex reprogramming of the expression of cardiac “fetal genes” such as β -myosin heavy chain (MHC), atrial natriuretic factor (ANF), and brain natriuretic peptide (BNP). The up-regulation of ANF and BNP is viewed as an event related to the antigrowth properties of those peptides. Little is known about factors that underlie this up-regulation in vivo. In the present work, the contribution of chronic hemodynamic overload on ANF and BNP gene expression was determined in N^ω-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor, induced hypertension which may develop with or without hypertrophy. The hypothesis states that chronic hemodynamic overload without attendant hypertrophy would not upregulate ANF and BNP gene expression. The studies were carried out in the context provided by other markers of cardiac hypertrophy including β -MHC, collagen III as well as the parameters associated with cardiac hypertrophy such as LV renin expression, plasma renin activity (PRA) and angiotensin converting enzyme (ACE) activity, given the coincidental activation of this renin-angiotensin system (RAS) and the development of hypertrophy.

Male Sprague-Dawley rats received L-NAME in the tap water (75mg/kg/day) or tap water at 4 weeks (n = 24) or 8 weeks (n = 24). Systolic blood pressure (SBP) was 140 $\pm$ 3 mmHg at the end of 4 weeks, and 173 $\pm$ 6 mmHg at the end of 8 weeks, which were

significantly ($p < 0.001$) higher than SBP in control rats (105 ± 1 mmHg) and (106 ± 2 mmHg) respectively. LV hypertrophy did not develop with a 4 week L-NAME treatment and 5 of 12 of rats had an increased ratio of left ventricular weight to body weight (LVW/BW) compared to controls (2.50 ± 0.10 versus 2.03 ± 0.04 , $P < 0.01$) after 8 week treatment, therefore only 5 of 12 (42%) of rats developed LV hypertrophy. With 8 week L-NAME treatment, LV ANF mRNA level, PRA, LV renin gene expression and LV ACE activity significantly correlated with the LVW/BW. Plasma and LV immunoreactive natriuretic peptide (irNP) were not significantly enhanced compared to controls. In addition, LV β -MHC mRNA and the ratio of β -MHC to α -MHC mRNA (β -MHC/ α -MHC mRNA) were significantly increased in both 4 and 8 week L-NAME treatment.

In conclusion, LV ANF gene expression in long-term L-NAME treatment is related to cardiac hypertrophy and not related to pressure overload, suggesting that ANF gene expression is a specific marker of cardiac hypertrophy. Long-term L-NAME treatment does not affect plasma and cardiac tissue irNP levels, suggesting that NO does not play an important role in NP production and release in vivo. Increased β -MHC gene expression is a sensitive marker of pressure overload even in the absence of LV hypertrophy. Cardiac hypertrophy correlated with PRA, LV renin expression and LV ACE activity, suggesting that the RAS components play a role in the development of hypertrophy in long-term L-NAME hypertension. Supported by CIHR and HSFO.

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List of Abbreviations

ACE	angiotensin converting enzyme
ACh	acetylcholine
ANF	atrial natriuretic peptide
Ang I	angiotensin I
Ang II	angiotensin II
ATPase	adenosine triphosphatase
AVP	arginine vasopressin
BNP	brain natriuretic peptide
cGMP	cyclic 3', 5'-guanosine monophosphate
CNP	C-type natriuretic peptide
DOCA	deoxycorticosterone acetate
EDCFs	endothelium-derived contracting factors
EDHF	endothelium-derived hyperpolarizing factor
EDRF)	endothelium-derived relaxing factor
ET-1	endothelin-1
ET_A	endothelin-1 type-A receptor
GFR	glomerular filtration rate
irANF	immunoreactive-ANF
irBNP	immunoreactive-BNP
LA	left atrium

L-NAME	N^ω-nitro-L-arginine methyl ester
L-NMMA	N^G-monomethyl-L-arginine
LV	left ventricle
LVEF	left ventricular ejection fraction
LVW / BW	ratio of left ventricular weight to body weight
MHC	myosin heavy chain
MI	myocardial infarction
NEP	neutral endopeptidase
NLA	nitro-L-arginine
NO	nitric oxide
NOS	nitric oxide synthase
NP	natriuretic peptide
NPR	natriuretic peptide receptor
PCR	polymerase chain reaction
PCWP	pulmonary capillary wedge pressure
PDEs	phosphodiesterases
PGI₂	prostacyclin
PGK	phosphoglycerate kinase
PRA	plasma rennin activity
RA	right atrium
RAS	rennin-angiotensin system
RT-PCR	reverse transcription-polymerase chain reaction

RV	right ventricle
SBP	systolic blood pressure
SHR	spontaneously hypertensive rats
SVR	systemic vascular resistance
TIVCC	thoracic inferior vena caval constriction
VPI	vasopeptidase inhibitor
VSMC	vascular smooth muscle cells
WKHA	Wistar-Kyoto-derived hyperactive
WKHT	Wistar-Kyoto-derived hypertensive
WKY	Wistar-Kyoto

Dedication

To my husband Dr. Xin Chen for his love, support and encouragement.

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Introduction

Cardiac hypertrophy is an important adaptive response to increased hemodynamic overload. Initially, the increase in cardiac mass serves to normalize wall stress and permits normal cardiovascular function at rest and during exercise in compensated hypertrophy. If the stimulus for cardiac hypertrophy is sufficiently intense or prolonged, decompensated hypertrophy and heart failure occur. Cardiac hypertrophy represents the most powerful risk factor for cardiovascular morbidity and mortality.

Chronic increases in hemodynamic overload that are imposed on the adult heart profoundly modify both the size and the structure of cardiac myocytes. These cells are terminally differentiated and have lost their ability to proliferate. Therefore, in response to hemodynamic overload, they hypertrophy without dividing, and simultaneously, the type and content of key proteins for cardiac contraction and relaxation change (Nadal-Ginard et al 1989; Schwartz et al 1992). This myocardial response to work overload is due to a complex reprogramming of the expression of cardiac genes, characterized in most cases by the re-expression of “fetal genes” such as β -myosin heavy chain (MHC), skeletal α -actin, atrial natriuretic factor (ANF), and brain natriuretic peptide (BNP). The mechanisms involved in these changes are still largely unknown (Chien et al 1991; Morgan et al 1991), but these processes are considered to be essential in the adaptation of cardiocytes to chronic work load.

Cardiac hypertrophy can be caused by pressure overload such as in systemic arterial hypertension. Several studies have demonstrated that administration of nitric oxide

synthase inhibitors such as N^ω-nitro-L-arginine methyl ester (L-NAME) and other L-arginine analogues cause acute and chronic hypertension in many animal species (Johnson and Freeman 1992; Ward and Angus 1993; Manning et al 1993; Hu et al 1994; Ribeiro et al 1992; Deng et al 1993; Li and Schiffrin 1994). However, it is surprising that despite the elevation of blood pressure, in the hypertension produced by L-NAME, there is with little cardiac hypertrophy (Deng et al 1993; Li and Schiffrin 1994; Jover et al 1993; Arnal et al 1992; Arnal et al 1993; Delacretaz et al 1994). Schiffrin has suggested that this finding could be the result of an inhibitory effect of L-NAME on growth (Schiffrin 1995; Li et al 1996) independent of the opposing action expected from the inhibition of generation of nitric oxide (NO) (Garg and Hassid 1989), which should in fact stimulate smooth muscle cell growth.

The fact that L-NAME can induce severe hypertension without hypertrophy provides a unique opportunity to study the expression of cardiac fetal gene program as induced by pressure overload. Increased ventricular gene expression of ANF and BNP is considered a sensitive indicator of cardiac hypertrophy. However, Ogawa and coworkers (Ogawa et al 1996) found that in aortic banded rats, low-dose of ACE inhibitor, ramipril, can prevent cardiac hypertrophy without affecting blood pressure. They further demonstrated the low-dose ramipril reversed or prevented the hypertrophy that resulted in decreased gene expression of ANF and BNP, but did not completely reverse the increase of gene expression of natriuretic peptide (NP) in the left ventricle, which suggested hypertension can maintain high levels of NP expression independent from hypertrophy (Ogawa et al 1996). In another DOCA-salt hypertension model, Yokota et al (Yokota et

al 1995) also reported that increased gene expression of NP was found in the non-hypertrophied right ventricle. These findings indicate that cardiac hypertrophy is accompanied by increased gene expression of ANF and BNP, but increased gene expression of ANF and BNP can occur without cardiac hypertrophy. The above evidence suggests that further studies are necessary to determine factors that modulate NP gene expression as well as the expression of other genes that are correlated with cardiac hypertrophy.

In the present work, we took advantage of L-NAME hypertension, a severe form of hypertension that develops with or without cardiac hypertrophy, to determine the effect of chronic pressure overload on gene expression of ANF, BNP, α - and β -MHC, collagen III, LV renin as well as other parameters associated with cardiac hypertrophy including plasma renin activity (PRA) and angiotensin converting enzyme (ACE) activity.

In the following paragraphs, the various systems involved in this study would be reviewed firstly in the physiological context of NP and NO and later on, in the context of L-NAME hypertension.

1.1 Natriuretic Peptides

1.1.1 Background

The discovery that the heart is an endocrine organ resulted from consideration of the functional morphology of cardiac muscle cells. Kisch (Kisch 1956) in 1956 observed

'very small bodies' on electron microscopic examination of guinea-pig atrial myocytes. Jamieson and Palade (Jamieson and Palade 1964) in 1964 provided a more detailed description of the granules which were found in mammalian atria but not ventricles.

In 1981, de Bold et al (de Bold et al 1981) homogenized the atria and ventricles of rats and intravenously injected either the atrial or ventricular homogenates into anesthetized rats. Injection of the atrial homogenate increased sodium excretion and urine volume thirty-fold and ten-fold, respectively. Injection of the ventricular homogenate altered neither natriuresis nor diuresis. The implication was that the atrial granules contained a natriuretic substance, later named atrial natriuretic factor (ANF).

The second cardiac natriuretic peptide, brain natriuretic peptide (BNP), was isolated from porcine brain in 1988 (Sudoh et al 1988) but the highest concentration of BNP was found in the heart where it acts as a cardiac hormone (Aburaya et al 1989; Nakao et al 1990).

The search for additional structurally related peptides led to the discovery of a third member of the family in 1990 (Sudon et al 1990). This natriuretic peptide was also isolated from the porcine brain and named C-type natriuretic peptide (CNP). It has been found in brain (Minamino et al 1991) and endothelial cells (Suga et al 1992). Thus, CNP may function as a neurotransmitter and paracrine hormone rather than as a cardiac hormone in mammals.

1.1.2 Biological Actions of Natriuretic Peptides

The biological effects of ANF and BNP are very similar, both of them causing diuresis, natriuresis, vasodilation, and suppression of the renin-angiotensin-aldosterone system (de Bold et al 1981; de Bold 1985; Nakao et al 1992; Brenner et al 1990; Lang et al 1992; Ruskoaho 1992). CNP appears to play a paracrine function, whereas, CNP acts as a potent vasodilator and has a potent growth inhibition action on vascular smooth muscle cells (Wei et al 1993; Porter et al 1992; Furuya et al 1993; Suga et al 1998).

The natriuretic and diuretic actions of natriuretic peptides involve both renal hemodynamic and direct inhibition of Na^+ reabsorption at the collecting duct. ANF stimulates dilatation of afferent renal arterioles and constriction of efferent arterioles, leading to increased pressure within the glomerular capillaries, which causes increased glomerular filtration (Marin-Grez et al 1986). Furthermore, ANF can suppress angiotensin II-induced proximal tubular sodium and water reabsorption to exert its natriuretic and diuretic action (Harris et al 1987).

ANF also reduces sympathetic tone. This reduction is probably caused by suppression of sympathetic outflow from the central nervous system and the release of catecholamines from autonomic nerve endings (Schultz et al 1988; Yang et al 1992).

BNP has very similar effects to ANF. CNP may function as one of the vascular mediators that regulate local vascular tone and growth through cGMP production by vascular smooth muscle cells (VSMC), which suggests that CNP could play some role in the pathogenesis of atherosclerosis (Suga et al 1998).

Apart from acting as a circulating hormone, ANF has functions in cardiac growth. Oliver and colleagues (Oliver et al 1997) reported that elevated blood pressure and marked cardiac hypertrophy with interstitial fibrosis were found in natriuretic peptide receptor-A (NPR-A) knockout mice. John et al (John et al 1995) found that mice homozygous for disruption of the pro-ANF gene have no circulating or tissue ANF, and those mice exhibit increased heart weight and blood pressure when maintained on intermediate salt diets. Transgenic mice overexpressing ANF have a low heart weight under normoxic conditions and a blunted right ventricular hypertrophy response to hypoxia-induced pulmonary hypertension (Kinger et al 1993). These observations suggest the possibility that ANF regulates the development of cardiac myocyte hypertrophy. However, the issue of whether the influence of ANF on cardiac hypertrophy is a direct effect of this peptide or secondary to the change of blood pressure levels was not demonstrated by these studies using knockout or transgenic mice. With regard to the direct effect of ANF on cardiac hypertrophy, Horio et al (Horio et al 2000) recently demonstrated that the blockade of endogenous ANF by HS-142-1, a NPR antagonist, increased protein synthesis and cell size of cardiac myocytes under basal and phenylephrine (PE)-stimulated conditions. In addition, the expression level of skeletal α -actin, β -MHC, and ANF genes was partially elevated by treatment with HS-142-1 under nonstimulated or PE-stimulated conditions. They further found that a cGMP-specific phosphodiesterase inhibitor and a cGMP analogue competitor prevented basal and PE-stimulated protein syntheses. These observations suggest that endogenous ANF inhibits cardiac myocyte hypertrophy under basal and PE-stimulated conditions probably through a

cGMP-dependent process and ANF may regulate cardiac myocyte growth as an autocrine factor (Horio et al 2000).

The availability of the Wistar-Kyoto-derived hyperactive (WKHA) inbred rat strain provides a way to determine whether there is a genetic link between ventricular ANF expression and cardiac mass (Masciotra et al 1999). WKHA inbred strain, derived from Wistar-Kyoto (WKY)/spontaneously hypertensive rat (SHR) hybrid crosses, exhibits increased behavioral activity in a novel environment as well as increased cardiovascular reactivity to stress compared to WKY rats (Hendley et al 1991; Deschepper et al 1997; Knardahl et al 1990). Masciotra et al (Masciotra et al 1999) observed that ventricular mass was higher in WKHA than in WKY rats, yet ventricular ANF mRNA was consistently lower in WKHA than WKY and that low abundance of ventricular ANF (either peptide or mRNA) correlated with high ventricular mass. Given that the WKHA and WKY rats are both normotensive, it is unlikely that mechanical forces would be responsible for differences between the two strains. In the hypertensive SHR and WKY-derived hypertensive (WKHT) strains (both of them have same degree of hypertension), it was found that ventricular mass was higher in SHR than in WKHT, and ventricular ANF mRNA was less abundance in SHR than in WKHT. Taken collectively, these results demonstrate that low ventricular ANF mRNA is associated genetically with high cardiac mass independently of blood pressure and are consistent with a protective role for ANF against LV hypertrophy (Masciotra et al 1999). Thus, augmented production of ANF in hypertrophied myocardium can be considered a compensatory mechanism against ventricular overload and hypertrophy.

1.1.3 Molecular Biology and Biochemistry of Natriuretic Peptides

ANF, the prototype of the natriuretic peptide family, was originally identified in mammalian atrial tissues by de Bold in 1981 (de Bold et al 1981). The structure of ANF and its amino acid sequences was identified in 1983 (Flynn and de Bold 1983) (see Fig 1). Like other peptide hormones, ANF is initially synthesized as preprohormone containing 151 amino acids. Cleavage of signal sequence generates proANF, which contains 126 amino acids. ProANF is the precursor of ANF and the major storage form of ANF in atrial granules. During the time of ANF secretion, proANF is further cleaved into the carboxyl-terminal 28-amino acid peptide, ANF (99-126), the bioactive circulating form of ANF, and N-terminal 98-amino acid peptide, ANF (1-98).

The ANF gene is present as a single-copy gene and is organized into three exons separated by two introns (Seidman et al 1984; Greenberg et al 1984). Exon 1 encodes the signal sequence and the first 16 amino acid residues of the proANF. Exon 2 encodes most of the proANF sequence, whereas the remaining carboxyl-terminal Tyr (in human) or Tyr-Arg-Arg (in rodents) residues are encoded by exon 3. In the 5' flanking region of the gene, many cis-acting regulatory elements are involved in ANF gene expression (Seidman et al 1984; Greenberg et al 1984). In addition, the second intron contains a potential glucocorticoid receptor binding site, which suggests ANF gene may be regulated by glucocorticoids (Seidman et al 1984; Nemer et al 1988).

Figure 1 Primary structures of the human natriuretic peptide family.

ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide. ANF consists of a 28 amino acid peptide and BNP are composed of a 32 amino acid peptide with a central ring structure similar to ANF. CNP-22, consisting of 22 amino acids, is highly homologous with ANF and BNP within the ring structure, but uniquely it lacks the carboxyl-terminal extension. Amino acids shaded are common to all three hormones.

Fig 1

Atria are the major site of expression of the ANF gene in adult heart. In ventricle, ANF mRNA levels are 150 times lower than in atria. However, in cardiac hypertrophy or in congestive heart failure, ventricular ANF mRNA and peptide levels are dramatically increased both in animal models and in humans. This indicates that the ventricles are a major site of ANF gene expression in certain pathophysiological conditions (Nemer et al 1988).

In humans the major circulating form of BNP consists of a 32 amino acid peptide with a central ring structure similar to ANF (Fig 1). The BNP gene is also present as a single-copy gene that is organized into three exons and two introns rather like the ANF gene (Seilhamer et al 1989). BNP is expressed in both the atria and ventricles (Hosoda et al 1991; Magga et al 1998).

CNP was isolated from the porcine brain (Sudon et al 1990). Two forms of CNP exist, CNP-53 and CNP-22, consisting of either 53 or 22 amino acids, respectively. CNP is highly homologous with ANF and BNP within the ring structure, but uniquely it lacks the further carboxyl-terminal extension (Fig 1). Production of CNP occurs in brain (Minamino et al 1991) and endothelial cells (Suga et al 1992).

1.1.4 Natriuretic Peptide Receptors

The three natriuretic peptides exert their effects through interaction with high-affinity natriuretic peptide receptors (NPRs) present in the membrane of target cells. Three NPRs (A, B, and C) have been identified in mammalian tissues. The NPR-A binds both

ANF and BNP, with preference for ANF, while the NPR-B preferentially binds CNP. As yet there is no receptor which preferentially binds BNP. The NPR-A is the most common type on large blood vessels. The NPR-B is found predominantly in the brain. Both of them are present in the adrenal glands and the kidney (Levin et al 1998). The NPR-A and NPR-B are structurally similar, with approximately 44% homology in the ligand-binding extracellular domain. The intracellular regions of the NPR-A and B contain the guanylyl cyclase catalytic domain, which is activated upon binding of the appropriate natriuretic peptide to the extracellular domain (Koller et al 1992). Binding of the natriuretic peptides to their receptors leads to an elevation in intracellular cGMP through guanylyl cyclases (GC), which catalyze the formation of the intracellular signal molecule cGMP from GTP. The NPRs are linked to the cGMP-dependent signaling cascade and mediate many of the cardiovascular and renal effects of the natriuretic peptides (Fig 2). cGMP exerts its biologic effects indirectly through cGMP-dependent protein kinase G or one or more phosphodiesterases (PDEs), or by direct action on effectors such as amiloride-sensitive sodium channels in the kidney (Levin et al 1998).

The NPR-C is involved in clearance of the peptides (Maack et al 1987). This receptor is quite abundant in vascular tissue. The NPR-C contains a very short intracellular domain and has no guanylyl cyclase activity (Fuller 1988). Circulating natriuretic peptides also are inactivated by cleavage by neutral endopeptidases present within renal tubular cells and vascular cells.

Figure 2 Physiological effects of NP and NO.

NP and NO share in common the formation of cGMP and thus exert their biological effects. NP, natriuretic peptide; ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; NPR, natriuretic peptide receptor; NEP, neutral endopepdase; NO, nitric oxide; NOS, NO synthase; cGMP, cyclic 3', 5'-guanosine monophosphate; L-NAME, N^ω-nitro-L-arginine methyl ester.

Fig 2

1.1.5 Regulation of Natriuretic Peptide Secretion

ANF secretion is determined largely by atrial muscle stretch (de Bold et al 1986; Lang et al 1985; Miki et al 1986). Initial speculation about the mechanism of release was based on the theory that atrial pressure was important in determining release of ANF. Edwards et al (Edwards et al 1988) designed an experiment that attempted to distinguish increased atrial pressure from increased atrial stretch as the cause of ANF release in an intact animal model. In the first part of the experiment, tamponade was produced by saline infusion through a pericardial catheter. This caused increases in right atrial pressure and pulmonary capillary wedge pressure (PCWP). However, these increases in intracavitary pressure were not associated with increased transmural pressure. This state of tamponade is not associated with increased plasma ANF concentration. In the second set of experiments, saline was not infused into the pericardium, the increased intracavitary atrial pressure that was produced due to great vessel constriction was associated with an increase in atrial transmural pressure, that is, stretch, and a substantial rise in ANF concentration was noted. This clearly shows that increased atrial stretch and not increased atrial pressure caused ANF release.

Although atrial stretch is regarded as the main stimulus for ANF release, when atrial stretch is maintained, the plasma ANF concentration reduced to baseline values within minutes (Lang et al 1985; de Bold et al 1986; Edwards et al 1988; Agnoletti et al 1987). Stretch-induced increase of ANF release seems as the endocrine response of the atria to acute volume load (de Bold et al 1991). It has been shown that the rapid decline in

ANF output after atrial stretch is due to the rapid depletion of an immediately releasable pool of newly synthesized peptide without apparent effect on increased ANF synthesis (Mangat and de Bold 1993).

The mechanisms of chronic increase of ANF release from the atria is different from acute stretch-stimulated ANF release. Many disorders, such as chronic heart failure and ventricular hypertrophy can stimulate chronic or long-term ANF release, which is related to increased ANF gene expression (Lee et al 1988).

Numerous humoral or endocrine factors have been shown to stimulate natriuretic peptide secretion in vitro, including adrenergic agonists (epinephrine and norepinephrine), endothelin-1 (ET-1), glucocorticoids, acetylcholine, Na-K-ATPase inhibitors, prostaglandins, thyroid hormone and angiotensin II. Among these factors, ET-1 is the most potent stimulator of ANF secretion. ET-1 stimulates ANF and BNP secretion from isolated atria (Bruneau et al 1994; Schiebinger et al 1990). The increased NP secretion stimulated by ET-1 is transient. The time course of ET-1-stimulated BNP secretion is different from that of ANF, showing that there are differences in the response of these two peptides to ET-1 stimulation (Bruneau et al 1994). Since ET-1 is produced in endothelial cells, it suggests that ET-1 functions as a paracrine hormone to modulate NP secretion. In a unique model of chronic myocardial unloading produced by thoracic inferior vena caval constriction (TIVCC) that results in reduced myocardial load and marked activation of endogenous ET and Ang II, Lisy et al (Lisy et al 2000) found that chronic mechanical unloading of the heart caused myocardial atrophy even in the presence of marked increases in circulating and myocardial ET and Ang II. No evidence for increased ANF

gene expression was observed despite activation of ET and Ang II, recognizing ANF to be a marker for myocardial hypertrophy and the importance of mechanical load but not neurohumoral stimulation as the principal regulator of ANF cardiac production (Lisy et al 2000).

Contradicting results have been reported from experiments attempting to define the effects of adrenergic agonist stimulation of ANF. Epinephrine and norepinephrine have been shown to increase ANF secretion (Schiebinger et al 1987; Shields et al 1989). What is less clear, however, is whether this is due to a predominantly α - or β - adrenergic receptor-mediated effect. Some studies suggest that an α -adrenergic receptor-, and not β -adrenergic receptor-mediated effect is mainly responsible for adrenergic stimulation of ANF (Ambler and Leite 1994). α_1 -adrenergic agonists also stimulate BNP secretion in cultured rat cardiac myocytes (Hanford et al 1994). α -specific drugs have also been shown to increase ANF secretion (Schiebinger et al 1987; Shields et al 1989; Bruneau et al 1996). Phenylephrine can also stimulate BNP secretion from atrial tissue by α -adrenergic stimulation and this secretion of BNP was more sustained than that of ANF (Bruneau et al 1996).

In addition to humoral or endocrine factors, Ca^{++} was found to modulate the basal ANF secretion. Using the isolated perfused atrial tissue, the basal ANF secretion is augmented in Ca^{++} -free medium (de Bold et al 1989), which was rapidly reversed when Ca^{++} was reintroduced. This result indicates that basal ANF release and excitation-contraction coupling do not share a common regulator in Ca^{++} and further suggests a dissociation between the mechanical activity of the atria and its endocrine function (de

Bold et al 1996).

1.1.6 Pathophysiology of Natriuretic Peptides

Patients with congestive heart failure have high plasma concentrations of ANF and the concentration correlates with the severity of cardiac dysfunction (Burnett et al 1986; Bates et al 1986; Raine et al 1986). Numerous studies have shown a statistically significant positive correlation between circulating ANF levels and cardiac filling pressures and inverse associations with indices of left ventricular function (Bates et al 1986; Raine et al 1986; Nagae et al 1986). However, the circulating form of ANF (the biologically active carboxyl-terminal fragment containing 28 amino acids) has a brief plasma half-life and therefore, one might anticipate that measurement of N-terminal portion of pro-ANF (N-ANF), which has a prolonged half-life in plasma, should better reflect cardiac dysfunction. Indeed N-ANF is elevated in patients with heart failure and is useful in the detection of patients with mild, even symptomless ventricular dysfunction and may prove superior to conventional ANF assays in dissecting the degree of cardiac dysfunction in those patients with established heart failure (Winters et al 1989; Lerman et al 1993). N-ANF was also found to be superior to ANF in predicting survival in a subgroup of patients with acute myocardial infarction (MI) from the CONSENSUS II trial (Omland et al 1993). The high sensitivity and predictive value make N-ANF an excellent screening tool of left ventricular function (Bonarjee et al 1995).

The concentration of BNP in plasma is lower than that of ANF in the physiological

state. However, its concentration increases markedly in congestive heart failure, in proportion to its severity and surpasses the plasma ANF concentration in severe cases (Mukoyawa et al 1991). The increased plasma BNP concentration is due to increased synthesis of BNP in the ventricles and its slower clearance from the circulation (Mukoyawa et al 1991). Furthermore, plasma BNP concentration is more remarkably elevated than plasma ANF concentration in acute myocardial infarction (Mukoyawa et al 1991). However, in mitral stenosis BNP levels are increased less obviously than ANF (Nakamura et al 1992), which suggests that BNP could provide a more accurate index of ventricular dysfunction than ANF. BNP is found to be more sensitive and specific than ANF for detecting a decrease in left ventricular ejection fraction (LVEF) (Choy et al 1994). A strong inverse correlation between BNP and LVEF was found in patients with Q-wave anterior wall MI on days 2, 7, 42 and 180 (Motwani et al 1993). Furthermore, increased BNP levels during the early period after MI can predict LVEF and ventricular remodeling (Arakawa et al 1994). Darbar et al (Darbar et al 1996) reported that plasma BNP measured after acute MI is a powerful neurohumoral predictor of cardiovascular mortality, whereas plasma ANF is better for identifying patients with symptomatic heart failure. Neither LVEF nor Killip class could be able to predict the development of these risks. The favorable results of BNP compared to ANF are likely because of its ventricular site of production in pathophysiological state, different gene regulation, and its ability to reflect regional wall stress that occurs with ischemia in the absence of changes in global hemodynamics (Omeland et al 1996). Collectively, measurement of both these peptides may be a superior, noninvasive method to identify risk in patients after acute MI. In addition,

patients with essential hypertension also have raised levels of ANF and BNP that may reflect increased left ventricular mass (Montorsi et al 1987; Kohno et al 1992; Takeda et al 1995).

Increased plasma ANF concentration and filling pressures is also observed in patients undergoing cardiac transplantation (Masters et al 1993). With return of cardiac function and normalization of filling pressures, plasma ANF concentration remains chronically abnormally elevated. The pharmacological actions of cyclosporine (a potent immunosuppressive agent) on smooth muscle endothelial cells leading to increased sensitivity to vasopressor hormones and overproduction of endothelin may likely explain the chronic elevation of plasma ANF concentration (Masters et al 1993).

1.1.7 Natriuretic Peptide Gene Expression in Volume and Pressure Overload

It is generally accepted that blood volume and sodium balance play a very important role in ANF release and gene expression. Nakayama et al (Nakayama et al 1984) found that ANF mRNA levels decreased when the rats were deprived of water, and the total content of the mRNA was about one-half to one-third of that in the control rats after 2 and 4 days of water deprivation. Takayanagi et al (Takayanagi et al 1985) further showed that 5 days of water or sodium deprivation in normal rats caused a 30% to 70% decrease in ANF mRNA in both atria and that this returned to control levels after administration of NaCl solution. In DOCA-salt hypertension, which is regarded as a

volume-expanded hypertension, several studies have shown that the ANF mRNA level was increased in the atria (Lattion et al 1986; Nunez et al 1989; Itoh et al 1991). Yokota et al (Yokota et al 1995) found that ANF mRNA increased 1.7 fold and BNP mRNA increased 2.8 fold in the LA after 5 week DOCA-salt treatment compared to controls. These results suggest that blood volume and sodium balance are important factors in the regulation of ANF gene expression in the atria.

Atrial ANF gene expression has been extensively studied in pressure overload. Spontaneously hypertensive rats (SHR) slowly develop a progressive increase in arterial pressure and this makes them a suitable model for studying the sequential alterations associated with pressure overload. Arai et al (Arai et al 1988) found that total contents of atrial ANF mRNA and immunoreactive-ANF (irANF) levels were similar in SHR compared to those of control rats. Kinnunen et al (Kinnunen et al 1991) measured atrial ANF mRNA and irANF levels from 2- and 21-month-old SHR and Wistar-Kyoto (WKY) rats. They found that atrial ANF concentrations in the SHR decreased significantly with increasing age and that ANF mRNA levels were similar in young and old SHR. Furthermore, atrial ANF mRNA levels in SHR did not differ significantly from the age-matched WKY rats at either age (Kinnunen et al 1991). In the pressure-overload by aortic banding, irANF concentration was found significantly decreased in the atria (Day et al 1987; Mercadier et al 1989). In the rats with aortic coarctation, irANF was decreased, and in rats whose coarctations were released, the concentration of irANF returned to the control level (Gu et al 1989). However, the level of ANF mRNA in atria remained unchanged during coarctation and after coarctation release (Gu et al 1989). These results

suggeste that the pressure overload does not play an important role in the activation of ANF gene expression in the atria in vivo.

In addition to atrial NP expression, ventricular NP gene expression was also studied with changes in hemodynamic overload. Lattion et al (Lattion et al 1986) measured LV ANF mRNA levels in two models of volume loading: DOCA-salt and aortocaval fistula. Both experimental conditions were accompanied by a highly significant increase in the LV ANF mRNA levels. The most striking induction of ventricular ANF expression occurred in the aortocaval fistula model, with a 11 fold increase in ANF mRNA, which was associated with the most pronounced ventricular hypertrophy. LV ANF mRNA increased 4 fold in the DOCA-salt model with moderate myocardial hypertrophy. Itoh et al (Itoh et al 1991) reported the ANF mRNA level in the ventricles of DOCA-salt rats exhibited a 7 fold increase compared to that of control rats. The de Bold group (Yokota et al 1995) found that pronounced LV hypertrophy accompanied by a 13.6 fold increase in ANF mRNA level, 3.5 fold increase in BNP mRNA level and peptide content after 5 weeks of DOCA-salt treatment. Recently, using the same model of DOCA-salt without unilateral nephrectomy as performed previously, Bianciotti and de Bold (Bianciotti and de Bold 2000) found that LV hypertrophy was associated with 3.3 fold increase in ANF mRNA level, 2.1 fold increase in BNP mRNA level compared to that of control rats. They further demonstrated that the selective endothelin-1 (ET-1) type-A receptor (ET_A) antagonist reduced NP gene expression in the ventricles and did not affect NP mRNA levels in the atria, suggesting that NP production in the ventricles but not in the atria is regulated by ET-1 in this DOCA-salt hypertension model (Bianciotti and de

Bold 2000).

Myocardial performance in SHR is impaired when ventricular hypertrophy is severe and heart failure develops. Ventricular ANF mRNA levels were markedly increased in SHR rats with ventricular hypertrophy (Arai et al 1988). When ANF gene expression was studied in young and old SHR, the total amount of ventricular irANF increased 41 fold, and ANF mRNA levels increased 12.9 fold from the age of 2 to 21 months (Kinnunen et al 1991). In SHR, treatment with minoxidil, a vasodilator, increased cardiac hypertrophy and produced a 2.1 fold increase in ventricular ANF mRNA compared to controls (Kinnunen et al 1990). Furthermore, a highly significant correlation was found between ventricular irANF content and ventricular hypertrophy in SHR, suggesting that the increased gene expression of ANF in the diseased ventricle is due to ventricular hypertrophy.

In addition to volume overload and genetic hypertension, pressure overload also increases ventricular level of ANF and BNP gene expression. Constriction of the abdominal aorta produced LV hypertrophy and increased ANF mRNA levels in the LV (Day et al 1987). Using the same abdominal aortic constriction model, Gu et al (Gu et al 1989) found increased irANF and ANF mRNA and their amounts increased in proportion to the intraventricular pressure and duration of coarctation. In a model of pressure overload produced by suprarenal aortic banding, Ogawa et al (Ogawa et al 1996) found that banding for 6 weeks caused LV hypertrophy and significantly increased plasma and ventricular irANF and irBNP concentration and ventricular ANF and BNP mRNA levels. Recently, Kaiser et al (Kaiser et al 1998) reported that in rats with pressure-induced

cardiac hypertrophy the ventricular level of ANF mRNA was increased 10 fold compared to controls. The increase was first noticeable at 24h post-banding and persisted until 28 days. A hypertrophy-associated increase in ventricular ANF has been shown to occur in models producing an increase in pulmonary arterial pressure and right ventricular hypertrophy. Right ventricular pressure overload produced by hypoxic pulmonary hypertension leads to substantial induction of right ventricular irANF and ANF mRNA (Raffestin et al 1990). Further, Adachi and colleagues (Adachi et al 1995) observed ANF and BNP gene expression by using an experimental model of acute RV hypertrophy produced by surgical coarctation of the main pulmonary artery in rats. In this model, the LV is considered to be free from any direct influence of mechanical load, therefore this model can differentiate whether gene induction is induced by hormonal or by direct hemodynamic mechanisms. They found that there were 20, 28, and 47 fold increases in the RV ANF mRNA levels at days 1, 3 and 7 and BNP mRNA in the RV was also increased at day 1 and remained at this level at day 7 after pulmonary artery banding (Adachi et al 1995). The increased expression of ANF and BNP mRNAs occurred exclusively in the RV free wall and in the junctional region between the RV free wall and the interventricular septum (IVS), whereas minimal expression of these genes was observed in LVs of both banded and sham-operated rats. These observations suggest that cardiomyocyte hypertrophy and its associated accumulation of ANF and BNP mRNAs are caused by myocyte stretch or local autocrine mechanisms rather than by imposed pressure and systemic hormonal factors do not play a role in this model (Adachi et al 1995).

1.1.8 Therapeutic Effects of Natriuretic Peptides

Several studies have shown the efficacy of natriuretic peptides in the treatment of some disorders, such as congestive heart failure, myocardial ischaemia, hypertension and renal insufficiency. Administration of ANF reduces blood pressure, promotes sodium excretion and improves hemodynamic index both in patients with essential hypertension and congestive heart failure (Weder et al 1987; Fifer et al 1990). Both ANF and BNP have been shown to increase coronary sinus blood flow and decrease coronary vascular resistance leading to dilation of epicardial and resistance vessels (Boche et al 1988; Okumura et al 1995). ANF infused during acute MI led to an increase in diuresis and natriuresis compared to placebo without any adverse hemodynamic effects (Nakamura et al 1990). Infusion of BNP into normal controls and patients with heart failure decreased both preload and afterload of the left ventricle by decreasing PCWP, right atrial pressure and systemic vascular resistance (SVR) (Yoshimura et al 1991).

The neutrophil is thought to play a role in ischaemia in reperfusion injury by contributing to endothelial cell damage (Cronstein et al 1989). Human neutrophils incubated in either ANF or BNP demonstrated suppressed adhesiveness, inhibition of elastase release, and inhibited detachment of human endothelial cells (Mastsumura et al 1996). Therefore, ANF and BNP, which increase in acute MI, modulate the neutrophil function and exert protective effects against neutrophil-induced endothelial cytotoxicity but the effects are suppressed due to their degradation by the neutrophil's own neutral endopeptidase (NEP), which destroys the natriuretic peptide's activity (Mastsumura et al

1996). Morishita et al (Morishita et al 1994) transfected natriuretic peptide gene constructs into vascular smooth muscle and endothelial cells and these transfected cells exhibited less DNA synthesis in response to mitogens. However, further studies are needed to apply this ANF transfection for in vivo gene therapy for vascular diseases.

Although natriuretic peptide infusion has beneficial effects in some disorders, the intravenous route of administration makes the peptides cumbersome treatment option due to their short half-life. Natriuretic peptides are degraded to a great extent by NEP. Inhibition of NEP thereby protects endogenous natriuretic peptides. However, NEP inhibitors alone have not been effective as chronic antihypertensive agents because NEP inhibition alone can cause a compensatory reflex activation of RAS, which causes a compensatory elevation in blood pressure (Robl et al 1997). Therefore, NEP inhibitors may achieve their full therapeutic potential when used along with an angiotensin converting enzyme (ACE) inhibitor. ACE inhibition blocks the conversion of angiotensin I to the potent vasoconstrictor, angiotensin II. In addition, ACE inhibitors potentate aspects of the kallikrein-kinin system, which result in vasodilation and cardioprotective properties (Kaplan 1997). Vasopeptidase inhibitors (VPIs) are recently developed single molecules that simultaneously inhibit the activity of NEP and ACE (Burnett 1999). Omapatrilat, a novel vasopeptidase inhibitor, demonstrates antihypertensive efficacy in low-, normal-, and high-renin animal models. Omapatrilat inhibits vasoconstriction and enhances vasodilation by means of several interrelated mechanisms and consequently lowers vascular tone. Omapatrilat has been shown to be more efficacious than an ACE inhibitor in achieving improvements in both cardiac function and survival in an animal model of heart failure.

Omapatrilat appears to be a safe, well tolerated, and effective antihypertensive agent in humans (Burnett 1999). VPIs represent a new class of pharmacological agent that alters the balance of local and circulating humoral factors in favor of vasodilation. VPIs offer the promise to be an important new advancement in the treatment of cardiovascular disease, specifically hypertension and heart failure.

1.2 Nitric Oxide (NO) and Nitric Oxide Synthase (NOS)

In 1980, Furchgott and Zawadzki demonstrated the role of the endothelium in the relaxation of the isolated rabbit aorta in response to acetylcholine (Furchgott and Zawadzki 1980). It is now known that the endothelium synthesizes and releases several relaxing factors, including endothelium-derived relaxing factor (EDRF), endothelium-derived hyperpolarizing factor (EDHF) and prostacyclin (PGI_2), as well as several contracting factors (endothelium-derived contracting factors, EDCFs). EDRF has been identified as nitric oxide (NO) (Palmer et al 1987), or a closely related nitroso compound. NO, the free radical gas, is generated by a family of enzymes known as NO synthases (NOS) that catalyze the conversion of the amino acid L-arginine into L-citrulline in the presence of O_2 and NADPH.

The three NOS isoforms originally identified are: in the brain (neuronal NOS, or nNOS) (Bredt et al 1991), in macrophages (inducible NOS, or iNOS) (Xie et al 1992) and in endothelial cells (endothelial NOS, or eNOS) (Lamas et al 1992; Nishida et al 1992). They share 50% to 60% homology of their amino acid sequence. It is now widely

accepted that activation of the soluble form of guanylate cyclase (Ignarro 1990), and the consequent increase in cGMP levels induces a sequence of protein phosphorylation associated with NO's biological effects. The soluble guanylyl cyclase consists of two subunits (α_1 and β_1) and both subunits contain the putative catalytic domain, which is conserved in the membrane-bound guanylyl cyclases (Koesting et al 1991). NO induces relaxation of vascular smooth muscle cells (Furchgott and Zawadzki 1980; Palmer et al 1987) and inhibits migration and proliferation of vascular smooth muscle cells (Sarker et al 1996; Garg et al 1989). NO also inhibits both platelet adhesion to endothelial cells and platelet aggregation (Radomski et al 1987; Radomski et al 1990) (see Fig 2).

1.3 L-NAME-induced Hypertension

1.3.1 Mechanisms Involved in L-NAME Hypertension

Several studies have demonstrated that administration of NOS inhibitors such as N^ω-nitro-L-arginine methyl ester (L-NAME) and other L-arginine analogues cause acute or chronic hypertension in many animal species (Johnson and Freeman 1992; Ward and Angus 1993; Manning et al 1993; Hu et al 1994; Ribeiro et al 1992; Deng et al 1993; Li and Schiffrin 1994). The mechanism mediating the hypertension induced by the inhibition of NOS is incompletely understood. Several investigators have reported evidence that sodium retention contributes to the development of hypertension in this L-NAME hypertension model (Salazar et al 1993; Tolins and Shultz 1994). The acute inhibition of

NOS results in a decrease in excretion of sodium associated with a reduction in renal blood flow and an increase in glomerular filtration (Bachmann and Mundel 1994; Manning et al 1994). However, some investigators have shown that sodium output increases after acute administration of L-NAME (Baylis et al 1990; Johnson and Freeman 1992).

The effects of chronic inhibition of NOS on sodium and water balance remain controversial. At low L-NAME doses, the development of hypertension depended entirely on concomitant dietary salt overload (Lahera et al 1991), an observation in agreement with those made by Salazar and coworkers (Salazar et al 1993), who showed induction of salt-dependent hypertension in dogs by chronic infusion of L-NAME, and by Tolins and Shultz (Tolins and Shultz 1994), who showed marked aggravation of hypertension induced by low-dose L-NAME in rats also receiving dietary salt excess. In addition, these results are consistent with those reported by Chen and Sanders (Chen and Sanders 1991), who suggested that spontaneous NO deficiency may underlie the salt-dependent hypertension observed in rats. Collectively, these studies support the view that low-grade NO inhibition subtly impairs the renal ability to excrete sodium, predisposing the animals to the development of hypertension on salt overload. Several other studies have shown that the overall sodium and water balance is unchanged during L-NAME administration (Manning et al 1993; Hu et al 1994; Liu et al 1998), which suggests that the retention of sodium is not important in this model and the hypertension associated with NO synthesis blockade is not a volume-dependent type of hypertension. Others have shown that L-NAME increases vascular resistance in the renal, mesenteric, and hindquarter vascular beds (Gardiner et al 1990). In addition, L-NAME administration has been associated with

increases in total peripheral resistance and decreases in cardiac output (Elsne et al 1992). Therefore, L-NAME administration may produce a vasoconstrictor type of hypertension.

Liu and coworkers (Liu et al 1998) reported that chronic blockade of NOS by the oral administration of L-NAME is associated with both central and peripheral deficiency of NO. Sander et al (Sander et al 1995) identified an important sympathetic neural component to the sustained hypertension produced by pharmacological inhibition of NO, because chronic sympathectomy greatly attenuates the development of L-NAME induced hypertension in the rat and has no discernible effect on endothelium-dependent vasodilatation. Sakuma et al (Sakuma et al 1992) also found that renal sympathetic nerve activity increased during acute L-NAME treatment. Taken together, these findings suggest that sympathetic activation contributes to chronic hypertension induced by inhibition of NO synthesis.

Several studies have shown that the long-term increase in blood pressure caused by the inhibition of NOS can be prevented or reversed by treatments with angiotensin converting enzyme (ACE) inhibitors (Morton et al 1993; Hropot et al 1994) and angiotensin II antagonists (Polloch et al 1993; Jover et al 1993), indicating that there is an interaction between NO and the renin-angiotensin system (RAS).

Bank and colleagues (Bank et al 1994) examined the effect of blockade of receptors for several endogenous vasoconstrictors on blood pressure and renal hemodynamics in rats after 3 week NO inhibition using nitro-L-arginine (NLA). Intravenous infusion of receptor antagonists for angiotensin II, thromboxane, epinephrine, and endothelin-1 had no significant effect on systemic blood pressure in the hypertensive

NLA-treated rats. If inhibition of NO synthesis resulted in overexpression of any of these endogenous vasoconstrictors, it would be expected that receptor blockade would result in a sharp fall in blood pressure. In their study, the nonspecific Ang II receptor antagonist saralasin was used, which presumably blocked both AT₁ and AT₂ receptors in the kidney and elsewhere. The failure of saralasin to reduce blood pressure in the rats with chronic NO inhibition therefore provides evidence that Ang II did not contribute to the rise in peripheral vascular resistance (Bank et al 1994). The weight of findings suggests that the increase in vascular smooth muscle tone caused by inhibition of nitric oxide synthesis can not be accounted for by overexpression of common endogenous vasoconstrictors. Rather, the generalized increase in vascular smooth muscle tone appears to be due to a direct effect of reduced nitric oxide availability, which may lead to an increase in intracellular calcium concentration or sensitivity (Bank et al 1994).

1.3.2 Renin-Angiotensin System in L-NAME Hypertension

The renin-angiotensin system (RAS) plays an important role in the regulation of blood pressure and fluid volume. According to the classical concept, liver-derived angiotensinogen is cleaved in the circulating blood by renin from the kidneys, to form angiotensin I (Ang I). Ang I is then converted to Ang II, a potent vasoconstrictor and stimulant of the release of aldosterone, by angiotensin-converting enzyme (ACE) located on the luminal side of the vascular endothelium

Arterial blood pressure is considered to be an important determinant of left

ventricular hypertrophy in most rat models of experimental hypertension. This was confirmed in the DOCA-salt model in which significant left ventricular hypertrophy correlated with systolic blood pressure (Xu et al 1995). However, this correlation did not exist in L-NAME rats which had little left ventricular hypertrophy, despite the fact that rats had the same arterial blood pressure as in DOCA-salt rats. Numerous studies have shown that the RAS is strongly implicated in the pathophysiology of L-NAME hypertension (Morton et al 1993; Polloch et al 1993; Jover et al 1993; Hropot et al 1994; Li et al 1996; Pechanova et al 1997; Takemoto et al 1997; Akucawa et al 1998; Bernatova et al 2000). However, the direct role of NO in inhibiting or stimulating renin synthesis remains very controversial. Some groups demonstrated that NO could stimulate renin secretion (Gardes et al 1992; Scholz et al 1993), but other groups have shown that NO suppresses renin secretion (Beierwaltes 1989; Vidal et al 1988; Sigmon et al 1992). It has been reported that plasma renin activity (PRA) can increase (Morton et al 1993), decrease (Polloch et al 1993; Knoblich et al 1996), or even remain unchanged (Liu et al 1998; Jover et al 1993; Manning and Hu 1994) during chronic inhibition of NOS. Arnal (Arnal et al 1993) and Xu (Xu et al 1995) further demonstrated that only the rats with hypertrophy observed in L-NAME hypertension were associated with increased PRA and there was a close correlation between the cardiac hypertrophy and increased PRA in L-NAME rats.

Chronic administration of L-NAME also increases cardiac tissue angiotensin converting enzyme (ACE) activity (Arnal et al 1993; Takemoto et al 1997). It has been reported that ACE activation contributes to the development of vascular and myocardial structural changes after left ventricular hypertrophy and failure in rats (Schieffer et al

1994; Weinberg et al 1994; Schunkert et al 1990; Hirsch et al 1991; Schunkert et al 1992). The results of several studies have shown that the long-term increase in blood pressure and cardiac hypertrophy caused by the inhibition of NOS can be prevented or reversed by treatments with ACE inhibitors (Morton et al 1993; Hropot et al 1994; Li et al 1996; Pechanova et al 1997; Takemoto et al 1997; Akucawa et al 1998; Bernatova et al 2000) and angiotensin II antagonists (Polloch et al 1993; Jover et al 1993). It is known that ACE inhibitors and calcium channel blockers are the most effective substances in reducing LV hypertrophy in hypertensive patients (Schlaich and Schmieder 1998). Akuzawa and co-workers (Akuzawa et al 1998) demonstrated that imidapril (ACE inhibitor) is more effective to prevent left ventricular hypertrophy and nephrosclerosis produced by the chronic inhibition of NO than amlodipine (calcium channel blocker), suggesting that in rats the inhibition of the RAS has a better effect than the blockade of calcium channels in preventing hypertensive tissue injury. One group (Li et al 1996) has shown that hypertension in spontaneously hypertensive rats (SHR) treated with L-NAME is endothelin independent based on the absence of response to chronic endothelin receptor antagonism. However, there is a renin-dependent component of the hypertension, as demonstrated by the fact that cilazapril, an ACE inhibitor, induced a marked regression of vascular hypertrophy although not lowering the blood pressure dramatically. This finding suggests that regression was a direct consequence of the pharmacological effect of cilazapril, whether via inhibition of angiotensin II generation, increased accumulation of kinins, or other effects of the ACE inhibitor but independent of the lowering of blood pressure. Pechanova et al (Pechanova et al 1997) have reported that captopril (ACE

inhibitor) completely prevented not only hypertension and left ventricular hypertrophy development but also increased RNA and DNA concentration in LV of rats during chronic L-NAME administration. Bernatova et al (Bernatova et al 2000) examined the effect of both spontaneous and captopril-induced recovery on hypertension, LV hypertrophy and protein composition of LV in rats with L-NAME-induced hypertension. They found that spontaneous regression of L-NAME-induced hypertension is not associated with regression of LV hypertrophy and LV hypertrophy was regressed only in captopril-treated animals. Taken together, these findings suggest that the RAS plays a significant role in the hypertension and hypertrophy induced by L-NAME treatment.

In addition to the circulating RAS, several lines of evidence indicate that components of the RAS are present in the myocardium (Dostal et al 1999) and Ang II is regulated independently of the circulating RAS (Dell'Italia et al 1997; de Lannoy et al 1998). Dostal et al (Dostal et al 1999) reported that cardiac RAS is activated in rats with cardiac hypertrophy. Ang II is found to stimulate hypertrophic growth of cardiac myocytes and proliferation of cardiac fibroblasts in vitro (Neri Serneri et al 1996). Treatment of aortic banded rats with ACE inhibitor which did not cause a reduction in blood pressure (Baker et al 1990; Linz et al 1989) resulted in the regression of heart hypertrophy. Hence, evidence for a dissociation between changes in blood pressure and cardiac mass gave indirect support for the possibility that the cardiac RAS acts as a stimulator of cardiac growth and causes changes in cardiac mass. These findings suggest that the cardiac RAS has a pivotal role in pressure overload and cardiac hypertrophy.

1.3.3 Microvascular Remodeling and Cardiac Hypertrophy in L-NAME Hypertension

It is widely acknowledged that structural changes in the heart and blood vessels occur in almost all forms of hypertension. A less-defined association is the cause-effect relationship between the level of cardiovascular hypertrophy and the magnitude of the hypertension. The development of cardiovascular structural changes is a feature commonly associated with experimental models of hypertension such as two-kidney one clip, spontaneously hypertensive rats (SHR), or Ang II infusion (Folkow 1982; Griffin et al 1991; Dostal and Baker 1992). Since NO has been shown to inhibit smooth muscle cell growth in vitro (Garg and Hassid 1989), it would be expected that the hypertrophy would be enhanced under L-NAME treatment since the potential tonic inhibitory effect of NO on smooth muscle cell growth is no longer present. However, it is surprising that despite the elevation of blood pressure, in the hypertension model induced by inhibition of NOS with L-NAME, there is little cardiac hypertrophy (Deng et al 1993; Li and Schiffrin 1994; Jover et al 1993; Arnal et al 1992; Arnal et al 1993; Delacretaz et al 1994). In addition, there is little or no detectable vascular hypertrophy, particularly of small, resistance-size arteries (Deng et al 1993; Li and Schiffrin 1994; Dunn and Gardiner 1995). Banting and colleagues (Banting et al 1997) reported that L-NAME treatment resulted in only a modest change in vascular resistance and no cardiac hypertrophy despite the presence of marked hypertension, suggesting either pressure alone is not a sufficient stimulus to induce cardiovascular growth processes or L-NAME may be nonspecifically inhibiting

cardiovascular growth processes. Schiffrin has suggested that this finding could be the result of an inhibitory effect of L-NAME on growth (Schiffrin 1995; Li et al 1996) independent of the opposing action expected from the inhibition of generation of NO (Garg and Hassid 1989), which should in fact stimulate cell proliferation. This potential direct or indirect inhibitory effect of L-NAME on cardiovascular growth remains to be explored, but the possibility should be considered in the light of studies that have shown that L-NAME has other actions independent of its ability to inhibit NO synthesis (Buxton et al 1993). The reported antimuscarinic action of L-NAME could be one underlying mechanism for a direct inhibitory effect of L-NAME on cell growth, since muscarinic receptor-mediated stimulation of DNA synthesis and growth factor-like activity has been described in brain-derived cells (Ashkenazi et al 1989). Alternatively, since muscarinic receptors play a crucial role in the hypothalamus to stimulate growth hormone release (Mazza et al 1994), the antimuscarinic effect of L-NAME could be exerted indirectly by interfering with growth hormone secretion or through other mechanisms that remain to be established.

Numaguchi et al (Numaguchi et al 1995) reported that chronic inhibition of NO synthesis with L-NAME for 8 weeks is associated with cardiac hypertrophy and structural changes of the coronary microvessels (thickening of the media and increased perivascular fibrosis) but not with those in the large coronary arteries. An impressive accumulation of collagen also observed in the media of coronary microvessels in animals with L-NAME treatment. The pathogenesis of microvascular remodeling may be related to the increased production of mitogen- or growth-promoting factors due to decreased NO synthesis (Garg

and Hassid 1989; Von der Leyen et al 1995) because the microvascular remodeling did not appear until week 4 despite the presence of arterial hypertension at week 2. This indicates that arterial hypertension may not be a prerequisite for the occurrence of L-NAME-induced microvascular remodeling (Numaguchi et al 1995). In this work, it was also found that cardiac hypertrophy (increased myocyte cross-sectional area) and myocardial fibrosis did occur and hydralazine did not affect the increase in the relative LV weight or myocyte hypertrophy even though it prevented arterial hypertension, suggesting that arterial hypertension is not the sole factor responsible for the development of cardiac hypertrophy in this model.

Arnal and coworkers (Arnal et al 1992; Arnal et al 1993) have demonstrated that increases in cardiac mass are normally lacking with hypertension induced by L-NAME. However, only 25% of rats developed cardiac hypertrophy that strongly correlated with increased plasma renin activity. These findings indicate that in the majority of rats, during the first 2 to 3 weeks of inhibition of NOS, the lack of development of cardiac hypertrophy may be due to the absence of neurohumoral activation (Jover et al 1993; Arnal et al 1992; Arnal et al 1993; Delacretaz et al 1994).

1.4 Myosin Heavy Chain in Pressure Overload and Cardiac Hypertrophy

In the early search for biochemical changes in cardiac hypertrophy, the relation between decreased mechanical performance and decreased myosin adenosine

triphosphatase (ATPase) activity was demonstrated in various animal models of hemodynamic overload (Sheuer et al 1979). A direct relationship between the rate of Ca^{++} -stimulated myosin ATPase activity and the maximum velocity of muscle shortening was also demonstrated (Barany 1967). Myosin is the transducer for conversion of chemical energy in the form of ATP to mechanical shortening caused by sliding of interdigitated thick and thin myofilaments of striated muscle. Each molecule of myosin is a hexamer consisting of two myosin heavy chains and two pairs of distinct myosin light chains. Hydrolysis of ATP occurs at the globular head region of the myosin heavy chain (MHC) molecule and results in force development by causing formation of cross bridges between associated actin and myosin molecules (Harrington et al 1984). In smaller mammals three different myosin isozymes, designated V1, V2, and V3, can be resolved by electrophoresis in nondenaturing gels and can be physiologically differentiated on the basis of different catalytic rates of ATP hydrolysis (Hoh et al 1978). Biochemically, these three isozymes result from the presence of two different myosin heavy chains termed α -MHC, and β -MHC. V1 is the $\alpha\alpha$ -homodimer and V3 is the $\beta\beta$ -homodimer, whereas V2 is the $\alpha\beta$ -heterodimer (Swynghedauw 1986).

α -MHC and β -MHC are known to be expressed in mammalian heart (Marhdavi et al 1982). The α -MHC has the highest ATPase activity, whereas β -MHC has the lowest ATPase activity, and the contractile velocity of the heart correlated with the relative amount of each MHC. While hearts expressing more α -MHC have a more rapid contractile velocity, hearts with more β -MHC allow for great economy in force generation (Holubarsch et al 1985). The MHC composition of the ventricular myocardium of small

mammals has been reported to be > 90% α -MHC (Mercadier et al 1981; Izumo et al 1987), whereas that of humans has been reported to be > 95% β -MHC (Gorza et al 1984; Mercadier et al 1983; Bouvagnet et al 1989). A number of stimuli have been shown to shift the MHC composition of the rodent heart. Elevation of thyroid hormone and exercise increase α -MHC, in contrast, α -MHC mRNA and protein levels are decreased significantly in patients with end-stage heart failure (Nakao et al 1997; Miyata et al 2000), whereas thyroid depletion, aging, and pressure overload increase β -MHC (Swynghedauw et al 1986).

β -MHC expression is dominant during fetal life. In certain species, expression of the β -MHC isogene is characteristic of the fetal myocardium and the myocardium subjected to pressure overload. In myocardial hypertrophy produced by pressure overload, there is marked up-regulation of β -MHC iso-mRNA and isoprotein relative to α -MHC iso-mRNA and isoprotein (Izumo et al 1987; Litten et al 1982; Mercadier et al 1981; Nagai et al 1987). Studies have indicated that this change in isogene predominance can be accounted for by a preferential increase in β -MHC isogene expression, with no change in α -MHC (Nagai et al 1987; Waspe et al 1990). It has been shown that MHC isozyme transition induced by pressure overload is mainly regulated by pretranslational level (Nagai et al 1987, Imamura et al 1991, Bochler et al 1992) since not only did β -MHC mRNA level correspond closely to the relative β -MHC synthesis but also both were linearly correlated (Nagai et al 1987).

Waspe et al (Waspe et al 1990) studied the molecular signals responsible for selective induction of the β -MHC in pressure overload hypertrophy. They found that α_1 -

adrenergic receptor stimulation increases the cellular contents of β -MHC iso-mRNA and β -MHC isoprotein during hypertrophy of cultured neonatal rat cardiac myocytes, but does not change the levels of α -MHC iso-mRNA or isoprotein. Therefore, activation of the α_1 -adrenergic receptor is the first identified molecular signal for increased β -MHC isogene expression in a model of cardiac hypertrophy (Waspé et al 1990).

1.5 Myocardial Fibrosis in Cardiac Hypertrophy

Myocardial fibrosis is known to occur in left ventricular hypertrophy associated with hypertension (Olivetti et al 1994) and contributes critically to the overall risk linked to hypertrophy (Frohlich 1999). This fibrosis consists of an increase in the interstitial and perivascular content of fibrillar collagen type I and type III (Pardo-Mindán and Panizo 1993). The remodeling of the collagen networks is associated with increased myocardial stiffness (Doering et al 1988) and abnormalities of cardiac function in both animal models and humans (McLenachan and Dargie 1990). Indeed, it has been suggested that a major factor determining the progression of the left ventricular hypertrophy to failure is the presence of cardiac fibrosis (Weber et al 1995).

In pressure overload induced by aortic banding, LV collagen III mRNA levels were significantly increased in banded rats which were associated with LV hypertrophy, and they returned to control levels after LV hypertrophy was reverted by ramipril, an ACE inhibitor (Ogawa et al 1996). This result suggests that the presence of cardiac fibrosis is related to LV hypertrophy and an ACE inhibitor can revert the increased collagen

deposition. In volume overload model of DOCA-salt, collagen III gene expression was elevated in the LV and it was not affected by endothelin-1 type-A (ET_A) receptor blockade, indicating that endothelin-1 (ET-1) does not signal through the ET_A receptor to regulate collagen III production in this model (Bianciotti and de Bold 2000).

1.6 The Interaction between Nitric Oxide (NO) and Natriuretic Peptides (NP)

The role of NO in NP production and release is still controversial both in vitro and in vivo.

In vitro, Melo et al (Melo and Sonnenberg 1996) reported that secretion of ANF from the heart was decreased during perfusion with arginine vasopressin (AVP) and acetylcholine (ACh) and to a lesser degree with Ang II. N^G-monomethyl-L-arginine (L-NMMA), another inhibitor of NOS, can overcome the inhibitory effect of AVP and ACh, which indicates that NO participates in mediating this response. This was further supported by the fact that both the NO donor SNP and L-arginine produced changes in ANF secretion similar to ACh and AVP. These results indicate that AVP and ACh inhibit ANF secretion in the isolated heart indirectly by stimulating NO and suggest a novel function of NO as a negative modulator of ANF secretion (Melo and Sonnenberg 1996). Skvorak et al (Skvorak and Dietz 1997) demonstrated that, in the perfused atria, when NO production was stimulated with ACh or inhibited by L-NAME and hydroquinone, there was no significant change in the ANF secretion. This indicates that NO does not

affect basal ANF secretion. During stretch, when the concentration of ET is increased concurrently with NO inhibition, the ANF secretion increased significantly. Therefore, it appears that the relative levels of both NO and ET interact to regulate ANF secretion. Taken together, these findings suggest that increased ANF secretion requires both an elevation in local ET concentration and reduced NO activity, whereas decreased ANF secretion requires both a reduced concentration of ET and elevated NO activity. Furthermore, the results show that increased NO activity can rapidly terminate ET-stimulated ANF secretion. It has been reported that the infusion of inhibitors of NO, methylene blue, oxyhemoglobin, or hydroquinone increased the basal release of ANF by different mechanisms (Sanchez-Ferrer et al 1990). Methylene blue enters into cells, and its action is mediated by inhibition of guanylate cyclase (Martin et al 1985). Oxyhemoglobin, a large molecule that is confined to the extracellular space, binds and inactivates NO (Martin et al 1985). The action of hydroquinone is attributed to the formation of superoxide ions, which destroy NO (Moncada et al 1986). The fact that the three drugs increase the secretion of ANF, despite their different chemical nature and mechanism of action, suggests that this similar effect is mediated by their common characteristic: interference with NO (Sanchez-Ferrer et al 1990). In contrast, inhibition of NO production by L-NMMA in co-cultures of mesothelial and atrial cardiocytes was demonstrated not to affect ANF release (Lew and Baertschi 1992).

In vivo, Leskinen and colleagues (Leskinen et al 1995) reported a dose-dependent increase in plasma irANF levels by L-NAME in conscious rats. This increase in irANF concentration in response to L-NAME infusions showed a positive linear correlation with

the increase in mean arterial pressure. In addition, acute volume expansion with 0.9% saline in the presence of L-NAME resulted in a greater increase in plasma ANF levels than that in the control group. However, acute volume expansion and pressure overload, which caused increases in right atrial pressure and mean arterial pressure, did not stimulate BNP release from the heart, despite the fact that plasma ANF levels increased markedly (Leskinen et al 1995). These findings suggest that L-NAME increases the basal and volume load-induced release of ANF in conscious rats, indicating that the secretion of ANF in response to stretch may be modulated by the locally released NO from the endothelium. Further, the effect of NO synthase inhibition and volume loading on ANF and BNP release shows that the regulation of BNP release differs from that of ANF (Leskinen et al 1995). However, long-term administration of L-NAME to rats showed no change in plasma ANF levels (Arnal et al 1993). There was also no change in plasma concentration of ANF in conscious dogs by using L-NNA, the inhibitor of NOS (Elsner et al 1992). Therefore, further studies are necessary to determine the role of NO in NP production and secretion.

1.7 Objectives of Study

The objective of this study was to determine the effect of chronic pressure overload as obtained in L-NAME hypertension with or without cardiac hypertrophy on the expression of genes that are related to cardiac hypertrophy including natriuretic peptides (NP), myosin heavy chain (MHC), collagen III and their relationship to the renin-

angiotensin system (RAS).

1.8 Hypothesis

We hypothesized that cardiac fetal gene program would be activated only in those L-NAME treated animals in which hypertrophy develops together with activation of the renin-angiotensin system (RAS).

Materials and Methods

2.1 Experimental Design and Animal Preparation

48 male Sprague-Dawley rats weighting 180-200g were divided into four groups: (1) 4 week L-NAME group (n = 12), rats received L-NAME (Sigma Chemical Co) in tap water for 4 weeks at a concentration adjusted according to the body weight such that rats received 75mg/kg/day. (2) 4 week control group (n = 12), rats were given tap water. (3) 8 week L-NAME group (n = 12), rats received L-NAME in tap water for 8 weeks the same dose as above. (4) 8 week control group (n = 12), rats were given tap water. Systolic blood pressure was measured every two weeks in prewarmed, slightly restrained animals by the tail-cuff method. The average of three pressures was recorded. At the end of the treatment period, the rats were killed by decapitation. Blood was collected in ice-chilled tubes containing EDTA and Aprotinin for the measurement of plasma concentration of ANF, BNP, Ang I and Ang II. Blood was also collected into ice-chilled tubes with EDTA to measure plasma renin and ACE activities. Blood was immediately centrifuged at 3000 rpm for 15minutes at 4°C. After centrifugation, the plasma was stored at -80°C until used for RIA. After blood collection, the heart was excised, rapidly weighted, and dissected in cold saline into right and left atrium and right and left ventricles with their respective septa as part of the left chambers. After the tissue weight was measured, the tissue was quickly wrapped in aluminum foil and snap-frozen in liquid nitrogen.

2.2 Extraction of Plasma and Tissue Sample

Plasma samples were acidified by adding 100 μ l/mL of 1 mol/L HCl and passed through Sep-pak C₁₈ cartridges (Millipore). The cartridges were prewetted with 5 ml of 80% ACN in 0.1% TFA and 10 ml of 0.1% TFA. The cartridges with the absorbed peptides were washed with 20 ml of TFA and eluted with 3 ml of 80% ACN in 0.1% TFA. Tissue samples were homogenized in 10 vol of an extracting mixture consisting of 0.1 N HCl, 1.0 mol/L acetic acid, and 1 % NaCl and centrifuged at 10,000g for 30 minutes at 4°C. The supernatants were then extracted with the use of Sep-Pak C18 cartridges as described above for plasma. The elutes from tissue or plasma were freeze-dried and processed for RIA to measure ANF, BNP, Ang I and Ang II.

2.3 Radioimmunoassays

Plasma and cardiac tissue concentration of immunoreactive ANF and BNP were measured by RIA, as previously described (Sarda et al 1989). Antisera raised against rat ANF₉₉₋₁₂₆ and rat BNP₆₄₋₉₅ were obtained from Peninsula Laboratories. Cross-reactivity of ANF and BNP antisera with BNP and ANF peptides, respectively, was less than 0.01%. Plasma renin activity (PRA) was measured by determining the level of angiotensin I (Ang I) generated during 1 hour of incubation at 37°C in the presence of dimercaprol and 8-hydroxyquinoline (NENTM Life Science Products, Inc). Plasma and cardiac tissue Ang I and II concentration were measured using RIA. The standards and antisera of Ang I and

Ang II were purchased from Advanced ChemTech. Both ^{125}I -Ang I and ^{125}I -Ang II were from Peninsula Laboratories. The Ang I antisera showed <0.01% cross-reactivity with Ang II peptide, and the Ang II antisera showed 1.7% cross-reactivity with Ang I peptide.

2.4 Measurement of Plasma and Cardiac Tissue ACE activity

Plasma and left ventricular ACE activity were measured by the rate of generation of His-Leu from Hip-His-Leu substrate using the fluorometric assay described by Cushman and Cheung (Cushman and Cheung 1971) and Reneland and Lithell (Reneland and Lithell 1994). Left ventricular tissue was homogenized in Tris-buffer at 4°C (100 mg tissue: 1 ml Tris-buffer) using a polytron. Part of the homogenate was diluted 1:1 in Tris buffer. The substrate solution contained 12.5 mM Hip-His-Leu (Sigma Chemical Co) in Tris buffer. The incubation mixture consisted of 20 µl diluted tissue homogenate and water 80 µl or 10 µl plasma and 90 µl water. The reaction was started by adding 100 µl of substrate solution to the incubation mixture. Thus the final assay conditions in the 200 µl assay incubation mixture were 5 mM substrate (Hip-His-Leu), and 1 mg homogenized tissue, or 10 µl plasma. After incubation at 60 min for tissue or 10 min for plasma at 37°C the reaction was stopped by adding of 1.45 ml 1.28 M NaOH. The enzymatic product His-Leu was turned to a fluorescent product by adding 100 µl of 2% o-phthaldialdehyde (Sigma Chemical Co) in methanol and incubating for 10 min at room temperature followed by 200 µl of 3 M HCl and incubation at room temperature for 30 min. After centrifugation at 1500 x g for 10 min, the relative fluorescence intensity was measured

using a spectrofluorometer with an excitation wavelength of 365 nm and an emission wavelength of 485 nm. Plasma ACE activity was calculated as nmol/mL/min and tissue ACE activity was expressed as nmol/g/min according to the standard curve which was constructed from His-Leu (Sigma Chemical Co) containing 0 to 10 μ M His-Leu (0.5 μ M increment) in 200 μ l Tris buffer.

2.5 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA samples (2 μ g of total RNA) were reverse transcribed with Super Script II RNase H⁻ Reverse Transcriptase and oligo(dT)₁₂₋₁₈ primer with the use of a reverse transcription kit (GIBCO BRL). After RT, an aliquot of the cDNA product was used for PCR amplification with renin and β -actin primers (β -actin primer used as an internal control). The sequence of renin and β -actin primers is shown in Table 1. PCRs were carried out in a final volume of 50 μ L containing PCR buffer (10 mmol/L Tris-HCl and 50 mmol/L KCl, pH 8.3), 2 mmol/L MgCl₂, 1.25 units of Ampli Taq DNA Polymerase (Perkin-Elmer), and 0.15 μ mol/L of the respective primers. The reaction was heated at 95°C for 10 minutes and cycled 35 times through a 60 seconds denaturing step at 95°C, a 30 seconds annealing step at 62°C, and a 60 seconds extension step at 72°C. After the final cycle, a 7 minutes extension step at 72°C was included. Aliquots (μ L) of the PCR product were electrophoresed on a 2% agarose gel and were visualized by ethidium bromide staining. To confirm the specificity of PCR products, we performed restriction enzyme analysis using Mob I, Kpn I and Msp I (GIBCO BRL). We also confirmed the

renin mRNA sequence using Big Dye Terminator Cyclosequencing (Applied Biosystem) with the assistance of the Biotechnology Department at the University of Ottawa. To check that our PCR conditions were in the linear assay range, we prepared a standard curve by performing several dilutions of total RNA (a detailed description of this method has been previously published by Malhotra et al ,1999). The quantification of renin and β -actin mRNA levels from the agarose gel was done using the Quantity One software package (BIO RAD).

2.6 RNA Extraction and Northern Blot Analysis

Atrial and ventricular tissue samples from individual rats ($n = 6$, each chamber, each group) were extracted using Trizol (GIBCO BRL). Total RNA from the atria (10ug) and ventricles (20ug) were electrophoretically separated in an agarose-formaldehyde gel and transferred to the nylon membranes (Hybond N+, Amersham) overnight. Membranes were prehybridized in 2.5x Denhardt's solution, 5x SSC, 50% formamide, 25 mmol/L KH_2PO_4 , pH 6.4, 0.2 SDS, and 0.2 mg/mL herring sssDNA for 3 hours at 42°C for cDNA probes or prehybridized in 5x Denhardt's solution, 6x SSC, 50 mmol/L KH_2PO_4 , 0.5% SDS, and 0.2 mg/mL herring sssDNA for 3 hours at 5°C below the calculated T_m for oligonucleotide probes. Hybridization was then carried out for 16 hours at the same temperature as for prehybridization in the presence of the radiolabeled probes. Five cDNA probes and two oligonucleotide probes were used. The cDNA probes used were as follows: (1) a 900-bp *EcoR* I/*Hind* III fragment containing the full-length rat ANF cDNA

Table 1 Primer sequences used in PCR

Gene	Primer Sequences	PCR Product, bp
Renin Sense:	5' –CTGGGAGGCAGTGACCCTCAACATTACCAG– 3'	372
Antisense:	5' –GAGAGCCAGTATGCACAGGTCATCGTTCCT– 3'	
β-actin Sense:	5' –TACAACCTCCTTGCAGCTCC– 3'	630
Antisense:	5' –GGATCTTCATGAGGTAGTCTGTC– 3'	

PCR, polymerase chain reaction.

(Flynn et al 1985), (2) a 595-bp *Sal* I fragment containing full-length rat BNP cDNA (Bruneau and de Bold 1994), (3) a 5-kb *EcoR* I/*Sal* I fragment of the mouse 28S rRNA cDNA probe, (4) a 2-kb *Bam*H I/*Bgl* II fragment of the mouse phosphoglycerate kinase (PGK) gene cDNA (Mori et al 1986), and (5) rat α 1-III collagen cDNA containing 1300 bp of the 3' noncoding and coding regions (Glumoff et al 1994). The two oligonucleotide probes were 39 and 24 base fragments specific for unique regions in the 3' untranslated regions of the rat α -MHC and β -MHC genes (McNally et al 1989; Kraft et al 1989). The cDNAs were labeled with 5'-[α - 32 P] dCTP (3000 Ci/mmol; Amersham) using the Megaprime DNA labeling system (Amersham). The oligonucleitides were labeled with [γ - 32 P] ATP (3000 Ci/mmol, Amersham) using a 5'-end-labeling kit (Amersham). At the end of hybridization, the membranes were washed twice at 42°C with 2x SSC and 1% SDS and twice at 55°C with 1x SSC and 0.1% SDS for the cDNA probes or were washed once at 30°C with 5x SSC and 0.1% SDS twice at the same temperature as the hybridization with 1x SSC and 0.1%SDS for the oligonuclotide probes. Before additional probing, bound counts were completely stripped from the membranes by washing twice in 10 mmol/L sodium citrate, pH 6.8, 0.25% SDS for 10 minutes at 100°C. Autoradiographs were scanned with an Ultrascan XL laser densitometer (LKB Productter) and LKB 2400 Gelscan XL software package. The scanning values of ANF, BNP, collagen III, α -MHC and β -MHC mRNA were normalized to 28S rRNA or PGK mRNA as internal controls to correct for differences in the amount of RNA applied and transfer efficiency.

2.7 Statistical Analysis

Results of quantitative studies were expressed as mean $\pm$ SEM. Statistical comparisons between groups were performed by unpaired Student's *t* test. Correlations between the two parameters were determined by linear regression analysis. Statistical significance was accepted at a value of $P < 0.05$.

Results

3.1 Systolic Blood Pressure

L-NAME treatment induced a progressive rise in systolic blood pressure (SBP) (Fig 3). SBP was 140 ± 3 mmHg at the end of 4 weeks, and 173 ± 6 mmHg at the end of 8 weeks, which were significantly ($p < 0.001$) higher than SBP in control rats (105 ± 1 mmHg) and (106 ± 2 mmHg) respectively. These results indicated that administration of L-NAME increased SBP in a time-dependent manner.

3.2 Body and Heart Weight

Despite the increase in systolic blood pressure, the average LV weight to body weight ratio (LVW/BW) of rats given L-NAME were not statistically different from those of controls both in 4 and 8 week groups, though LVW/BW was higher (2.22 ± 0.08 mg/g) in 8 week L-NAME treatment than that in control (2.03 ± 0.04 mg/g). RV weight to body weight ratio (RVW/BW) was significantly decreased 0.50 ± 0.01 versus 0.55 ± 0.02 , $P < 0.01$ in 4 week rats, and 0.49 ± 0.01 versus 0.56 ± 0.01 , $P < 0.01$ in 8 week rats. LA weight to body weight ratio (LAW /BW) and RA weight to body weight ratio (RAW/BW) were not significantly different in either 4 or 8 week groups compared to their respective control groups.

Figure 3 Changes in systolic blood pressure in rats given tap water (Control, n = 12), or N^ω-nitro-L-arginine methyl ester (L-NAME 75 mg/kg/day, n = 12) in tap water at 4 and 8 weeks of treatment. Values are means $\pm$ SEM. ** P < 0.01, *** P < 0.001 vs. Control.

Fig 3

Fig 4 shows LVW/BW in 4 and 8 week L-NAME treatment. Fig 5 represents the relation between SBP and the LVW/BW in 8 week L-NAME treatment. LVW/BW correlated with SBP ($n = 12$, $r = 0.75$, $P < 0.05$). 5 of 12 of rats had an increased LVW/BW compared to controls (2.50 ± 0.10 versus 2.03 ± 0.04 , $P < 0.01$) and therefore that only 5 of 12 (42%) of rats developed LV hypertrophy. SBP was much higher in the rats with LV hypertrophy (192 ± 5 mmHg) than in those without (160 ± 6 mmHg, $P < 0.01$).

3.3 Plasma and Cardiac Tissue Renin-Angiotensin System

Plasma renin activity (PRA) was decreased in 4 week L-NAME rats (2.95 ± 0.54 versus 7.59 ± 0.74 , $P < 0.001$) and had a tendency to be increased in 8 week L-NAME rats compared to control, but the difference did not reach statistical significance (Fig 6). Plasma and LV Ang I and Ang II were not significantly different in L-NAME 4 and 8 week groups compared to control groups.

Fig 7 shows the relation between PRA and the LVW/BW in 8 week L-NAME rats. LVW/BW correlated with PRA ($n = 12$, $r = 0.66$, $P < 0.05$).

Figure 4 The ratio of left ventricular weight to body weight (LVW/BW) in rats given tap water (Control, n = 12), or N^ω-nitro-L-arginine methyl ester (L-NAME 75 mg/kg/day, n = 12) in tap water at 4 and 8 weeks of treatment.

Figure 5 Relationship between systolic blood pressure and LVW/BW in rats given tap water (Control, n = 12), or N^ω-nitro-L-arginine methyl ester (L-NAME 75 mg/kg/day, n = 12, r = 0.75, P < 0.05) in tap water at 8 week treatment.

Figure 6 Plasma renin activity (PRA) in rats given tap water (Control, n = 12), or N^ω-nitro-L-arginine methyl ester (L-NAME 75 mg/kg/day, n = 12) in tap water at 4 and 8 weeks of treatment. Values are mean $\pm$ SEM. * P < 0.001 vs. Control.**

Figure 7 Relationship between plasma renin activity and the LVW/BW in rats given tap water (Control, n = 12), or N^ω-nitro-L-arginine methyl ester (L-NAME 75 mg/kg/day, n = 12) in tap water at 8 week treatment. Correlation (r = 0.66, P < 0.05) between plasma renin activity and LVW/BW in L-NAME rats.

Fig 6

Fig 7

3.4 LV Renin Gene Expression

Ethidium bromide staining of renin and β -actin PCR products and their quantitative analysis are shown in Fig 8. LV renin mRNA level was not significantly different in 4 week treatment (0.29 ± 0.02 versus 0.34 ± 0.01) and it was increased in 8 week treatment (0.49 ± 0.08 versus 0.93 ± 0.31), but did not reach statistical significance. However, LV renin mRNA level correlated with the LVW/BW ($n = 6$, $r = 0.67$, $P < 0.05$) in 8 week L-NAME treatment (Fig 9).

3.5 Plasma and Cardiac Tissue ACE Activities

L-NAME 4 week treatment did not affect plasma and LV ACE activity. However, 8 week rats showed a significant increase in plasma ACE activity (8.33 ± 0.08 versus 7.43 ± 0.08 , $P < 0.001$) and LV ACE activity (13.48 ± 1.09 versus 8.32 ± 0.36 , $P < 0.01$) (Fig 10 and Fig 12). Plasma ACE activity was not associated with the LVW/BW (Fig 11), whereas, LV ACE activity correlated with the LVW/BW ($n = 6$, $r = 0.77$, $P < 0.05$) in 8 week L-NAME treatment (Fig 13).

Figure 8 Ethidium bromide-stained RT-PCR of LV products (left) from rats given tap water (Control, n = 6), or N^ω-nitro-L-arginine methyl ester (L-NAME 75 mg/kg/day, n = 6) in tap water at 4 and 8 weeks of treatment. 2 μg of total RNA was reverse transcribed, and the resultant cDNA was amplified with PCR using renin specific primer. Primer for β-actin used as an internal control. The specificity of PCR product was confirmed by restriction enzyme analysis. The relative expression of renin from LV (right) of control and L-NAME rats. Values are mean ± SEM.

Figure 9 Relationship between LV renin mRNA level and the LVW/BW in rats given tap water (Control, n = 6), or N^ω-nitro-L-arginine methyl ester (L-NAME 75 mg/kg/day, n = 6) in tap water at 8 week treatment. Correlation ($r = 0.67$, $P < 0.05$) between LV renin mRNA level and LVW/BW in L-NAME rats.

Renin Gene Expression in LV

Fig 8

Fig 9

Figure 10 Plasma angiotensin converting enzyme (ACE) activity in rats given tap water (Control, n = 12), or N^ω-nitro-L-arginine methyl ester (L-NAME 75 mg/kg/day, n = 12) in tap water at 4 and 8 weeks of treatment. Values are mean ± SEM. * P < 0.001 vs. Control.**

Figure 11 Relationship between plasma ACE activity and the ratio of LV weight to body weight (LVW/BW) in rats given tap water (Control, n = 12), or N^ω-nitro-L-arginine methyl ester (L-NAME 75 mg/kg/day, n = 12) in tap water at 8 week treatment.

Fig 10

Fig 11

Figure 12 Left ventricular ACE activity in rats given tap water (Control, n = 12), or N^ω-nitro-L-arginine methyl ester (L-NAME 75 mg/kg/day, n = 12) in tap water at 4 and 8 weeks of treatment. Values are mean $\pm$ SEM. ** P < 0.01 vs. Control.

Figure 13 Relationship between LV ACE activity and the ratio of LV weight to body weight (LVW/BW) in rats given tap water (Control, n = 6), or N^ω-nitro-L-arginine methyl ester (L-NAME 75 mg/kg/day, n = 6) in tap water at 8 week treatment. Correlation (r = 0.77, P < 0.05) between LV ACE activity and LVW/BW in L-NAME rats.

Fig 12

Fig 13

3.6 irANF and irBNP Cardiac Tissue and Plasma Concentration

In 4 week L-NAME treatment, LA irANF was decreased (115.0 ± 11.9 versus 182.5 ± 18.9 , $P < 0.05$). LV irBNP was increased (20.5 ± 1.7 versus 10.0 ± 0.6 , $P < 0.01$) (Fig 14). LA irBNP and LV irANF were comparable to controls. No significant changes in irANF and irBNP were observed in plasma and in the RA and RV. In 8 week treatment, levels of irANF and irBNP of L-NAME rats were similar to those of controls in plasma, atria and ventricles (Fig 15).

3.7 ANF and BNP Cardiac Gene Expression

Representative Northern blot of cardiac tissues (RA, LA, RV, and LV) with ANF, BNP, PGK, and 28S rRNA probes and their quantitation by densitometric scanning are shown in Fig 16, Fig 17, Fig 18, and Fig 19 respectively. Relative ANF mRNA level was significantly decreased both in RA (0.61 ± 0.08 versus 1.30 ± 0.25 , $P < 0.05$) and LA (0.71 ± 0.11 versus 1.43 ± 0.20 , $P < 0.01$) in 4 week L-NAME rats and no significant change in 8 week treatment, whereas, BNP mRNA level was comparable to controls in both the 4 and 8 week treatment groups. RV ANF and BNP mRNA were not significantly different in 4 and 8 week L-NAME groups compared to control groups. In the 8 week treatment group, ANF mRNA level correlated with LVW/BW ($n = 6$, $r = 0.64$, $P < 0.05$), (Fig 20 top), whereas, BNP mRNA was not significantly associated with LVW/BW (Fig 20 bottom).

Figure 14 Cardiac irANF and irBNP in rats given tap water (Control, n = 12), or N^ω-nitro-L-arginine methyl ester (L-NAME 75 mg/kg/day, n = 12) in tap water at 4 and 8 weeks of treatment. Values are mean ± SEM. * P < 0.05, ** P < 0.01 vs. Control.

Figure 15 Plasma irANF and irBNP in rats given tap water (Control, n = 12), or N^ω-nitro-L-arginine methyl ester (L-NAME 75 mg/kg/day, n = 12) in tap water at 4 and 8 weeks of treatment. Values are mean ± SEM.

Fig 14

Fig 15

Figure 16 Representative northern blot of ANF and BNP mRNA levels in the right atrium (top) in rats given tap water (Control, n = 6), or N^ω-nitro-L-arginine methyl ester (L-NAME 75 mg/kg/day, n = 6) in tap water at 4 and 8 weeks of treatment. 10 µg of total RNA was loaded in each lane and separated on a 1.2% agarose gel. For each group, a single lane of a single membrane used for hybridizations with ³²P-labeled probes is shown. Probes hybridized to single bands of the expected size for each mRNA: ANF (0.9 kb), BNP (0.9 kb), 28S (4.7 kb), and PGK (1.9 kb). 28S and PGK used as internal controls. Relative abundance of ANF and BNP mRNA levels in the right atrium (bottom) in control and L-NAME rats at 4 and 8 weeks of treatment. Values are mean ± SEM. * P < 0.05 vs. Control.

NP Gene Expression in RA

Fig 16

Figure 17 Representative northern blot of ANF and BNP mRNA levels in the left atrium (top) in rats given tap water (Control, n = 6), or N^ω-nitro-L-arginine methyl ester (L-NAME 75 mg/kg/day, n = 6) in tap water at 4 and 8 weeks of treatment. 10 µg of total RNA was loaded in each lane and separated on a 1.2% agarose gel. For each group, a single lane of a single membrane used for hybridizations with ³²P-labeled probes is shown. Probes hybridized to single bands of the expected size for each mRNA: ANF (0.9 kb), BNP (0.9 kb), 28S (4.7 kb), and PGK (1.9 kb). 28S and PGK used as internal controls. Relative abundance of ANF and BNP mRNA levels in the left atrium (bottom) in control and L-NAME rats at 4 and 8 weeks of treatment. Values are mean ± SEM. * P < 0.05 vs. Control.

NP Gene Expression in LA

Fig 17

Figure 18 Representative northern blot of ANF and BNP mRNA levels in the right ventricle (top) in rats given tap water (Control, n = 6), or N^ω-nitro-L-arginine methyl ester (L-NAME 75 mg/kg/day, n = 6) in tap water at 4 and 8 weeks of treatment. 20 µg of total RNA was loaded in each lane and separated on a 1.2% agarose gel. For each group, a single lane of a single membrane used for hybridizations with ³²P-labeled probes is shown. Probes hybridized to single bands of the expected size for each mRNA: ANF (0.9 kb), BNP (0.9 kb), and PGK (1.9 kb). PGK used as an internal control. Relative abundance of ANF and BNP mRNA levels in the right ventricle (bottom) in control and L-NAME rats at 4 and 8 weeks of treatment. Values are mean ± SEM.

NP Gene Expression in RV

Fig 18

Figure 19 Representative northern blot of ANF and BNP mRNA levels in the left ventricle (top) in rats given tap water (Control, n = 6), or N^ω-nitro-L-arginine methyl ester (L-NAME 75 mg/kg/day, n = 6) in tap water at 4 and 8 weeks of treatment. 20 µg of total RNA was loaded in each lane and separated on a 1.2% agarose gel. For each group, a single lane of a single membrane used for hybridizations with ³²P-labeled probes is shown. Probes hybridized to single bands of the expected size for each mRNA: ANF (0.9 kb), BNP (0.9 kb), and PGK (1.9 kb). PGK used as an internal control. Relative abundance of ANF and BNP mRNA levels in the left ventricle (bottom) in control and L-NAME rats at 4 and 8 weeks of treatment. Values are mean ± SEM.

NP Gene Expression in LV

Fig 19

Figure 20 Relationship between relative abundance of LV ANF mRNA level and the ratio of LV weight to body weight (LVW/BW) (top) in rats given tap water (Control, n = 6), or N^ω-nitro-L-arginine methyl ester (L-NAME 75 mg/kg/day, n = 6, $r = 0.64$, $P < 0.05$) in tap water at 8 week treatment. Relationship between relative abundance of LV BNP mRNA level and the ratio of LV weight to body weight (LVW/BW) (bottom) in rats given tap water (Control, n = 6), or N^ω-nitro-L-arginine methyl ester (L-NAME 75 mg/kg/day, n = 6, $r = 0.48$, $P = 0.08$) in tap water at 8 week treatment.

Fig 20

3.8 α -MHC and β -MHC Cardiac Gene Expression

Representative Northern blot of cardiac tissues (RV and LV) using rat α -MHC and β -MHC oligonucleotide probes and their quantitative analysis are shown in Fig 17 and Fig 18. LV α -MHC mRNA was significantly elevated (3.89 ± 0.24 versus 2.75 ± 0.19 , $P < 0.05$) in 4 week L-NAME rats and no change in 8 week rats. RV α - and β -MHC mRNA levels were comparable to controls both in 4 and 8 week treatment. LV β -MHC mRNA was significantly increased both in 4 week (0.66 ± 0.10 versus 0.18 ± 0.03 , $P < 0.01$) and 8 week (0.91 ± 0.07 versus 0.25 ± 0.05 , $P < 0.001$) L-NAME rats. The ratio of LV β -MHC to α -MHC mRNA (β -MHC/ α -MHC mRNA) was also significantly increased in 4 week (0.17 ± 0.02 versus 0.07 ± 0.01 , $P < 0.01$) and 8 week (0.38 ± 0.05 versus 0.12 ± 0.03 , $P < 0.01$).

3.9 Collagen III Cardiac Gene Expression

Northern blot analysis of collagen III in RV and LV and their quantitative evaluation are shown in Fig 19. RV and LV collagen III mRNA levels did not change in 4 and 8 week L-NAME treatment. LV collagen III mRNA did not correlate with the LVW/BW ($n = 6$, $r = 0.34$, $P = 0.22$) in 8 week L-NAME treatment.

Figure 21 Representative northern blot of α -MHC and β -MHC mRNAs in the right ventricle (top) in rats given tap water (Control, n = 5 to 6), or N^o-nitro-L-arginine methyl ester (L-NAME 75 mg/kg/day, n = 5 to 6) in tap water at 4 and 8 weeks of treatment. 20 μ g of total RNA was loaded in each lane and separated on a 1.2% agarose gel. For each group, a single lane of a single membrane used for hybridizations with ³²P-labeled probes is shown. Probes hybridized to single bands of the expected size for each mRNA: α -MHC and β -MHC (7.2 kb), and PGK (1.9 kb). PGK used as an internal control. Relative abundance of α -MHC and β -MHC mRNA levels in the right ventricle (bottom) in control and L-NAME rats at 4 and 8 weeks of treatment. Values are mean $\pm$ SEM.

MHC Gene Expression in RV

Fig 21

Figure 22 Representative northern blot of α -MHC and β -MHC mRNAs in the left ventricle (top) in rats given tap water (Control, n = 6), or N^ω-nitro-L-arginine methyl ester (L-NAME 75 mg/kg/day, n = 6) in tap water at 4 and 8 weeks of treatment. 20 μ g of total RNA was loaded in each lane and separated on a 1.2% agarose gel. For each group, a single lane of a single membrane used for hybridizations with ³²P-labeled probes is shown. Probes hybridized to single bands of the expected size for each mRNA: α -MHC and β -MHC (7.2 kb), and PGK (1.9 kb). PGK used as an internal control. Relative abundance of α -MHC and β -MHC mRNA levels in the left ventricle (bottom) in control and L-NAME rats at 4 and 8 weeks of treatment. Values are mean $\pm$ SEM. ** P < 0.01, *** P < 0.001 vs. Control.

MHC Gene Expression in LV

Fig 22

Figure 23 Representative northern blot of collagen III mRNAs in the right and left ventricle (top) in rats given tap water (Control, n = 5 to 6), or N^ω-nitro-L-arginine methyl ester (L-NAME 75 mg/kg/day, n = 5 to 6) in tap water at 4 and 8 weeks of treatment. 20 µg of total RNA was loaded in each lane and separated on a 1.2% agarose gel. For each group, a single lane of a single membrane used for hybridizations with ³²P-labeled probes is shown. Probes hybridized to single bands of the expected size for each mRNA: collagen III (5.3 kb) and PGK (1.9 kb). PGK used as an internal control. Relative abundance of collagen III mRNA levels in the right and left ventricle (bottom) in control and L-NAME rats at 4 and 8 weeks of treatment. Values are mean ± SEM.

Collagen III Gene Expression in RV

Collagen III Gene Expression in LV

Fig 23

Discussion

NO released from L-arginine by endothelial cells activates vascular smooth muscle soluble guanylate cyclase, resulting in cGMP generation and vascular relaxation (Ignarro 1990; Furchgott and Zawadzki 1980; Palmer et al 1987). Long-term oral administration of L-NAME, an inhibitor of NO synthase, is an experimental model producing hypertension (Johnson and Freeman 1992; Ward and Angus 1993; Manning et al 1993; Hu et al 1994; Ribeiro et al 1992; Deng et al 1993; Li and Schiffrin 1994). The present study confirms that chronic administration of L-NAME (75 mg/kg/day) induces progressive systemic arterial hypertension in Sprague-Dawley rats in vivo. Arterial hypertension induced by L-NAME is due to the actions of this compound at several levels including: (1) Decreased NO synthesis. Long-term administration of L-NAME causes a marked decrease in the arterial wall content of cGMP (the second messenger of NO) (Arnal et al 1992; Baylis et al 1992) and a decrease in urinary secretion of the stable products of NO (NO_2^- and NO_3^-). Moreover, L-NAME-induced hypertension is reversed or prevented by short-term administration of excess L-arginine but not D-arginine (Hu et al 1994). (2) Activation of sympathetic nerves. Chronic sympathectomy greatly attenuates the development of L-NAME induced hypertension in the rat and has no discernible effect on endothelium-dependent vasodilation (Sander et al 1995). (3) Activation of renin-angiotensin system. Several studies have shown that the long-term increase in blood pressure and cardiac hypertrophy caused by the inhibition of NOS can be prevented or reversed by treatments with angiotensin converting enzyme (ACE) inhibitors (Morton et al 1993; Hropot et al

1994; Li et al 1996; Pechanova et al 1997; Takemoto et al 1997; Akucawa et al 1998; Bernatova et al 2000) and angiotensin II antagonists (Polloch et al 1993; Jover et al 1993), suggesting that the renin-angiotensin system is involved in the pathophysiology of L-NAME hypertension. (4) Effect on vascular resistance. L-NAME treatment has been shown to induce increases in the renal, mesenteric, and hindquarter vascular beds (Gardiner et al 1990), and in total peripheral resistance (Elsne et al 1992), suggesting that hypertension associated with NO synthesis blockade is a vasoconstrictor type of hypertension.

4.1 Left Ventricular (LV) Hypertrophy Induced by Chronic Inhibition of NO Synthesis

Arterial blood pressure plays an important role in LV hypertrophy in most rat models of experimental hypertension. LV hypertrophy is thus considered to be a basic and universal adaptive response to hemodynamic overload. However, LV hypertrophy also represents the most powerful risk factor for cardiovascular morbidity and mortality (Koren et al 1991). In the present study, we found that LV hypertrophy did not develop after 4 week L-NAME treatment and 42% of the rats (5 of 12) developed LV hypertrophy after 8 week L-NAME treatment. This is consistent with previous studies which showed that a relative increase in LV mass ranging from 9% to 30% in chronic L-NAME administration (Arnal et al 1993; Moreno et al 1996; Takemoto et al 1997; Pechanova et al 1997; Sladek et al 1996). Systolic blood pressure was much higher in the rats with LV hypertrophy (192

mmHg) than in those without hypertrophy (160 mmHg). Fig 5 shows that the systolic blood pressure in rats with LV hypertrophy was more than 170 mmHg, suggesting that higher pressure overload is required to produce LV hypertrophy in L-NAME rats as compared to other types of hypertension. Taken collectively, our and previous studies demonstrate that changes in LV mass are absent or modest in L-NAME treatment even in the face of severe sustained systemic arterial hypertension.

Several mechanisms are likely to be involved in blunted hypertrophic response. Firstly, in addition to NO inhibition, L-NAME may have growth-inhibitory properties independent of the effects on NO generation (Schiffrin 1995; Li et al 1996). Secondly, in contrast to the expected inhibitory effects of L-NAME on tissue cGMP content (Baylis et al 1992; Arnal et al 1992; Arnal et al 1993; Delacretex et al 1994), LV cGMP content in response to chronic L-NAME treatment remains unchanged (Arnal et al 1992). Thirdly, the absence of enhancement of endothelin-1, a hypertrophic agent, in small arteries in normal Sprague-Dawley rats treated with L-NAME (Sventek et al 1997) and SHR treated with L-NAME (Li et al 1996) may account for the absence of increased severity of hypertrophy of small vessels in these rats and may be a mechanism whereby L-NAME inhibits cardiovascular growth (Li et al 1996).

4.2 Changes of MHC Gene Expression in L-NAME Hypertension

The basic property of the heart is its ability to adapt to altered hemodynamic loads (Bugaisky et al 1992). On a short-term basis, the heart can cope with an enhanced load by

increasing its ability to develop pressure via Frank-Starling mechanisms. However, when the overload is repeated or long lasting, a second mechanism becomes activated, leading to an altered expression of cardiac genes. The basis for phenotypic adaptation lies in the ability of a cell to increase its protein turnover.

The phenotypic transition in the heart that has been most extensively studied is that of myosin heavy chain (MHC). Changes in the relative amount of α - and β -MHC are believed to be responsible in part for altered cardiac performance during hemodynamic overload and cardiac hypertrophy. Studies have shown that a high proportion of α -MHC is associated with a faster, but less economical force development, whereas, a high proportion of β -MHC is associated with relative slow, economical tension development during isometric contraction (Alpert et al 1982; Holubarsch et al 1985). In the present study, we found that LV β -MHC mRNA and the LV β -MHC/ α -MHC mRNA were significantly increased in both 4 week L-NAME rats (hypertension without hypertrophy) and 8 week L-NAME rats (hypertension with or without hypertrophy), suggesting that pressure overload alone is a stimulator of β -MHC gene expression. This result is consistent with de Bold group's finding (Ogawa et al 1996) that in the suprarenal aortic banding (pressure overload) rats, the increased ventricular β -MHC gene expression did not respond to a low-dose ramipril, a treatment that reverses LV hypertrophy but not blood pressure. These findings suggest that pressure overload plays a major role in inducing β -MHC mRNA and pressure overload exposed on LV wall may directly induce β -MHC gene expression before cardiac hypertrophy occurs. Imamura et al (Imamura et al 1991) further demonstrated that in the pressure overload produced by aortic coarctation,

the first adaptational response of the heart is the MHC isoform transition; cardiac hypertrophy is a later response to hemodynamic overload. We also found that enhanced β -MHC mRNA response to increased hemodynamic overload was not associated with any change of α -MHC gene expression. This finding is consistent with the observation of Nagai et al (Nagai et al 1987) who observed that change in isogene predominance could be accounted for a preferential increase in β -MHC isogene expression, with no change in α -MHC.

In the present experiments, β -MHC mRNA did not change in the RV, demonstrating that the change in β -MHC gene expression is limited to the involved ventricle in terms of pressure overload. This finding is supported by the observation that in aortic coarctated and pulmonary artery banded rats, the significant inductions of the β -MHC mRNA and corresponding protein are limited to the involved and overloaded ventricles (Imamura et al 1990). In summary, the present study shows that increased β -MHC gene expression is a sensitive marker of pressure overload even in the absence of cardiac hypertrophy.

4.3 Renin-Angiotensin System in L-NAME Hypertension

Previous studies have demonstrated that the RAS is strongly involved in the pathophysiology of L-NAME hypertension (Morton et al 1993; Polloch et al 1993; Jover et al 1993; Hropot et al 1994; Li et al 1996; Pechanova et al 1997; Takemoto et al 1997; Akucawa et al 1998; Bernatova et al 2000). In the present study, we observed the PRA

was significantly decreased in 4 week L-NAME rats. This decreased renin secretion could be attributed either to an increase in renal perfusion pressure or to the suppression of the potential direct stimulation of renin release by NO (Gardes et al 1992; Scholz et al 1993). We also found that PRA and LV renin expression significantly correlated with the relative heart weight (LVW/BW) in the 8 week L-NAME treatment. These results are consistent with previous reports (Arnal et al 1993; Xu et al 1995), suggesting that the RAS plays a significant role in the development of cardiac hypertrophy in this model. This rise in PRA is probably related to the development of the nephroangiosclerosis in the kidney (Baylis et al 1992; Xu et al 1995). Hropot and colleagues (Hropot et al 1994) found that chronic treatment with L-NAME over 6 weeks decreased glomerular filtration rate (GFR) and renal plasma flow which was accompanied by a moderate increase in urine and protein excretion, and by a significant decrease in potassium excretion. Baylis et al (Baylis et al 1992) also demonstrated pronounced proteinuria during the chronic blockade of NO synthesis.

In the present study, we found that L-NAME 4 week treatment did not affect plasma or LV ACE activity. However, plasma and LV ACE activity were increased in 8 week treatment. In addition, LV ACE activity was found to significantly correlate with the relative heart weight in 8 week L-NAME treated rats. These findings agree with previous reports by Arnal et al (Arnal et al 1992) who showed that rats that did not develop cardiac hypertrophy after the fourth week of L-NAME administration did not show increased cardiac ACE activity. While a subset of rats that developed cardiac hypertrophy had increased cardiac ACE activity (Arnal et al 1993). Takemoto et al (Takemoto et al 1997)

reported that cardiac hypertrophy occurred by the eighth week of L-NAME treatment and an increase in tissue ACE activity were found as early as the first week. Thus, activation of tissue ACE preceded the development of myocardial structural changes. They further found that this structural change was markedly reduced by the administration of ACE inhibitor but not of hydralazine.

These above results strongly suggest that the activation of plasma and cardiac ACE contribute to cardiac hypertrophy in the L-NAME-induced hypertension model. However, the mechanisms by which ACE is activated after long-term administration of L-NAME are not evident from the present study. It is possible that the activation of ACE would increase the synthesis of angiotensin II, which in turn would directly induce vascular smooth muscle proliferation (Itoh et al 1993) and myocyte hypertrophy (Weinberg et al 1994; Schunkert et al 1990), and would also induce the release of hypertrophic substances such as platelet-derived growth factor (Naftilan et al 1989) and transforming growth factor- β (Gibbons et al 1992). In our study, plasma and LV Ang II were not significantly different in L-NAME 4 and 8 week groups. There was also no significant correlation between plasma Ang II or LV Ang II with LV hypertrophy. However, numerous studies have demonstrated that hypertension and cardiac hypertrophy caused by long term inhibition of NOS synthesis can be prevented or reversed by treatments with angiotensin converting enzyme (ACE) inhibitors (Morton et al 1993; Hropot et al 1994; Li et al 1996; Pechanova et al 1997; Takemoto et al 1997; Akucawa et al 1998; Bernatova et al 2000). It is possible that the mechanisms by which the ACE inhibitor exerts its beneficial effects on cardiovascular remodeling in this model may be

due to the effect of ACE inhibitors on the inhibition of hydrolysis of kinin, which is a potent vasodilator and acts in part via release of NO (Farhy et al 1993; DeBlois et al 1992). In a study of neointima formation after balloon injury to the intima of rat arteries, Farhy et al (Farhy et al 1993) found that two different ACE inhibitors, ramipril and enalapril, prevent neointima formation and the kinin antagonist Hoe 140 reduced the inhibitory effect of ramipril and enalapril by 73% and 62%, respectively. Losartan, the Ang II receptor antagonist, also inhibited neointima formation but was less effective than ACE inhibitors. It was also found that inhibition of neointima formation by ramipril was blocked by the NO synthesis inhibitor L-NAME, indicating that the effect of ACE inhibitors on neointima formation is mediated in part by inhibition of kinin degradation and the effect of kinin may be due to stimulation of NO synthesis. Taken together, these findings indicate that the protective effect of ACE inhibitors is due to both blockade of Ang II formation and kinin degradation. Therefore, the increased plasma and LV ACE activities may play a crucial role in cardiac hypertrophy via increased kinin breakdown.

4.4 Natriuretic Peptide Gene Expression in L-NAME Hypertension

It is known that the adaptive changes in myocardial function that occur after the initiation of hemodynamic stress are associated with alterations in most cases by the re-expression of β -MHC, skeletal α -actin, ANF, and BNP. These processes are regarded as essential adaptation to chronically hemodynamic overload.

In the present study, we found in L-NAME-induced hypertension which is

considered to be a model of vasoconstrictor type of hypertension, the ANF mRNA level in RA and LA was significantly decreased in L-NAME 4 week rats and BNP mRNA levels were comparable to controls in both atria. This is in agreement with a report on abdominal aortic constriction rats, in which the atrial irANF was decreased and the level of ANF mRNA in atria remained unchanged during coarctation (Gu et al 1989). Atrial ANF mRNA levels were found to be similar in SHR compared to those of WKY (Arai et al 1988). These findings and previous studies suggest that the ability of stimulating NP gene expression in the atria in vivo in response to chronic pressure overload is limited and pressure overload in rats may be accompanied by a lower ability of the atria to synthesize NP in response to increased blood pressure. Furthermore, the decreased ANF mRNA level suggests that L-NAME may inhibit ANF gene expression in both atria in the early stage of hypertension without hypertrophy and do not affect BNP gene expression.

Increased plasma and LV irANF and irBNP were found in volume overload hypertension induced by DOCA-salt (Yokota et al 1995; Bianciotti and de Bold 2000), and in pressure overload hypertension produced by aortic banding (Ogawa et al 1996). In L-NAME hypertension, we observed that plasma and LV irANF and irBNP were unchanged compared to controls after 8 week treatment, which are consistent with previous studies in which long-term L-NAME treatment to rats showed no change in plasma irANF levels (Arnal et al 1993), suggesting that NO does not play an important role in NP release.

In the present work, we observed that a 4 week L-NAME treatment (hypertension without hypertrophy) had no effect on ANF and BNP gene expression. However, LV

ANF gene expression significantly correlated with the relative heart weight in 8 week L-NAME treated rats. Our results are consistent with previous studies in which cardiac hypertrophy was associated with increased ANF and BNP gene expression in volume overload (Yokota et al 1995; Bianciotti and de Bold 2000) and pressure overload (Day et al 1987; Ogawa et al 1996; Kaiser et al 1998). Furthermore, Masciotra et al (Masciotra et al 1999) found that low ventricular ANF gene expression is linked genetically to increased cardiac mass in genetic WKHA rats independent of blood pressure. Altogether, these findings indicate that increased LV ANF gene expression in long-term L-NAME treatment is related to cardiac hypertrophy and not related to pressure overload, suggesting that ANF gene expression is a specific marker of cardiac hypertrophy.

Ogawa and coworkers (Ogawa et al 1996) found that in aortic banded rats, low-dose of ACE inhibitor, ramipril, decreased cardiac hypertrophy without affecting blood pressure. They further observed that a low-dose of ramipril reversed or prevented hypertrophy that resulted in decreased gene expression of ANF and BNP, but did not completely reverse the increase of gene expression of NP in the left ventricle, which suggested hypertension can maintain high level of NP expression independent from hypertrophy (Ogawa et al 1996). The discrepancy between the present study and this previous study might be due to the difference of the level of endothelin-1 (ET-1) gene expression between the different models of hypertension. ET-1 has been shown to induce ANF and BNP gene expression in cultured ventricular cardiocytes (Shubeita et al 1990; McDonough et al 1993; Nakagawa et al 1995). Plasma ET-1 concentration and ET-1 mRNA level in LV were increased after aortic banding (Ito et al 1994). In contrast,

vascular ET-1 gene expression was not enhanced either in normal Sprague-Dawley rats treated with L-NAME (Sventek et al 1997) or SHR treated with L-NAME (Li et al 1996), suggesting that endothelin system does not make an important contribution leading to elevated blood pressure and increased NP expression after chronic L-NAME treatment.

The myocardial fibrillar collagens such as collagen type III and I, are major components of the cardiac extracellular matrix (Boluyt et al 1994). Increases in fibrillar collagen in the interstitium contribute to tissue stiffness and thus increases in collagen gene expression likely are related to impaired cardiac function (Boluyt and Bing 2000). In the present study, we found that 4 and 8 week L-NAME treatment did not affect collagen III mRNA level and there was no significant correlation between collagen III expression and the relative heart weight in 8 week L-NAME treated rats, suggesting that cardiac hypertrophy induced by L-NAME probably did not associated with the increase of cardiac extracellular matrix.

In summary, we found that: (1) 4 week L-NAME treatment is associated with hypertension without hypertrophy. 8 week L-NAME treatment leads to severe hypertension but only 42% of rats develop anatomical cardiac hypertrophy. (2) LV ANF mRNA level was found to correlate with the relative heart weight (LVW/BW) in 8 week L-NAME treated rats. (3) 8 week L-NAME treatment does not affect irANF and irBNP level in plasma or ventricles. (4) PRA, LV renin gene expression and LV ACE activity correlated with the relative heart weight (LVW/BW) in 8 week L-NAME treatment. (5) β -MHC mRNA was significantly increased in 4 and 8 week L-NAME treatment.

Taken together, this research work shows that L-NAME hypertension can not be

described as a hypertension model without hypertrophy because it is recognized that a subset of experimental animals with the highest hemodynamic overload do develop hypertrophy. When this occurs, a re-induction of the cardiac fetal gene program is observed as in the case in other cardiac hypertrophies, including those clinically observed in humans. Pressure overload per se does not produce this re-induction and its best marker is the upregulation of β -MHC. Pressure overload likely requires neurohumoral activation, including the activation of RAS and perhaps of other system not addressed in this work.

4.5 Conclusions

In the present study, using the L-NAME hypertension model, a severe form of hypertension without hypertrophy, we have shown that: (1) LV ANF gene expression in long-term L-NAME treatment is related to cardiac hypertrophy and not related to pressure overload, suggesting that ANF gene expression is a specific marker of cardiac hypertrophy. (2) Long-term L-NAME treatment does not affect plasma or cardiac tissue irNP levels, suggesting that NO does not play an important role in NP production and release in vivo. (3) Increased β -MHC is a sensitive marker of pressure overload even in the absence of cardiac hypertrophy. (4) Cardiac hypertrophy correlated with PRA, LV renin expression and LV ACE activity, suggesting that the RAS components play a role in the development of hypertrophy in long-term L-NAME hypertension.

4.6 Future Directions

Since L-NAME reduces the hypertrophic response, it may be advantageous to determine if L-NAME analogues can be found to reduce the cardiac hypertrophic response without inducing hypertension.

Since this and other investigations (ACE inhibition and ET receptor blockade) performed in our laboratory showed that ventricular but not atrial NP gene expression depends on endocrine/paracrine mechanisms, the future of the endocrine heart should be investigated in mechanical terms (stretch-transcription coupling).

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