VENOUS CONTRACTION TO ENDOTHELIN-1
IN CONGESTIVE HEART FAILURE

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IN CONGESTIVE HEART FAILURE

This dissertation is submitted by Vikram Reddy and has been examined and approved by an appointed committee of the faculty of the School of Graduate Studies of the Medical College of Georgia.

The signatures which appear below verify the fact that all required changes have been incorporated and that the dissertation has received final approval with reference to content, form and accuracy of presentation.

This dissertation is therefore in partial fulfillment of the requirements for the degree Doctor of Philosophy.

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Endothelin-1 (ET-1) is produced by endothelial cells and can stimulate either the ET\textsubscript{A} or the ET\textsubscript{B} receptors. The role of ET-1 and the identity of the endothelin receptors involved in mediating tone in the mesenteric small veins of the Golden Syrian hamster are not known. ET-1 induces venoconstriction, thereby increasing the preload to the heart in congestive heart failure. However, mechanisms mediating contraction to ET-1 in the mesenteric small veins of the cardiomyopathic hamsters in the early and late stages of CHF are not known. Therefore, mechanisms mediating ET-1 induced contraction were determined in the mesenteric small veins of the Golden Syrian and cardiomyopathic hamsters in the early and late stages of CHF.

Baseline intraluminal diameter of small veins was measured before and after treatment with either ET\textsubscript{A} or ET\textsubscript{B} receptor antagonists. ET-1 induced contraction was higher in the early stage of CHF, while it was decreased in the late stage of CHF. Blockade of the ET\textsubscript{A} receptor decreased ET-1 induced contraction in the mesenteric small veins from the control and cardiomyopathic hamsters in both the early and late stage of CHF. ET\textsubscript{B} receptor blockade decreased the ET-1 induced contraction in the control and cardiomyopathic hamsters in the early, but not late, stage of CHF. Therefore, ET-1 induced contraction in the mesenteric small veins is mediated by the ET\textsubscript{A} receptors alone in the late stage of CHF, while both the ET\textsubscript{A} and ET\textsubscript{B} receptors mediate vasoconstriction in the controls and in the early stage of CHF.
Stimulation of ET-1 receptors is associated with an increase in calcium levels within the vascular smooth muscle cells. It is not known whether the increase in reactivity to ET-1 in the early stage of CHF or the decrease in reactivity to ET-1 in the late stage of CHF is due to problems with mobilization of the intracellular calcium levels within the vascular smooth muscle cell. Following ET-1, calcium levels within the vascular smooth muscle cell were increased to a larger extent in the early stage of CHF, than in the late stage of CHF, in agreement with the vascular reactivity data. Calcium levels were also measured before and after treatment with either ET_A or ET_B receptor antagonists. Blockade of the ET_A receptor inhibited the ET-1 induced increase in calcium levels in the mesenteric small veins from the control and cardiomyopathic hamsters in both the early and late stage of CHF. However, ET_B receptor blockade inhibited the ET-1 induced increase in calcium levels in only the control and cardiomyopathic hamsters in the early stage of CHF. These results indicate the absence of a functional responses mediated by the ET_B receptor in the late stage of CHF.

Studies have shown that NO can modulate the contraction to ET-1 in the vasculature. Baseline intraluminal diameter of small veins were measured before and after treatment with N-nitro-L-arginine (LNA), a specific inhibitor of nitric oxide synthase. LNA decreased the contraction to ET-1 in the early stage of CHF, but increased contraction to ET-1 in the late stage of CHF. This indicates that NOS mediates a vasodilatory effect that counteracts contraction to ET-1 in the late stage, but contributes to the vasoconstrictor effect of ET-1 in the late stage of CHF.

NOS activity was measured to identify the NOS isoforms contributing to the modulation of ET-1 induced vascular reactivity. Total NOS activity was significantly
increased in the cytosolic fraction of small veins from hamsters in the late stage of CHF and in the particulate fraction in hamsters in the early stage of CHF. In the late stage, the increase in NOS activity was inhibitable by 1400W, an iNOS selective inhibitor, suggesting that an increase in iNOS decreases the contraction to ET-1.

In summary, in the early stage of CHF, there is an increase in the vascular reactivity to ET-1 associated with an increase in intracellular calcium levels and partially mediated by NOS. This may increase preload and impair myocardial function in CHF.

There is an absence of ET\textsubscript{B} receptor-mediated responses in the late stage of CHF, associated with very high plasma ET-1 levels and impaired intracellular calcium signaling. NOS activity is significantly enhanced in the mesenteric small veins from the cardiomyopathic hamsters in the late phase of CHF, and this increase in NOS activity is at least partially dependent on iNOS and may contribute to impaired venous contraction to ET-1 in cardiomyopathic hamsters. This may serve as a compensatory mechanism to decrease the preload to the failing heart.

INDEX WORDS: Congestive Heart Failure, Endothelin, Veins, Nitric Oxide Synthase
DEDICATION

This work is wholeheartedly dedicated to those selfless hamsters who sacrificed themselves for science.
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LIST OF ABBREVIATIONS

C Control hamsters
CHF Congestive Heart Failure
ET Endothelin
ET-1 Endothelin-1
ET\textsubscript{A} Endothelin A receptor
ET\textsubscript{B} Endothelin B receptor
iNOS inducible nitric oxide synthase
NO Nitric oxide
NOS Nitric oxide synthase
LNA Nitro-L-arginine
M\textsubscript{1} and M\textsubscript{2} Cardiomyopathic hamsters
I. INTRODUCTION

A. Statement of the Problem

Congestive heart failure is characterized by impaired myocardial contractility and reduced cardiac output. Increased ventricular preload, which can occur as a result of loss of venous compliance and increased venous tone, further impairs cardiac performance in congestive heart failure. Endothelin-1 is a potent venous vasoconstrictor capable of increasing preload. However, little is known about the role of ET-1 in regulation of venous tone in congestive heart failure. This problem was addressed utilizing a hamster model of cardiomyopathy that exhibits overt congestive heart failure.

The cardiomyopathic hamster was studied in both the early stage and late stage of congestive heart failure. In humans, early stage congestive heart failure is a compensatory stage characterized by myocardial hypertrophy and/or dilation with compensatory mechanisms maintaining myocardial function at a level at which patients maintain normal physical activity. The cardiomyopathic hamster exhibits a similar stage in which myocardial hypertrophy is present but hamsters remain physically active. Late stage congestive heart failure in humans is a decompensated stage in which myocardial function is depressed and patients exhibit marked limitation of physical activity often associated with severe edema. The cardiomyopathic hamster also exhibits similar traits of severe edema and reduced physical activity in the late stage of congestive heart failure.
Studies in humans with congestive heart failure found increased plasma endothelin-1 levels and beneficial effects of endothelin receptor antagonists suggesting an upregulation of the endothelin system in congestive heart failure. Another interesting finding in humans with congestive heart failure is that the inducible nitric oxide synthase (iNOS) may be expressed in late stages. If iNOS was present in late stage congestive heart failure, it would be capable of producing long-lasting generation of nitric oxide (NO), a vasodilator substance that can inhibit contraction to ET-1. The present series of studies utilized the cardiomyopathic hamster to determine mechanisms mediating venous contraction to ET-1 in the early and late stages of congestive heart failure. It was hypothesized that venous contraction to ET-1 is enhanced in the early stage and impaired in the late stage of congestive heart failure. The mechanisms mediating changes in venous contraction to ET-1 were also determined by addressing the role of ET$_A$ and ET$_B$ receptors, changes in intracellular Ca$^{2+}$ and NOS.

B. Specific Aims

Contraction to ET-1 in the mesenteric small veins of cardiomyopathic hamsters in different stages of heart failure has not been determined. Moreover, clinical studies indicate that treatment of CHF patients with an ET$_A$ receptor antagonist may have beneficial effects in reducing preload. Additionally, in the late stage of CHF, inducible nitric oxide synthase (iNOS) levels are reportedly elevated. Thus, it is hypothesized that ET-1-induced contraction of isolated mesenteric veins is increased in the early stage of CHF, while it is decreased in the late stage of CHF. Stimulation by ET-1
produces an increase in intracellular calcium by various mechanisms. Subsequently, it can be hypothesized that calcium signaling in response to ET-1 stimulation in mesenteric small veins is increased in the early stage of CHF, while it is decreased in the late stage of CHF.

Nitric oxide (NO), a product of the nitric oxide synthase action on L-arginine and oxygen, is a potent vasodilator responsible for maintaining a basal vasodilatory tone. The NO pathway can extrude or sequester intracellular calcium or terminate the elevation in intracellular calcium produced by ET-1-agonism. In CHF, the imbalance between ET-1 and NOS activity can play an important role in maintaining venous vascular tone. Consequently, it can be hypothesized that NOS activity contributes to decreased contraction to ET-1 in mesenteric veins isolated from hamsters in the late stage of CHF.

The central hypothesis of this thesis is that ET-1-induced venous contraction is increased in the early stage of heart failure as a result of increased intracellular calcium levels, and decreased in the late stage of heart failure due to induction of iNOS.

To evaluate these hypotheses, the following aims will be investigated:

Hypothesis I: ET-1-induced contraction of isolated mesenteric veins is increased in the early stage of CHF, while it is decreased in the late stage of CHF.

Aim 1. Compare concentration-dependent contraction to ET-1 and ET-3 in mesenteric veins isolated from early and late stage cardiomyopathic and Golden Syrian hamsters.
Aim 2. Determine the role of $\text{ET}_A$ and $\text{ET}_B$ receptors in contraction to ET-1 in mesenteric veins isolated from early and late stage cardiomyopathic and Golden Syrian hamsters using the $\text{ET}_A$ selective inhibitor, A-127722, and the $\text{ET}_B$ selective inhibitor, A-192621.

Sub-hypothesis II: Changes in the intracellular calcium signal contributes to altered contraction to ET-1 in isolated mesenteric small veins.

Aim 3: Compare ET-1 induced changes in intracellular calcium in mesenteric small veins isolated from early and late stage cardiomyopathic and Golden Syrian hamsters.

Aim 4: Determine the role of $\text{ET}_A$ and $\text{ET}_B$ receptors in ET-1-induced changes in intracellular calcium signal in mesenteric small veins isolated from early and late stage cardiomyopathic and Golden Syrian hamsters using the $\text{ET}_A$ selective inhibitor, A-127722, and the $\text{ET}_B$ selective inhibitor, A-192621.

Sub-hypothesis III: NOS activity contributes to decreased contraction to ET-1 in isolated mesenteric veins from the late stage of CHF, while it does not modulate the contraction to ET-1 in early stage of CHF.

Aim 5. Determine the role of NOS activity in ET-1 induced contraction in mesenteric veins isolated from early and late stage cardiomyopathic and Golden Syrian hamsters using N-nitro-L-arginine.

Aim 6. Compare NOS activity in the mesenteric small veins of the early and late stage cardiomyopathic and Golden Syrian hamsters.
The central hypothesis can be shown schematically as follows:

```
Control  Early CHF  Late CHF
ET-1     ET-1     ET-1
ET_A     ET_A     ET_A
ET_B     ET_B     ET_B
Ca++     Ca++     Ca++
NOS      NOS      NOS
Contraction  NO  Contraction  NO  Contraction  NO
```

C. Review of the Literature

**Congestive Heart Failure**

Heart failure is the mechanical failure of the heart to maintain systemic perfusion proportional to the requirements of the metabolically active tissues that may be caused by an impaired ability of the cardiac muscle to contract, or by an increased workload on the heart. CHF is characterized by decreased peripheral perfusion, increased peripheral vascular resistance, impaired cardiac performance and decreased cardiac output.

The development and progression of myocardial failure can be characterized by the activation of circulating neuro-hormonal systems that modulate both vascular tone and renal retention of salt and water. Furthermore, the peripheral circulation, in response to heart failure, undergoes local changes that are fundamental to the patho-physiology of this disease state. Stimulation of the renin-angiotensin-aldosterone system leads to vasoconstriction and an increase in blood volume due to retention of salt and water. The sympathetic nervous system is activated via low and high pressure baroreceptors, as an
early compensatory mechanism to provide inotropic support to maintain cardiac output. Sustained sympathetic stimulation can activate the renin-angiotensin-aldosterone system and other neurohormones, leading to increased venous and arterial tone, increased plasma norepinephrine levels, progressive retention of salt and water, and subsequent edema. In the heart, sustained sympathetic tone is associated with myocyte apoptosis, hypertrophy, focal myocardial necrosis, and enhanced sympathetic activity causes a down regulation of the β receptors in the heart. The atrial and brain natriuretic peptides increase in response to volume expansion and pressure overload of the heart and act as antagonists of the renin-angiotensin-aldosterone system. Vasopressin and endothelins are also increased in severe CHF.

The failing heart is accompanied by a change in ventricular shape and dimension, a process known as remodeling. Remodeling may be regional or global, and is brought about by an increase in myocardial mass, an increase in ventricular volume, a change in ventricular shape, and interstitial growth. The failing heart compromises stroke volume, and one adaptive compensatory mechanism to maintain it is to enlarge the ventricular volume so that a greater stroke volume results from a reduced ejection fraction. Remodeling also includes an increase in myocardial and interstitial mass that increases wall thickness to reduce wall stress and increase contractility, according to the law of Laplace^{124}. In the adult, cardiac myocytes cannot multiply, and so the myocyte compartment can be enlarged only by hypertrophy of existing cells^{3,101,102}. Myocardial interstitial fibrosis occurs in heart failure of both ischemic and dilated cardiomyopathic causes^{9}. A two- to threefold increase in myocardial collagen content alters ventricular filling properties particularly by increasing diastolic stiffness, whereas a fourfold or
greater increase affects systolic function also. The principal initial stimulus to myocyte hypertrophy is mechanical stretch, whereas the stimuli to fibrosis are humoral. Remodeling is detrimental: dilation of the ventricle leads to increased wall stress, thereby increasing metabolic requirements which can not be met by the increased angiogenesis, and therefore ischemia, particularly of the sub-endocardium, may occur, and excess fibrosis impairs contractility, reduces capillary density, and may be a precursor of arrhythmias. Remodeling becomes self-propagating as the ventricular enlargement may not adequately restore stroke volume and ventricular hypertrophy may not adequately reduce wall stress.

Hamster Model of Cardiomyopathy

The hamster model of cardiomyopathy is genetic where the cardiomyopathy is transmitted as an autosomal recessive gene expressed in 100% of the affected lines. Cardiomyopathic hamsters develop heart failure that is characterized by cardiac hypertrophy and dilation. Basal central venous pressure and the increase in central venous pressure produced by ET-1 are higher in conscious cardiomyopathic compared to control hamsters (unpublished observations from Dr. Fuchs laboratory).

Cardiomyopathic hamsters exhibit characteristics similar to human congestive heart failure. These include similar hemodynamic profiles such as elevated end-diastolic pressure and systemic vascular resistance, increased plasma catecholamines, increased myocardial dopamine, decreased myocardial norepinephrine, and enhanced fibrosis of the epicardium. Furthermore, the acute myocarditis observed in young patients who later developed cardiomyopathy has histological similarities to the myocardial changes
observed during the development of cardiomyopathy in hamsters. These similarities make the hamster a good model for comparison to humans.

**Endothelins**

The vascular endothelial cells, in response to various stimuli, play an important role in mediating contraction and relaxation of the adjacent vascular smooth muscle cells. Endothelium-derived factors such as nitric oxide (NO) and endothelin (ET) are important regulators of vascular tone.

The endothelins are a family of 21 amino acid peptides. Of the three ET isopeptides: ET-1, ET-2, and ET-3, ET-1 is the only isoform that is constitutively released from the endothelium. A series of proteolytic cleavages ending with the removal of 18 C-terminal amino acids from big-ET by a unique endothelin converting enzyme yields ET-1. ET-1 is released preferentially on the basal side of the endothelial cells, implying that ET-1 acts primarily on the adjacent endothelial and vascular smooth muscle cells in an autocrine and paracrine manner.

ET-1 is the most potent of the known vasoconstrictors. ET-1 exerts its effects through either the ETA or the ETB receptors. The ETA receptors are located on the smooth muscle cells, while the super-high affinity ETB receptors are located on the endothelial cells (ETB1) and the high affinity ETB receptors are located on the smooth muscle cells (ETB2). The ET receptors are G-protein coupled receptors. Endothelin-deficient and endothelin-receptor-deficient mice have demonstrated some crucial roles of ET in normal embryonic development: ET-1 deficient mice have craniofacial and cardiac abnormalities and die of respiratory failure after birth, while
ET$_B$ receptor-deficient mice exhibit aganglionic megacolon which resembles Hirschsprung's disease$^{129}$.

The ET$_A$ and ET$_B$ receptors located on the vascular smooth muscle cells mediate vasoconstriction through activation of phospholipase C (PLC), phospholipase D (PLD), phospholipase A$_2$ (PLA$_2$), and/or activation of Ca$^{2+}$ channels. Activated PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to inositol 1,4,5-triphosphate (IP$_3$) and sn1,2- diacylglycerol (DAG). IP$_3$ induces calcium release from intracellular stores, whereas DAG activates protein kinase C (PKC)$^{29}$. ET-1 causes a rapid rise in IP$_3$ levels within 10 seconds of stimulation$^{5,73,74,111,119,120,134,138,175,184}$. There are two different components that can activate PLC in response to ET-1 stimulation: a pertussis toxin-insensitive pathway most likely involving the activation of PLC-$eta$ by a member of the G$_q$ family$^{75,111,179,188}$, and a pertussis toxin-sensitive pathway involving the activation of PLC-$eta$ by receptor-mediated G$_i$ activation$^{139,160,161}$.

ET-1 stimulation produces a rapid, biphasic rise in DAG levels which is sustained for up to 20 minutes$^{42,111,176}$. DAG can be produced via phosphoinositol hydrolysis or via phosphatidylcholine hydrolysis by PLC and/or phosphatidylcholine hydrolysis by PLD and phosphatidic acid phosphohydrolase. The sustained levels of DAG correspond to sustained PLD activation following ET-1 stimulation$^{90,200}$ and play an important role in PKC activation$^{162}$.

PLA$_2$ activation liberates arachidonic acid from the membrane phospholipids, and leads to the synthesis of arachidonic acid metabolites including leukotrienes, prostaglandins, thromboxane, epoxides, and hydroxyeicosatetraenoic acids$^{6,80,116,156}$. ET-1 activation of PLA$_2$ involves a PKC-sensitive pathway in vascular smooth muscle$^{90}$. 


The arachidonic acid metabolites can modulate the activity of $\text{Ca}^{2+}$ channels, or can activate gene expression.

In vascular smooth muscle, binding of ET-1 to its receptor leads to a biphasic increase in $[\text{Ca}^{2+}]_{\text{i}}$, with an initial rapid, transient phase due to $\text{Ca}^{2+}$ release from the sarcoplasmic reticulum, followed by a sustained, elevated plateau phase due to $\text{Ca}^{2+}$ influx from the extracellular space. Significant increases in IP3 levels occur within 10 seconds following ET-1 stimulation, and it is well documented that IP3 production is responsible for $[\text{Ca}^{2+}]_{\text{i}}$ mobilization in vascular smooth muscle cells. Intracellular $\text{Ca}^{2+}$ release occurs through the IP3 receptor channel and the ryanodine receptor channel. Sustained contractile actions of ET-1 are mediated by extracellular $\text{Ca}^{2+}$ and ET-1 stimulates $\text{Ca}^{2+}$ entry into the vascular smooth muscle cells. Extracellular $\text{Ca}^{2+}$ influx pathways are highly variable among species, and among vascular beds, and may be mediated by L-type $\text{Ca}^{2+}$ channels (some studies have shown no involvement of L-type $\text{Ca}^{2+}$ channels in similar preparations), Ni$^{2+}$ sensitive T-type $\text{Ca}^{2+}$ channels, and/or non-selective cation channels. $\text{Ca}^{2+}$ released in response to IP3 can activate $\text{Ca}^{2+}$ activated Cl$^-$ channels as well as $\text{Ca}^{2+}$ activated K$^+$ channels. Both these channels are involved in the sustained depolarization of the membrane necessary to activate L-type $\text{Ca}^{2+}$ channels. The ET$_{B1}$ receptors located on the endothelial cells mediate vasodilation through the release of NO, prostacyclin, and/or endothelium derived hyperpolarizing factor (EDHF).

**Venous Contraction Produced by Endothelin**

The veins, unlike arteries, are capacitance vessels, and therefore control the preload to the heart. Venous contraction increases the preload to the heart thereby...
increasing the workload of the heart. Venous systems such as the mesenteric veins serve a large capacitance function containing most of the circulating blood. ET-1 induced alterations in their tone results in significant changes in blood volume distribution, cardiac output, and blood pressure. The distribution of ET receptor subtypes in vascular tissue varies considerably with animal species and between vascular beds. While the vasoconstrictor responses to ET-1 in resistance arteries are largely mediated by ET\(A\) receptors, vasoconstrictor responses in large caliber arteries and veins also involve ET\(B_2\) receptors. Vasoconstrictor responses in mesenteric veins involve ET\(A\) and ET\(B_2\) receptors, while stimulation of the ET\(B_1\) receptors results primarily in the release of NO. In normal subjects, concentration of ET-1 in venous plasma is slightly higher than in arterial blood. Studies in vitro consistently show that the maximal responses and potency for ET-1 in veins are greater than those of the corresponding arteries.

Role of Endothelin in Heart Failure

Plasma ET-1 levels are elevated in humans with heart failure, as well as in experimental animal models with CHF, and a correlation exists between the plasma level and the severity of the heart failure. In addition to elevated plasma ET-1 levels, the levels of big ET-1 are increased as well in CHF. The elevated endothelin levels in congestive heart failure may cause severe systemic vasoconstriction by increasing intracellular calcium levels, and the felodipine-sensitive calcium influx may in turn increase ET synthesis and release from the vasculature. The elevated ET-1 levels can stimulate the endothelial cells to release superoxide radicals (via a NADPH oxidase dependent pathway) which can contribute to endothelial dysfunction, and decreased
endothelial NO availability due to peroxynitrite formation. In a positive feedback manner, oxidative stress can increase synthesis of big ET-1 by activation of the ET-1 promoter.

Endothelins produced locally act locally, while plasma endothelin levels indicate the spillover of endothelin from the local tissue. Plasma concentration of ET-1 is of prognostic significance and is increased in proportion to the symptomatic and hemodynamic severity of heart failure. Endothelin concentration is also correlated with indices of severity such as the pulmonary artery capillary wedge pressure, right atrial pressure, morbidity, and mortality.

Infusing ET-1 into animals causes an increase in systemic vascular resistance and has a negative inotropic effect. Furthermore, ET-1 exerts long-term effects including the induction of myocardial hypertrophy, and causing cellular injury in cardiomyocytes. Prolonged upregulation of the endothelin system may be maladaptive as evidenced by the improvement in the survival rate with long-term treatment of CHF with an endothelin receptor antagonist. Furthermore, there is an improvement in the left ventricular dysfunction, and ventricular remodeling. An endothelin receptor antagonist can have beneficial effects in CHF by countering the toxic effects of ET-1 on the cardiomyocytes, decreasing the hypertrophic effects on the cardiomyocytes, lowering the long-term ET-1-induced inotropy, and by diminishing the arrhythmogenic potential. The use of the non-specific ET receptor antagonist, bosentan, has been shown to decrease systemic arterial pressure, pulmonary arterial pressure, pulmonary capillary wedge pressure, and right atrial pressure in a dose-dependent fashion in humans with CHF.
Nitric Oxide

Nitric oxide (NO) is one of the smallest biologically active messenger molecules which plays an important role in a plethora of physiological and patho-physiological situations. The biological actions of NO can be either cGMP-dependent or cGMP-independent. NO, upon diffusion into adjacent cells, displaces iron out of the plane of the porphyrin ring of the heme group of the soluble guanylate cyclase, thus activating it. This, along with the reduction in intracellular Ca\(^ {2+}\) concentration due to enhanced sequestration and extrusion, mediate relaxation of vascular smooth muscle. Additionally, NO inhibits platelet adhesion and aggregation, chemotaxis, and signal transduction pathways in central and peripheral nervous systems. Some of the cGMP-independent effects of NO include activation of calcium-dependent potassium channels, modulation of cyclooxygenase (COX) activity, inhibition of the cytochrome P-450 enzymes as well as modulation of gene transcription and translation, and cytotoxicity. In both arteries and veins, endothelial-derived NO is produced by eNOS under basal conditions. NO released by endothelial cells is a major endogenous vasodilator counterbalancing the vasoconstriction produced by the sympathetic nervous system, the renin-angiotensin system, and the endothelin system.

NO is produced from the guanidino nitrogen of L-arginine in a reaction catalyzed by a group of nitric oxide synthase (NOS) isozymes. The three isozymes of NOS are nNOS (type I), iNOS (type II), and eNOS (type III). Co-factors required by all NOS isozymes include reduced nicotinamide-adenine-dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and (6R)-5,6,7,8-
tetrahydrobiopterin (BH4)\textsuperscript{59,97,127,173}. nNOS is a calcium/calmodulin dependent dimer whose NO production is tightly coupled to the intracellular Ca\textsuperscript{2+} levels\textsuperscript{17-19}. Endothelial NOS (eNOS) is a calcium/calmodulin dependent monomer bound to the cell membrane with a myristoylate bridge on the N-terminal glycine whose NO production is tightly coupled to the intracellular Ca\textsuperscript{2+} levels and state of phosphorylation\textsuperscript{28,114,154,155}. An increase in intracellular Ca\textsuperscript{2+} levels from either extra- or intracellular sources can increase NO production from the endothelium, and consequently, increase endothelium-dependent relaxation of the vessels in various vascular beds. NO derived from eNOS maintains a basal vasodilatory tone, regulates blood flow to the organs, and inhibits adhesion and activation of circulating red blood cells\textsuperscript{43,87,96,130,131,136,163,166}. The basal production of NO as well as NO production upon endothelial stimulation has been reported to be decreased in veins\textsuperscript{53,185}. iNOS is a dimer\textsuperscript{7} which tightly binds calmodulin\textsuperscript{22}, consequently not requiring exogenous calmodulin for biological activity, and can maintain an elevated NO production that is relatively independent of the intracellular Ca\textsuperscript{2+} levels. Unlike nNOS and eNOS which produce NO in short spurts, iNOS produces a long-lasting generation of NO\textsuperscript{114,115} whose rate is only limited by the availability of extracellular L-arginine\textsuperscript{115,151}.

NOS is a potential source of superoxide anion generation in vascular tissue in the absence of L-arginine or lack of cofactors\textsuperscript{84}. Enzymatic uncoupling of L-arginine oxidation and oxygen reduction by the oxygenase and reductase domains of eNOS leads to production of superoxide anion. Suboptimal concentrations of L-arginine and/or reduced availability of the cofactors may cause eNOS uncoupling\textsuperscript{77,125,157}. Superoxide anion generation in vascular tissue results in scavenging of NO, production of
peroxynitrite and reduced NO-dependent vasodilation\textsuperscript{54,186,198}. Reactive oxygen species may also decrease eNOS expression\textsuperscript{122}.

**Modulation of ET-1 actions by NOS**

Stimulation of the production of NO\textsuperscript{15,91}, as well as experimentally induced elevation of intracellular cGMP\textsuperscript{20,51,107} inhibit the production of endothelin. Administration of exogenous NO or endothelial derived NO can mitigate the sustained vasoconstrictor potency of ET-1\textsuperscript{104}. Activation of ET\textsubscript{B1} receptors increases production of NO, which modulates the vasoconstrictor effect of ET-1\textsuperscript{1,16,36-38,44,63,92,106,117,149,192}, and consequently, any tendency to over produce ET-1 in the endothelial cells would, under normal conditions, be offset by the increased release of NO which will reduce the generation of ET-1, while diminishing its vasoconstrictive and growth-stimulating effects\textsuperscript{187}. This feedback effect is not limited to NO, as ET-1 also stimulates the release of prostacyclin and endothelium derived hyperpolarizing factor (EDHF)\textsuperscript{47,113}.

**Role of NO in Heart Failure**

Several studies have assessed the role of NO and NOS in the heart. NO, by a cGMP-mediated mechanism\textsuperscript{100}, inhibits the positive inotropy of the β-adrenergic stimulation in patients with left ventricular dysfunction\textsuperscript{58}, and in patients with severe heart failure\textsuperscript{33}. This increase in NO is related to the endogenous cardiac generation by iNOS rather than eNOS\textsuperscript{33}. Even though heart failure is associated with iNOS expression\textsuperscript{27,41,56}, the spatial and temporal expression and activity of iNOS remains controversial and dependent on the severity of the left ventricular dysfunction with experimental studies showing that after an early rise\textsuperscript{135}, cardiac NO production diminishes after the transition to cardiac failure\textsuperscript{118}. Mechanical strain originating from
severely depressed cardiac function suppresses iNOS expression, leading to decreased NO production in cardiac failure\textsuperscript{201}.

The role of endothelium derived NO in CHF is complex with some studies showing an increase in NO production in the coronary arteries of dogs post-rapid ventricular pacing\textsuperscript{118}, and others showing a decreased production of NO in the same model\textsuperscript{191}. In the early stages of a genetic cardiomyopathy model, superoxide anions contribute to impaired endothelium-dependent relaxation to acetylcholine in coronary small arteries\textsuperscript{55}. Increased production and release of free radicals has been shown to inactivate NO, thus impairing vascular relaxation\textsuperscript{142}, as well as to impair the regulation of mean arterial pressure by NOS\textsuperscript{55}. iNOS has been shown to be upregulated in the mesenteric arteries in CHF. However, instead of generating large quantities of NO, iNOS generates superoxide due to a deficiency in its substrate, L-arginine\textsuperscript{103}. Studies have shown an inverse relationship between the contribution of endogenous NO to basal vasomotor tone and the severity of heart failure\textsuperscript{69}. Consequently, NO donors can restore vascular relaxation, and thus decrease the preload to the heart in CHF\textsuperscript{89}. 
II. MATERIALS AND METHODS

A. Animals

Male Golden Syrian control (C) and cardiomyopathic (M) hamsters (BIO 14.6) were obtained from Biobreeders (Fitchburg, MA), and housed individually in the AALAC approved Medical College of Georgia animal care facility. All procedures were approved by the CAURE and comply with NIH and APS standards.

B. Measurement of vascular reactivity in isolated mesenteric small veins.

Hamsters were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). The skin over the abdominal skeletal muscle was shaved and an incision was made from the right lateral side to the left lateral side below the rib cage, leaving the diaphragm intact. Heparin (100 units) was administered into the left ventricle with an insulin syringe and allowed to circulate for at least 2 minutes to prevent coagulation. The small intestine was isolated approximately 2 cm below the stomach and tied on either end with 4-0 silk suture to prevent leakage of intestinal contents. The section of intestine was removed with the mesentery intact and placed in chilled oxygenated (20% O₂, 5% CO₂, balance N₂) Krebs-Ringer bicarbonate solution (mM composition: NaCl, 118.3; KCl, 4.7; CaCl₂, 2.5; MgSO₄, 1.2; KH₂PO₄, 1.2; NaHCO₃, 25; dextrose, 11.1).

With the aid of a dissecting scope (Olympus SZ30), forceps and microscissors (Fine Science Tools, Foster City, CA), a fifth order branch of the superior mesenteric...
vein was dissected free of the surrounding adipose and connective tissue. All the vessels were 1-2 mm in length and within the range of 300-400 µm in intraluminal diameter. The isolated small veins were be transferred to a vessel bath containing oxygenated Krebs solution (Medical Instruments, University of Iowa, Iowa City, IA), mounted between two glass micropipettes (100 µm diameter tip) with 10-0 ophthalmic suture, and flushed with Krebs solution to remove any blood from the lumen. The vessel bath was transferred to the stage of an inverted light microscope (Olympus CK2). A constant intraluminal pressure of 3 mmHg was applied through the glass pipettes. An intraluminal pressure of 3 mmHg is consistent with that used in small veins by others in the literature. In preliminary studies, maximal contraction of 60-75% in response to ET-1 was observed in small veins at this pressure. Although venous pressure is slightly elevated in cardiomyopathic compared to control hamsters, an intraluminal pressure of 3 mmHg will be used to allow for appropriate comparisons of vascular contraction to ET-1.

A camera attached to the light microscope projected the vessel image onto a video monitor (Sony). Intraluminal diameter was measured in microns with a video dimension analyzer (Living Systems Instrumentation, Burlington, VT) that is capable of tracking the vessel wall on the basis of optical density. Intraluminal diameter was recorded continuously on a Grass polygraph. All vessels were allowed to equilibrate for at least 30 min before performing concentration-response curves. In all vessels pretreated with inhibitors, baseline diameters were recorded before and after the inhibitor were added to the Krebs solution to determine its effect on basal tone. When possible, more than one vessel was obtained from the same hamster, but a single experiment was performed only
once in the same hamster. Additionally, only one concentration-response curve was performed per vessel.

A cumulative concentration-response curve to ET-1 (10^{-11} to 10^{-8} M) was performed by adding ET-1 to Krebs buffer that was continuously circulated through the vessel bath. To evaluate the role of the ET\textsubscript{A} and ET\textsubscript{B} receptors in contraction to ET-1, mesenteric small veins were pretreated for 20 min with either A-127722 (30 nM) or A-192621 (30 nM) respectively, followed by a concentration-response curve to ET-1. A-127722 and A-192621 were supplied by Dr. Jerry Wessale of Abbott Laboratories. To ensure the viability of the endothelium, acetylcholine (10^{-5} M) was added at the end of the concentration-response curve. To evaluate the role of NOS in contraction to ET-1, mesenteric small veins were pretreated for 20 min with LNA (0.1 mM), following which a concentration-response curve to ET-1 was performed.

Determination of Plasma ET-1 Levels

With EDTA as an anticoagulant, blood (2 ml) was collected from the inferior vena cava, centrifuged at 1500 x g, and the supernatant plasma was stored at -80\degree C. At the time of the assay, the plasma samples were thawed, and ET-1 levels were measured using the QuantiGlo human ET-1 immunoassay kit.

Measurement of Ca^{2+} flux using Fura-2

The mesentery was removed from pentobarbital-anesthetized hamsters (60 mg/kg IP), and with the aid of a dissecting scope (Olympus SZ30), forceps and micro-scissors (Fine Science Tools, Foster City, CA), a fifth order branch of the superior mesenteric vein was dissected free of the surrounding adipose and connective tissue. The isolated small vein was transferred to a mount containing oxygenated Krebs solution. The vessel
was slit along its length, exposing the luminal surface of the vessel, and pinned flat with two titanium weights. Subsequently, the mount was transferred to the stage of the microscope connected to the calcium imaging system. The vessel was loaded with fura-2 AM (30µM) for 40 min at room temperature, and washed twice with Krebs solution, and allowed to equilibrate for 30 min. Ca$^{2+}$ flux in the smooth muscle cells of the vessel was measured at 37°C either in the presence or absence of ET-1 by continuously monitoring fluorescence at excitation wavelengths of 340 nm and 380 nm. Light from an ultraviolet light lamp passed to the cells through a rotating wheel containing 340- and 380-nm interference filters. The fluorescence emission at 510 nm was recovered using a digital camera and subtraction of background fluorescence and calculation of the ratio of fura 2 fluencesces (a relative measure of average myoplasmic free Ca$^{2+}$ concentration [Ca$^{2+}$]$_i$) were performed with Ultra-View software.

ET-1 was added to the mount containing the vessel at a concentration previously shown to produce maximum contraction of the isolated vessel (10$^{-10}$ M). Additionally, a mesenteric small vein was isolated from each hamster, and a concentration-response curve to ET-1 was performed. The response to ET-1 was used to assign cardiomyopathic hamsters vessels to the M1 (maximum contraction >50%) or M2 (maximum contraction < 50%) group.

To evaluate the role of the ET$_A$ and ET$_B$ receptors in ET-1-induced changes in intracellular calcium, mesenteric small veins were pretreated for 20 min with either A-127722 (30 nM) or A-192621 (30 nM) respectively, following which the change in the intracellular calcium levels due to the addition of ET-1 (10$^{-9}$ M) was measured.
Determination of NOS Activity

NOS activity was determined by assaying the conversion of L-[^3H]arginine to L[^3H]citrulline. The mesentery was removed from pentobarbital-anesthetized hamsters (60 mg/kg IP). A mesenteric small vein was isolated from each hamster, and a concentration-response curve to ET-1 was performed. The response to ET-1 was used to assign cardiomyopathic hamster vessels to the M1 or M2 group as described above. The remaining mesenteric veins were dissected and snap-frozen in a 7.4 pH medium composed of TRIS (50 mM), EDTA (0.1 mM), EGTA (0.1 mM), sucrose (250 mM), BME (0.1%), Glycerol (10%). The frozen samples were homogenized on ice in the same pH 7.4 medium containing phenylmethylsulfonyl flouride (1 mM), leupeptin (500 ng/mL), pepstatin A (500 ng/mL) and aprotinin (500 ng/mL). A fraction of the crude homogenate was used for the NOS assay, while the remainder was centrifuged at 100,000 g for 30 min at 4°C. The supernatant and pellet fractions were then collected and used for the NOS assay. Protein concentrations in the homogenate, supernatant and pellet fractions were determined by the Bradford assay.

The fractions (30 µL) of each were incubated at room temperature for 30 min with a reaction mixture (25 µL) containing Tris (100 mM), NADPH (1 mM), tetrahydrobiopterin (10 µM), CaM (0.1 µM), CaCl₂ (2 mM), FMN (1 µM), FAD (1 µM) and L[^3H]arginine (10 µM) in the absence or presence of LNA (1 mM) or 1400W (100 nM). The reaction was terminated by the addition of stop buffer (400 µL, pH 5.5) composed of HEPES (50 mM) and EDTA (5 mM). The samples were then applied to 1 mL columns (Bio-Rad A6 50W-X8) pre-equilibrated with stop buffer. The columns were
eluted with 1.5 mL of distilled water, and the radioactivity in the eluates was measured by liquid scintillation with a Beckman LS 6500 scintillation counter.

NOS activity was reported as pmole citrulline/mg protein. Total NOS activity was defined as that inhibited by LNA (1 mM). iNOS activity was defined as that inhibited by 1400 W (100 nM).

Data Analysis and Statistics

The % Δ ID of the isolated small veins was calculated with the following formula:

$$%ΔID = \frac{ID_{preET-1} - ID_{postET-1}}{ID_{preET-1}} \times 100$$

where ID is the intraluminal diameter of the vessel. EC50 values were calculated using the program GraphPad Prism.

NOS activity was calculated as follows:

$$\left\{ \frac{(Total\ counts - LNA.inhibitable\ counts)}{Arginine.\ Activity/Micromoles.Arginine} \right\} \div \frac{[protein] \times Sample.Volume}{Sample.Volume}$$

Intracellular calcium levels were calculated using the following formula:

$$[Ca^{2+}]_i = \frac{F_{340} - Background_{340}}{F_{380} - Background_{380}}$$

where F is the fluorescence at either 340 or 380 nm.

Dose-response curves, EC50 values, NOS activities, and intracellular calcium levels were analyzed among and between groups by a repeated measures analysis of variance. If an analysis of variance demonstrated a significance of p<0.05, Fisher’s PLSD for multiple comparisons were completed. Data were represented as mean ± SEM with a significance level of p<0.05.
III. RESULTS

A. Is ET-1 induced contraction of isolated mesenteric veins increased in early stage and decreased in late stage congestive heart failure?

Contraction to ET-1 and ET-3 in mesenteric veins from control and cardiomyopathic hamsters.

Concentration-response curves to ET-1 and the respective EC₅₀ values in isolated mesenteric small veins from the Golden Syrian control (C) and cardiomyopathic (M) hamster are shown in Figure 1. ET-1 produced a concentration-dependent contraction in veins from C and M hamsters. However, the ET-1-induced contraction in mesenteric veins from M hamsters was significantly different from that produced in the C veins. Mesenteric veins from one group of cardiomyopathic hamsters (M1) exhibited contraction greater than controls, while other vessels (M2) displayed contraction that was less than in controls. The Kolmogorov-Smirnov one-sample test established a statistical difference (p<0.01) between the two groups. Small veins exhibiting a maximum contraction to ET-1 that was greater than 50% were placed in the M1 group, while those exhibiting a maximum contraction less than 50% were placed in the M2 group.

The average maximal response to ET-1 was 61±4% in C veins, 74±2% in M1 veins and 23±3% in M2 veins. EC₅₀ for ET-1 (x10⁻¹⁰ M) was 2.5±0.15, 0.93±0.08, and
EC50 compared to C. Baseline intraluminal diameter was similar between groups.

Intraluminal diameters were 350±18, 375±15, and 367±14 in C, M1 and M2 groups, respectively. In all three groups, acetylcholine (ACh 10^-5 M) was added at the end of the concentration-response curve to ET-1 to confirm the presence of functional endothelium. As shown in Figure 2, relaxation to ACh was observed in all groups.

Although this physiological measurement of vascular contraction was used to categorize hamsters as either M1 or M2, differences in the hamster's physical characteristics were also observed. Hamsters in the M2 group appeared to exhibit lower physical activity (walking, running or standing on hind legs), more edema, and greater difficulty breathing than hamsters in the M1 group. These findings cannot be compared quantitatively, but are signs of a later stage of heart failure, while hamsters in the M1 group were in an earlier stage of heart failure.

In a subset of M1 and M2 hamsters, concentration-response curves to ET-1 were performed in two mesenteric small veins from the same hamsters. In all cases, vessels from a single hamster were either M1 or M2. The combination of an M1 and an M2 vessel from a single hamster was never observed.

Concentration-response curves to ET-3 (10^-11 to 10^-8 M) were performed in small veins from C, M1, or M2 hamsters. ET-3 had no effect on small veins from C (n=3), M1 (n=3) or M2 (n=3) hamsters.
Figure 1: Dose-response curve to ET-1 in control (C) and cardiomyopathic (M1 and M2) hamsters. Values represent mean ± SEM. * p < 0.05 vs. C.
Figure 2: Venous relaxation to acetylcholine (ACh) at the end of the dose-response curve to ET-1 in control (C) and cardiomyopathic (M1 and M2) hamsters. Values represent mean ± SEM. * p < 0.05 vs. C.
Contribution of ET\textsubscript{A} receptors to contraction to ET-1.

1. **Effect of ET\textsubscript{A} receptor blockade on the response to ET-1 in C hamsters.**

To determine if the ET\textsubscript{A} receptor contributes to the ET-1 induced contraction in the isolated mesenteric small veins, concentration-response curves to ET-1 were performed in the presence of A-127722 (30 nM), a selective ET\textsubscript{A} receptor antagonist as shown in Figure 3. Baseline intraluminal diameter was not affected by pretreatment with A-127722. After pretreatment with A-127722, contraction to ET-1 at concentrations of $10^{-10}$ to $3 \times 10^{-8}$ M was significantly decreased in the C hamsters to 27 ± 4%.

2. **Effect of ET\textsubscript{A} receptor blockade on the response to ET-1 in the M hamsters.**

To determine if the ET\textsubscript{A} receptor contributes to the ET-1 induced contraction in the isolated mesenteric small veins of M hamsters, concentration-response curves to ET-1 were performed in the presence of A-127722 (30 nM). Based on the contraction to ET-1 in the absence of the inhibitor, hamsters were assigned to the M1 or M2 group, and all experiments were performed in pairs of vessels from the same hamster. Baseline intraluminal diameter was not affected by pretreatment with A-127722 in veins from either M1 or M2 hamsters. After pretreatment with A-127722, the concentration-dependent contraction to ET-1 at concentrations of $10^{-10}$ to $3 \times 10^{-8}$ M was greatly reduced in the M1 and M2 hamster veins to 11 ± 5% and 4 ± 3% respectively (Figure 4).
Figure 3: Dose-response curve to ET-1 in control (C) hamsters in the absence or presence of the ETA receptor antagonist A-127722. Values represent mean ± SEM. * p < 0.05 vs. no pre-treatment.
Figure 4: Dose-response curve to ET-1 in cardiomyopathic (M1 and M2) hamsters in the absence or presence of the ET\textsubscript{A} receptor antagonist A-127722. Values represent mean ± SEM. * p < 0.05 vs. no pre-treatment.
Contribution of ET$_B$ receptors to contraction to ET-1.

3. **Effect of ET$_B$ receptor blockade on the response to ET-1 in C hamsters.**

To determine if the ET$_B$ receptor contributes to the ET-1 induced contraction in the isolated mesenteric small veins, concentration-response curves to ET-1 were performed in the presence of A-192621 (30 nM), a selective ET$_B$ receptor antagonist as shown in Figure 5. A-192621 had no effect on baseline intraluminal diameter, but decreased the concentration-dependent contraction to ET-1 to 34±7%.

4. **Effect of ET$_B$ receptor blockade on the response to ET-1 in M hamsters.**

To determine if the ET$_B$ receptor contributes to the ET-1 induced contraction in the isolated mesenteric small veins of M hamsters, concentration-response curves to ET-1 were performed in the presence of A-192621 (30 nM). Pretreatment with A-192621 did not alter baseline intraluminal diameter in either the M1 or M2 groups. A-192621 had no significant effect on contraction to ET-1 in the M2 group (%contraction was 38±5%). However, the maximal contraction to ET-1 was significantly reduced in the M1 group to 50±7% (Figure 6).

A summary of the effect of ET$_A$ and ET$_B$ receptor blockade on maximum contraction to ET-1 in the C, M1 and M2 groups is shown in Figure 7. Both ET$_A$ and ET$_B$ receptors contribute to contraction to ET-1 in C and M1 groups, while contraction to ET-1 in small veins from M2 hamsters is exclusively dependent on ET$_A$ receptors.
**Figure 5:** Dose-response curve to ET-1 in control (C) hamsters in the absence or presence of the ET$_B$ receptor antagonist A-192621. Values represent mean ± SEM. *p < 0.05 vs. no pre-treatment.
Figure 6: Dose-response curve to ET-1 in cardiomyopathic (M1 and M2) hamsters in the absence or presence of the ET<sub>B</sub> receptor antagonist A-192621. Values represent mean ± SEM. * p < 0.05 vs. no pre-treatment.
Figure 7: Summary of ETA and ETB receptor blockade on the maximal contraction to ET-1. in control (C) and cardiomyopathic (M1 and M2) hamsters. Values represent mean ± SEM. * p < 0.05 vs. no pre-treatment.
Heart weight/body weight ratio in the C and M hamsters.

To determine if the degree of myocardial hypertrophy was different in the M1 compared to the M2 group, heart weight/body weight (HW/BW) ratio was measured. As shown in Figure 8, HW/BW was significantly increased in both the M1 and M2 groups compared to the C group. However, there was no significant difference in the HW/BW between the M1 and M2 groups. The age of hamsters from each group are also indicated in Figure 8. There were no significant differences between the groups.

Venous plasma ET-1 levels.

To determine if differences in plasma ET-1 levels were different in the M1 compared to the M2 group, venous plasma ET-1 levels were measured. Plasma ET-1 levels were significantly elevated in both M1 and M2 groups compared to C hamsters (Figure 9). Additionally, plasma ET-1 levels were significantly increased in the M2 compared to the M1 group.
Figure 8: Heart weight to body weight ratio in control (C) and cardiomyopathic (M1 and M2) hamsters. Values represent mean ± SEM. * p < 0.05 vs. C.
Age

C (n=9)  362±16
M1 (n=12)  380±18
M2 (n=16)  367±14
Figure 9: Plasma ET-1 levels in control (C) and cardiomyopathic (M1 and M2) hamsters. Values represent mean ± SEM. * p < 0.05 vs. C.
B. Are the changes in venous contraction to ET-1 observed in M1 and M2 hamsters due changes in intracellular calcium signaling?

**Change in intracellular calcium in response to ET-1.**

To determine the change in intracellular calcium induced by ET-1, the change in the ratio of $F_{340\text{nm}}/F_{380\text{nm}}$ was measured in the mesenteric small veins of the C, M1 and M2 hamsters. The fluorescence imaging of a typical vessel from a C hamster is shown pre- and post-ET-1 in Figure 10. Pre-ET-1, the vascular smooth muscle cells in the mesenteric vein exhibit a fluorescence that is higher at 380 nm than at 340 nm. Post-ET-1, the fluorescence pattern changes to exhibit greater fluorescence at 340 nm than at 380 nm. The change in ratio of $F_{340\text{nm}}/F_{380\text{nm}}$ over time is shown in Figure 11. In a representative vessel from a C hamster, ET-1 induced a rapid rise in intracellular calcium levels followed by a sustained plateau phase. The change in the ratio of $F_{340\text{nm}}/F_{380\text{nm}}$ was elevated in the M1 vs C group and was reduced in the M2 vs C group. A summary of the maximum % increase in ratio of $F_{340\text{nm}}/F_{380\text{nm}}$ produced by ET-1 in all three groups is shown in Figure 12. At the end of the experiment, when all the veins were subjected to KCl, there was no significant change in the fluorescence ratios for mesenteric veins from C, M1 and M2 hamsters.
Figure 10: Screen captures of the fluorescence at 340 ($F_{340}$) and 380 ($F_{380}$) nm in a control (C) mesenteric vein before (pre ET-1) and after (post ET-1) treatment with ET-1. There is a significant increase in $F_{340}$ post ET-1, while there is a significant decrease in $F_{380}$ post ET-1.
Figure 11: A temporal change in intracellular calcium as indicated by a change in the ratio of $F_{340nm}/F_{380nm}$ in control (C) and cardiomyopathic (M1 and M2) mesenteric veins.
Figure 12: Maximal change in intracellular calcium as indicated by a change in the ratio of F_{340nm}/F_{380nm} in control (C) and cardiomyopathic (M1 and M2) mesenteric veins upon ET-1 agonism. Values represent mean ± SEM. * p < 0.05 vs. C.
% Change (Ratio $F_{240\,nm}/F_{380\,nm}$)

- C
- M1
- M2
Role of $\text{ET}_A$ receptors in regulating intracellular calcium levels in response to ET-1.

To determine the contribution of the $\text{ET}_A$ receptor to the ET-1 induced change in intracellular calcium levels, changes in the ratio of $F_{340\text{nm}}/F_{380\text{nm}}$ produced by ET-1 were measured in the absence and presence of A-127722 (30 nM). Baseline ratio of $F_{340\text{nm}}/F_{380\text{nm}}$ was not affected by pretreatment with A-127722. After pretreatment with A-127722, the change in ratio of $F_{340\text{nm}}/F_{380\text{nm}}$ to ET-1 was decreased in the C hamster vessels as shown in Figure 13.

Baseline ratio of $F_{340\text{nm}}/F_{380\text{nm}}$ was not affected by pretreatment with A-127722 in vessels from the M1 and M2 groups. After pretreatment with A-127722, the change in ratio of $F_{340\text{nm}}/F_{380\text{nm}}$ to ET-1 was significantly reduced in the M1 and almost abolished in the M2 group as shown in Figure 13.

Role of $\text{ET}_B$ receptors in regulating intracellular calcium levels in response to ET-1.

To determine the contribution of the $\text{ET}_B$ receptor to the ET-1 induced change in intracellular calcium levels, changes in the ratio of $F_{340\text{nm}}/F_{380\text{nm}}$ upon ET-1 agonism were measured in the presence of A-192621 (30 nM). Baseline ratio of $F_{340\text{nm}}/F_{380\text{nm}}$ was not affected by pretreatment with A-192621. After pre-treatment with A-192621, the change in ratio of $F_{340\text{nm}}/F_{380\text{nm}}$ to ET-1 was decreased in the C hamsters as shown in Figure 13.

Baseline ratio of $F_{340\text{nm}}/F_{380\text{nm}}$ was not affected by pretreatment with A-192621 in M1 or M2 vessels. After pretreatment with A-192621, the change in ratio of $F_{340\text{nm}}/F_{380\text{nm}}$ to ET-1 was decreased in the M1 hamster veins but was not significantly altered in the M2 hamster veins.
Figure 13: Summary of the maximal changes in intracellular calcium as indicated by a change in the ratio of $F_{340nm}/F_{380nm}$ in control (C) and cardiomyopathic (M1 and M2) mesenteric veins upon ET-1 agonism in the absence, or presence of the $ETA$ receptor antagonist A-127722 or the $ETB$ receptor antagonist A-192621. Values represent mean ± SEM. * $p < 0.05$ vs. no pre-treatment.
C. Does NOS modulate contraction to ET-1 in mesenteric veins isolated from hamsters in the early and late stage of CHF?

**Contribution of NOS to ET-1 induced contraction.**

To determine if NOS modulates ET-1 induced contraction in the isolated mesenteric small veins, concentration-response curves to ET-1 were performed in the presence of LNA (0.1 mM), a nonselective inhibitor of NOS. Baseline intraluminal diameter of small veins from C hamsters was not affected by pretreatment with LNA. Pre-treatment with LNA significantly enhanced contraction to ET-1 in small veins from C hamsters (Figure 14).
Figure 14: Dose-response curve to ET-1 in control (C) hamsters in the absence or presence of the NOS inhibitor LNA. Values represent mean ± SEM. * p < 0.05 vs. no pre-treatment.
% Contraction vs. ET-1 (log M) for different conditions:

- ▣ C (n=5)
- ▢ C+LNA (n=5)

* indicates statistical significance compared to the control group.
As shown in Figure 15, pretreatment with LNA shifted the concentration-response curve to ET-1 to the right indicating reduced sensitivity to ET-1 in the presence of LNA in vessels from M1 hamsters. Maximum contraction to ET-1 was unaltered. LNA increased the concentration-dependent contraction to ET-1, and the maximal contraction to ET-1 was increased in veins from M2 compared to C hamsters (Figure 15). Baseline intraluminal diameter was not affected by pretreatment with LNA in either the M1 or the M2 group.

NOS activity in the mesenteric small veins.

LNA (1 mM)-inhibitable NOS activity was measured in supernatant (Figure 16), pellet (Figure 17) and homogenate (Figure 18) samples from the C, M1 and M2 hamsters. As shown in Figure 16, there was a significant increase in the NOS activity in the supernatant of small veins from M2, but not M1, hamsters compared to C. In the pellet samples, NOS activity was elevated in small veins from M1, but not M2, hamsters compared to C. There was no significant difference in the NOS activity in the homogenate samples from the three groups. Comparing the fractions within groups, it was noted that in small veins from C and M1 hamsters, NOS activity was similar in the supernatant and homogenate and was higher in the pellet. However, in small veins from M2 hamsters, NOS activity was similar in the homogenate and pellet, but was higher in the supernatant.
Figure 15: Dose-response curve to ET-1 in cardiomyopathic (M1 and M2) hamsters in the absence or presence of the NOS inhibitor LNA. Values represent mean ± SEM. * p < 0.05 vs. no pre-treatment.
% Contraction vs. ET-1 (log M)

- ▲ M1 (n=6)
- △ M1+LNA (n=6)
- ● M2 (n=6)
- ○ M2+LNA (n=6)

* Statistical significance
Figure 16: NOS activity in the supernatant fraction of the control (C) and cardiomyopathic (M1 and M2) mesenteric small veins. Values represent mean ± SEM. * p < 0.05 vs. C.
Figure 17: NOS activity in the pellet fraction of the control (C) and cardiomyopathic (M1 and M2) mesenteric small veins. Values represent mean ± SEM. * p < 0.05 vs. C.
Figure 18: NOS activity in the homogenate fraction of the control (C) and cardiomyopathic (M1 and M2) mesenteric small veins. Values represent mean ± SEM. * p < 0.05 vs. C.
Homogenate

NOS Activity (pmol citrulline/mg protein)

C (n=4)  M1 (n=4)  M2 (n=4)
Effect of inhibition of iNOS on the total NOS activity in the mesenteric small veins.

To determine iNOS activity in the homogenate, total NOS activity that was inhibitable by 1400W (100 nM), a specific inhibitor of iNOS, was measured in the supernatant (Figure 19), pellet (Figure 20) and homogenate (Figure 21). The % inhibition of NOS activity produced by 1400W was significantly higher in supernatant of small veins from M2 compared to M1 or C hamsters. The effect of 1400W was more variable in the pellet and homogenate fractions, and there were no significant differences between groups.
Figure 19: Inhibition of NOS activity by the selective iNOS inhibitor 1400W in the supernatant fraction of the control (C) and cardiomyopathic (M1 and M2) mesenteric small veins. Values represent mean ± SEM. * p < 0.05 vs. C.
Supernatant

% Inhibition

C (n=4)  M1 (n=4)  M2 (n=4)

*
Figure 20: Inhibition of NOS activity by the selective iNOS inhibitor 1400W in the pellet fraction of the control (C) and cardiomyopathic (M1 and M2) mesenteric small veins. Values represent mean ± SEM. * p < 0.05 vs. C.
Pellet

% Inhibition

C (n=4)  M1 (n=4)  M2 (n=4)
Figure 21: Inhibition of NOS activity by the selective iNOS inhibitor 1400W in the homogenate fraction of the control (C) and cardiomyopathic (M1 and M2) mesenteric small veins. Values represent mean ± SEM. * p < 0.05 vs. C.
Homogenate

% Inhibition

C (n=4) M1 (n=4) M2 (n=4)
IV. DISCUSSION

Systolic function of the heart is dependent on four major determinants: the contractility of the myocardium, the preload of the ventricle (which is dependent on the end diastolic volume and resultant fiber length of the pre-contractile myocardium), the afterload to the ventricles (which is the impedance to ventricular ejection), and finally, the heart rate. Alterations of any of these determinants can result in inadequate cardiac function. In most instances, heart failure can be attributed to the depression of myocardial contractility either due to a loss functional muscle as a consequence of myocardial infarction or by processes diffusely affecting the myocardium. The heart may also fail as a pump because the preload can be excessively elevated as in valvular regurgitation or loss of venous compliance. Excessive afterload or a heart rate that is too slow or too rapid can also result in inadequate pump function. While the normal heart can tolerate wide variations in preload, afterload, and heart rate, the diseased heart often has limited reserve for such alterations.

When the heart fails, a number of adaptations occur both in the heart and systemically. If the stroke volume of either ventricle is decreased by depressed contractility or excessive afterload, end-diastolic volume and pressure in that chamber will rise, increasing the end-diastolic myocardial fiber length, resulting in greater systolic shortening. However, under chronic conditions, ventricular dilation will occur. While this may restore resting cardiac output, the resulting chronic elevations of diastolic
pressure will be transmitted to the atria and to the pulmonary and systemic venous circulation. Ultimately, increased capillary pressure may lead to transudation of fluid with resulting pulmonary or systemic edema. Reduced cardiac output, particularly if associated with reduced arterial pressure or perfusion of the kidneys, will also activate several neural and humoral systems. Increased activity of the sympathetic system will stimulate myocardial contractility, heart rate, and venous tone; the latter change results in an increase in the effective central blood volume, which serves to further elevate preload. Though these adaptations are designed to increase cardiac output, they may themselves be deleterious. Sympathetic nervous system activation also increases peripheral vascular resistance; this adaptation is designed to maintain perfusion to vital organs, but when excessive, it may reduce renal and other tissue blood flow. Peripheral vascular resistance is also a major determinant of left ventricular afterload, so that excessive sympathetic activity may dramatically decrease cardiac function.

One of the more important effects of lower cardiac output is reduction of renal blood flow and glomerular filtration rate, which leads to sodium and fluid retention. The renin-angiotensin-aldosterone system is also activated, leading to further increases in peripheral vascular resistance and left ventricular afterload as well as sodium and fluid retention. Heart failure is also associated with increased circulating levels of arginine vasopressin, which also serves as a vasoconstrictor and inhibitor of water excretion. While release of atrial natriuretic peptide is increased in heart failure owing to elevated atrial pressures, there is evidence of resistance to its natriuretic and vasodilating effects.

Heart failure may be right-sided or left-sided. Left heart failure is characterized by low cardiac output and elevated pulmonary venous pressure; dyspnea is the prominent
Right heart failure is characterized by signs of fluid retention - edema, hepatic congestion, and ascites. However, signs and symptoms of left and right heart failure co-exist, and left ventricular dysfunction is the primary cause of right ventricular failure.

A. ET-1 induced contraction of isolated mesenteric veins is increased in the early stage of CHF, while being decreased in the late stage of CHF.

In congestive heart failure, systemic vasoconstriction contributes to the maintenance of perfusion pressure in the presence of reduced cardiac output. The endothelin system and other neuro-humoral systems mediate systemic vasoconstriction, and other mechanisms that can compensate for the failing heart. In the early stage of CHF in cardiomyopathic hamsters, there is an increase in plasma ET-1 levels. Plasma ET-1 levels have also been shown to be elevated in humans with heart failure\textsuperscript{60,99,171}, as well as in experimental animal models with CHF\textsuperscript{93,183}, and a correlation exists between the plasma level and the severity of the heart failure\textsuperscript{60}. The elevated plasma ET-1 levels in the early stage of CHF may represent a spillover of ET-1 from the endothelial cells, and may represent an increase in ET-1 synthesis or a decrease in ET-1 clearance. The elevated plasma endothelin levels have been shown to greatly increase the contractility of vascular smooth muscle and cardiac muscle\textsuperscript{50,143,203}. The elevated endothelin levels in congestive heart failure\textsuperscript{60} may cause severe systemic vasoconstriction by increasing intracellular calcium levels, and the endothelial cell calcium influx may in turn increase ET synthesis and release from the vasculature\textsuperscript{196}. Furthermore, vascular reactivity to ET-1 in the early stage of CHF is significantly higher than in the control hamsters. Studies have shown that ET-1 induced alterations in venous tone results in significant changes in
blood volume distribution, cardiac output, and blood pressure\textsuperscript{110,190}. The increased plasma ET-1 levels and the increased vascular reactivity to ET-1 in the mesenteric veins may provide a compensatory attempt to increase the preload to the heart, and consequently to increase cardiac output and systemic perfusion.

However, these compensatory mechanisms may aggravate rather than alleviate the symptoms of congestive heart failure by increasing cardiac work and myocardial oxygen consumption. ET-1 has been shown to exert long-term effects including the induction of myocardial hypertrophy\textsuperscript{50,70,108,143,158}, and causing cellular injury in cardiomyocytes\textsuperscript{128,168}. Prolonged up-regulation of the endothelin system has been shown to be maladaptive as evidenced by the improvement in the survival rate with long-term treatment of CHF with an endothelin receptor antagonist\textsuperscript{146}. Furthermore, there is an improvement in the left ventricular dysfunction, and ventricular remodeling\textsuperscript{146}. The late stage of CHF in the cardiomyopathic hamster was characterized by levels of plasma ET-1 that were higher than in the early stage. This finding is in agreement with clinical studies suggesting that plasma ET-1 levels correlate with the degree of CHF. A decrease in vascular reactivity to ET-1 was observed in the mesenteric small veins of hamsters in the late stage of CHR. In the presence of dramatically elevated ET-1 plasma levels, this finding suggests an up-regulation of the endothelin system superimposed over a failure of the mesenteric venous vasculature to respond to endothelin and a decrease in the ET\textsubscript{B} receptor function (especially since the ET\textsubscript{B} receptors are involved in clearance).

The distribution of ET receptor subtypes in vascular tissue varies considerably with animal species and between vascular beds. While the vasoconstrictor responses to ET-1 in resistance arteries are largely mediated by ET\textsubscript{A} receptors, vasoconstrictor
responses in large caliber arteries and veins also involve $\text{ET}_{B2}$ receptors$^{30,174}$. In the control hamsters, vasoconstrictor responses to ET-1 in the mesenteric small veins were mediated by both the $\text{ET}_A$ and $\text{ET}_B$ receptors. These results were in agreement with other studies$^{71}$. In the cardiomyopathic hamsters in the early stage of CHF, contraction to ET-1 was also mediated by the $\text{ET}_A$ and $\text{ET}_B$ receptors. However, in the cardiomyopathic hamsters in the late stage of CHF, contraction to ET-1 in the mesenteric small veins was mediated by the $\text{ET}_A$ receptors alone.

$\text{ET}_B$ receptors have been known to act as clearance receptors. Studies have shown that within several hours of exposure to ET-1, there is a rapid down-regulation of $\text{ET}_B$ receptor mRNA. Others have shown that exposure of vascular smooth muscle cells to ET-1 for a long time causes a marked decrease in ET-1 binding sites, indicating down-regulation of endothelin receptors. Internalization of the endothelin receptor complex is believed to be the mechanism for the down-regulation. After internalization, the ligand-receptor complex is thought to be sequestered in lysosomes, where the acidic environment promotes ligand disassociation. In the cardiomyopathic hamsters in the late stage of CHF, exposure to extremely high plasma ET-1 levels may lead to a decrease in $\text{ET}_B$ receptor protein synthesis and internalization of $\text{ET}_A$ and $\text{ET}_B$ receptors. The down-regulated $\text{ET}_A$ receptors may account for the inadequate vascular reactivity to ET-1, while the absence of an $\text{ET}_B$ receptor mediated contraction to ET-1 may be accounted for by $\text{ET}_B$ receptors that are either downregulated or non-functional. To prevent progression of heart failure, $\text{ET}_A$ receptor antagonist use may decrease the increased preload to the heart while preventing functional receptor down-regulation that is seen in the later stages of CHF.
B. Calcium signaling in response to ET-1 stimulation in the isolated mesenteric small veins is increased in the early stage of CHF while it is decreased in the late stage of CHF.

ET-1 stimulation of the isolated mesenteric small vein of the Golden Syrian hamster caused a rapid increase in the intracellular calcium levels. The increase in intracellular calcium level was biphasic, with an initial rapid, transient phase, followed by a sustained, elevated plateau phase. Studies have shown that binding of ET-1 to its receptor leads to a biphasic increase in \([\text{Ca}^{2+}]_i\), with an initial rapid, transient phase due to \(\text{Ca}^{2+}\) release from the sarcoplasmic reticulum, followed by a sustained, elevated plateau phase due to \(\text{Ca}^{2+}\) influx from the extracellular space\(^{94,165}\). Significant increases in IP\(_3\) levels have also been shown to occur within 10 seconds following ET-1 stimulation\(^5\), and it is well documented that IP\(_3\) production is responsible for \([\text{Ca}^{2+}]_i\) mobilization in vascular smooth muscle cells\(^{126,144}\). Intracellular \(\text{Ca}^{2+}\) release has been shown to occur through the IP\(_3\) receptor channel and the ryanodine receptor channel\(^{78,178}\). Sustained contractile actions of ET-1 are mediated by extracellular \(\text{Ca}^{2+}\) and ET-1 stimulates \(\text{Ca}^{2+}\) entry\(^{165}\) into the vascular smooth muscle cells\(^{126}\). Extracellular \(\text{Ca}^{2+}\) influx pathways are highly variable among species, and among vascular beds, and may be mediated by L-type \(\text{Ca}^{2+}\) channels\(^{49,67,73,74,177,205}\) (some studies have shown no involvement of L-type \(\text{Ca}^{2+}\) channels in similar preparations\(^{10,25,169}\)), Ni\(^{2+}\) sensitive T-type \(\text{Ca}^{2+}\) channels\(^{10,21}\) and/or non-selective cation channels\(^{35,184,194}\). \(\text{Ca}^{2+}\) released in response to IP\(_3\) can activate \(\text{Ca}^{2+}\) activated Cl\(^-\) channels as well as \(\text{Ca}^{2+}\) activated K\(^+\) channels\(^{48,65,83,177}\). Both these channels have been shown to be involved in the sustained depolarization of the membrane necessary to activate L-type \(\text{Ca}^{2+}\) channels.
The initial rapid, transient phase of the biphasic response to ET-1 stimulation in the isolated mesenteric small veins of the Golden Syrian hamster may be due to Ca\(^{2+}\) release from the sarcoplasmic reticulum as a result of IP\(_3\)-induced mobilization, while the sustained, elevated plateau phase may be due to Ca\(^{2+}\) influx from the extracellular space through either dihydropyridine-sensitive voltage-dependent L-type Ca\(^{2+}\) channels or receptor-operated, non-selective cation channels that are insensitive to dihydropyridine\(^{165}\). Unpublished observations in Dr. Fuchs' laboratory suggest that contraction to ET-1 in mesenteric veins is largely dependent on Ni\(^{2+}\)-sensitive T-type calcium channels, and that L-type calcium channels also contribute to enhanced contraction to ET-1.

ET-1 stimulation of the isolated mesenteric small vein from the cardiomyopathic hamsters in the early and late stages of CHF also caused an increase in intracellular calcium levels. However, the change in intracellular calcium level in the early stage of CHF was higher than in the control, while the change in the late stage of CHF is significantly lower than in the controls. These results are in agreement with the vascular reactivity studies, indicating that in the early stage of CHF, enhanced intracellular calcium signaling contributes to the augmented response to ET-1, while in the late stage of CHF there is an inability of the vascular smooth muscles to translate the ET-1 stimulation into an appropriate increase in intracellular calcium levels.

ET-1 induced increase in intracellular calcium levels were mediated by the ET\(_A\) and ET\(_B\) receptors in the control and cardiomyopathic hamsters in the early stage of CHF, while the cardiomyopathic hamsters in the late stage of CHF showed the absence of an ET\(_B\) receptor mediated increase in intracellular calcium levels. This is in agreement with
the vascular reactivity data where the ET\textsubscript{A} and ET\textsubscript{B} receptors mediated the response to ET-1 in the control and cardiomyopathic hamsters in the early stage of CHF, while only the ET\textsubscript{A} receptors were involved in the contraction to ET-1 in the late stage of CHF. Presently, the mechanisms mediating these changes are unknown, but may involve an alteration in receptor binding or receptor downregulation. Alternatively, calcium channel expression or activity could contribute to this effect.

C. NOS contributes to the decreased contraction to ET-1 in mesenteric veins isolated from hamsters in the late stage of CHF.

In both arteries and veins, endothelial-derived NO is produced by eNOS under basal conditions\textsuperscript{39,64,132,133}. NO released by endothelial cells is a major endogenous vasodilator counterbalancing the vasoconstriction produced by the sympathetic nervous system, the renin-angiotensin system, and the endothelin system\textsuperscript{2,42,57,82,121,137,140,180}. However, the basal production of NO as well as NO production upon endothelial stimulation has been reported to be decreased in veins\textsuperscript{53,185}. Vascular reactivity experiments in the presence of LNA, a selective inhibitor of NOS suggest a role for NOS in modulating the contraction to ET-1. As observed in many other vascular beds, LNA enhanced contraction to ET-1 in small veins from control hamsters, suggesting that NOS modulates the contraction to ET-1. In cardiomyopathic hamsters in the early stage of CHF, LNA reduced vascular reactivity to ET-1, suggesting that NOS actually contributes to contraction. This paradoxical finding could be due to the ability of reactive oxygen species produced by NOS in the absence of substrates or cofactors to decrease relaxation.
Upon NOS inhibition by LNA, superoxide production in NOS substrate-cofactor starvation states is diminished thereby allowing relaxation.

In the cardiomyopathic hamsters in the late stage of CHF, LNA enhanced the maximal contraction to ET-1 suggesting that NOS was inhibiting development of maximal tone. Studies have shown the contractile state of a vessel may influence the endothelium-dependent regulation of vascular tone. It has been shown that in baseline conditions, NO has no effect on ET-1 release, whereas receptor-operated ET-1 release is blunted by NO. Thus, factors that influence vessel wall tension and endothelial ET-1 production may be important in controlling vascular tone.

Stimulation of the production of NO$^{15,91}$, as well as experimentally induced elevation of intracellular cGMP$^{20,51,107}$ has been shown to inhibit the production of endothelin. Administration of exogenous NO or endothelial derived NO can also mitigate the sustained vasoconstrictor potency of ET-1$^{104}$. Activation of ET$_B$ receptors increases production of NO, which can also modulate the vasoconstrictor effect of ET-1$^{1,16,36-38,44,63,92,106,117,149,192}$. Consequently, any tendency to over produce ET-1 in the endothelial cells would be offset by the increased release of NO which will reduce the generation of ET-1, while diminishing its vasoconstrictive and growth-stimulating effects$^{187}$. Some studies have shown that NO can alter the binding capacity of the endothelin receptors to ET-1 by modifying free thiol groups present on cysteine residues of the endothelin receptor. In the late stage of CHF, NO may be decreasing the binding capacity of the endothelin receptors for ET-1. Pre-treating the vessel with LNA might be reversing this modulation, and allowing for an increased vascular reactivity to ET-1.
NOS activity was found to be higher in the late stage of CHF, and iNOS contributed to the increase in NOS activity. iNOS is known to be up-regulated in inflammation. iNOS produces a long-lasting generation of NO \( ^{114,115} \) whose rate is only limited by the availability of extracellular L-arginine \( ^{115,151} \). The late stage of CHF is characterized by systemic inflammation. Elevated ET-1 levels can also exert long-term effects like cellular injury \( ^{128} \), in turn producing inflammation \( ^{168} \). The elevated ET-1 levels can also stimulate the endothelial cells to release superoxide radicals (via a NADPH oxidase dependent pathway) which can contribute to endothelial dysfunction \( ^{34} \). Under conditions of inflammation, elevated iNOS activity can lead to sustained elevations in NO which could lead to dilator actions as well as nitrosylation of the endothelin receptors.

A schematic representation of how the results of this work supported and added to the hypothesis are show below:

Further work in this area include: estimating ET receptor densities and their role in mediating the changes seen in early vs. late CHF, determining ET receptor gene...
expression and ET-1 production in early/late CHF, evaluating the role of ET_A receptor antagonists in protecting hamsters from progression into late stage CHF, evaluating calcium channel differences in the early and late stage of CHF which could account for the differences in calcium flux, elucidating the role of reactive oxygen species in mediating decreased relaxation in the early stage of CHF, and determining the role of nitrosylation of ET receptors in their down-regulation.
V. SUMMARY AND CONCLUSIONS

1. Early-Stage Heart Failure

a. Increased plasma ET-1 levels (which represent spillover from the tissue) suggest an elevated ET-1 production in the early stages of heart failure.

b. In the early stage of heart failure, there is an increased contractile response in the veins to ET-1 mediated by the $E_{TA}$ receptors.

c. ET-1 stimulation produces a larger increase in the intracellular calcium levels in veins from cardiomyopathic compared to controls indicating an enhanced calcium flux in the early stage of heart failure.

d. NOS enhances the contraction to ET-1 in the early stage of heart failure.

e. Total NOS activity is increased in the particulate fraction of veins from the cardiomyopathic hamsters

THUS, these data suggests that the increase in contraction to ET-1 in the early stages of CHF may be due to an increase in the tissue levels of ET-1, an increase in the contractile response as mediated by the $E_{TA}$ receptors, an increase in the calcium flux, and a NOS dependent contraction.

2. End-Stage Heart Failure

a. In the late stage of heart failure, the contraction to ET-1 is decreased.
b. The decrease in contraction to ET-1 is despite plasma levels of ET-1 higher than in the early stage of CHF.

c. The contraction to ET-1 is mediated mostly by the ET\textsubscript{A} receptors with markedly decreased contribution from the ET\textsubscript{B} receptors.

d. Upon ET-1 agonism, there is a decreased calcium flux in the vascular smooth muscle of the mesenteric veins from the late stages of CHF.

d. NOS inhibition leads to an increased contraction to ET-1 in the late stage of CHF indicating NOS modulation of the ET-1 system.

e. NOS activity is increased in the supernatant from the mesenteric veins of the late stage of CHF.

f. iNOS activity represents most of the increased NOS activity in the late stage of CHF.

**THUS**, these data suggest that the overwhelming increase in the NOS activity due to iNOS activation combined with the decreased contraction to ET-1 due to either the decreased contribution of the ET\textsubscript{B} receptors or the decreased calcium flux on ET-1 agonism are responsible for the decreased venous reactivity to ET-1 in the late stages of CHF.
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