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PHARMACOLOGICAL MODULATION OF MYOCARDIAL TUMOR NECROSIS FACTOR \( \alpha \) SECRETION BY PHOSPHODIESTERASE INHIBITORS IN HEART FAILURE PRONE RATS

DISSertation

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Graduate School
The Ohio State University

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1996

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ABSTRACT

Congestive heart failure (CHF) affects four million Americans. Phosphodiesterase (PDE) inhibitors are potent inotropic and vasodilator agents which are currently used in acute treatment of congestive heart failure. PDE inhibitors also able to block tumor necrosis factor α (TNF) which is elevated in some CHF patients. TNF is able to produce cardiac depression in septic shock and could contribute to cardiac deterioration seen in CHF. The effect of PDE inhibitors on myocardial TNF production was investigated in this study. PDE inhibitors were able to block unstimulated and induced TNF secretion in Sprague Dawley rat left ventricle (LV). The type III PDE inhibitor, amrinone, was able to block myocardial TNF secretion in heart failure prone (HF) rats, in vitro, and the potency of the drug was not altered with age. In the control animals the amount of TNF secreted altered the potency of amrinone, indicating that hearts which secrete more TNF were more sensitive to amrinone inhibition. The type IV PDE inhibitor, Ro-20,1724, was more potent than amrinone, but had a lower efficacy compared to amrinone. When amrinone was used in vivo in 15 months old HF rats, it was able to block myocardial TNF content but TNF secretion was not altered. This may be a function of amrinone dose or the dosing schedule. However, this was the first study that demonstrates the ability of PDE inhibitors, particularly amrinone, to modulate myocardial TNF in HF rats.
DEDICATION

To Daniel and My Family
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STATEMENT OF THE PROBLEM

Despite a variety of pharmacological tools available to treat CHF, few have shown promise in prolonging life span. Therefore a shift in focus in CHF research has occurred to identify and modulate endogenous substances which may contribute to the progression to end stage failure. TNF has been identified as a cardiac suppressant which is elevated in CHF. However, the site of production, the time course of production and factors stimulating TNF production during the progression to end stage CHF are unknown.

Hypotheses:

1. TNF is produced by the heart to act locally to alter cardiac function and structure.

2. Cardiac TNF production increases with age in a rat model of CHF, but not in normotensive or hypertensive controls.

3. PDE type III inhibitors block cardiac production of TNF in vitro and in vivo which may be an additional mechanism in their utilization in CHF.
Specific Aims:

1. To determine and compare the local site of production of TNF in Sprague Dawley (SD) rats, Spontaneous Hypertensive rats (SHR) and heart failure (HF) rats. If TNF is produced in rat myocardium then an additional specific aim would be to determine whether TNF is synthesized by cardiac myocytes.

Rational and Significance: TNF causes cardiac suppression during sepsis in humans. Incubation of cardiac myocytes with TNF results in inhibition of isoproterenol induced contraction and cAMP accumulation. Although TNF effects on cardiac tissue have been established, a local site of production has not been investigated.

2. To determine and compare the effect and potency (IC₅₀) of different classes of PDE inhibitors on myocardial basal and LPS-induced TNF production/secretion from SD rats.

Rational and Significance: PDE inhibitors were shown to block TNF secretion from various tissues, but the effect of PDE inhibitors on TNF production from cardiac tissue has not being investigated. Rat cardiac tissue contains predominantly type III and some type I, II, and IV PDE. We decided to study
the effect and the potency of various inhibitors on TNF secretion from the heart. Comparison of various potencies will allow us to determine the most potent type of PDE inhibitor that can block TNF synthesis in vitro.

3. To compare the effect and potency of type III and IV PDE inhibitors on myocardial TNF production from SD, SHR, HF rats at 6, 12, 18 months of age.

Rational and Significance: The effect of type III and IV PDE inhibitors on TNF production in HF animals had not been demonstrated. It is possible that during the progression to end stages of heart failure the potency and/or efficacy of PDE inhibitors shifts compared to control. The comparison of potencies and the effect of PDE inhibitors on TNF secretion may provide information for doses needed for in vivo treatment of rats.

4. To demonstrate the effect of specific PDE inhibitors in vivo on TNF levels and modulation of CHF after chronic administration to HF rats.

Rational and significance: TNF may play a role in CHF. The improvement in HF rats with PDE treatment will provide an indication that TNF inhibition does contribute to prevention of end stages of CHF after acute or chronic use.
Congestive cardiac/heart failure (CHF) could be defined as:

A state in which the heart fails to maintain adequate circulation for the needs of the body despite a satisfactory filling pressure. (Paul Wood 1950)

A pathophysiological state in which an abnormality of cardiac function is responsible for the failure of the heart to pump blood at a rate commensurate with the requirements of the metabolizing tissue. (Eugene Braunwald 1980)

A clinical syndrome caused by an abnormality of the heart and recognized by a characteristic pattern of hemodynamic, renal, neural and hormonal response. Ventricular dysfunction with symptoms. (Philip Poole-Wilson 1993)

(Barnett et al., 1993)

These are only some of the definitions which have been developed throughout the years to explain heart failure. However, to date, there is no specific denotation that is being used to define CHF, because CHF is a disorder that has a very broad pathophysiology and etiology. CHF is a major cause of mortality and morbidity in most developed countries (Garg AND Yusuf, 1993), and is the number one contributor to cardiovascular mortality (Garg et al.,
In the United States about 4 million people are affected (Ho et al., 1993), and it is estimated that 400,000 new cases of CHF are diagnosed each year (Garg and Yusuf, 1993). In people 35-64 years of age annual incidents were estimated to be 3 per 1000 compared to 10 in 1000 in people 65-94 years of age (Kannel, 1989). The Framingham study showed a median survival time of 1.7 years in men and 3.2 years in women (Ho et al., 1993) once they reached end stage CHF. In younger individuals more men develop CHF, whereas among those above age 75 more women develop CHF. The age adjusted death rate is highest among African American men followed by African American women then caucasian man and caucasian women (Garg and Yusuf, 1993).

When the heart is not pumping efficiently, the blood supply to the rest of the body is inadequate. Initially, as a result of poor perfusion of the body during exercise, the cardiac output and cardiac filling pressure increase. This elevation of cardiac work will lead eventually to a decrease in cardiac performance which at the later stages causes a decreased cardiac function even at rest. The depressed cardiac function can lead to activation of compensatory neurohormonal mechanisms eg., an increase in sympathetic tone, an increase in activity of the renin-angiotensin system and an increase in arginine vasopressin. These will lead to an excessive vasoconstriction and
sodium and water retention later leading to an increase in impedance. Although initially the compensatory mechanisms are a means of helping the failing heart, eventually the mechanisms cause excessive cardiac stimulation and additional mechanical load which adds to the cardiac burden and contributes to further decline in function (Barnett et al., 1993). Some of the etiological factors that can produce CHF are long standing hypertension, ischemic heart diseases, valvular diseases, acute myocardial infarction, viral myocarditis and dilated cardiomyopathies. There are many signs and symptoms associated with cardiac failure. These include, exercise intolerance, cardiac enlargement, dyspnea, orthopnea, peripheral edema, tachycardia (Roffman, 1995) and cachexia are some of the complications that patients in CHF might experience.

The severity of CHF can be assessed using the New York Heart Association functional classification. The degree of fatigue and dyspnea correlates with severity of the disease. Class I patients are those with asymptomatic left ventricular dysfunction, class II patients are those with dyspnea at exercise, class III patients are those with dyspnea with minimal exertion and class IV patients are those with dyspnea at rest (Roffman, 1995). An additional assessment was made by Forrester which classified patients by the measurements of the cardiac index (Roffman, 1995). The therapeutic goals for treatment of patients with CHF are to: improve signs and symptoms
associated with volume overload, increase functional capacity and improve survival and prevent or delay the onset of CHF.

The therapeutic approaches used in CHF include: cardiac glycosides, diuretics, vasodilators, angiotensin converting enzyme (ACE) inhibitors, adrenergic receptor antagonists, phosphodiesterase inhibitors and sympathomimetics. However, even with this many options of pharmacological intervention, the mortality rate of patients with end stages of CHF is high. This indicates that research in CHF will need to discover new ways to treat this disorder using existing drugs or discover new drugs that will provide better treatment. To discover the new approaches that will lead to that perfect cure scientists need to identify new factors which contribute to the progressive nature of CHF.

**ETIOLOGY**

The heart's function is to receive blood into the ventricles during diastole and to pump blood into the aorta during systole. When the heart fails to perform one or both of these functions, the various organs are not receiving a sufficient amount of blood for their metabolic needs (Grossman, 1993). Systolic dysfunction occurs when the heart is unable to eject blood efficiently.
This can occur as a result of myocardial ischemia when 20% or more of the left ventricle is lost. Myocardial ischemia involves occlusion of the coronary arteries leading to an acute decrease in cardiac contractility. An increase in hydrogen ion and inorganic phosphate in the ischemic myocardium is associated with desensitization of the myofilaments to calcium leading to a decrease in contractile function (Kihara et al., 1989). Systolic dysfunction can occur because of administration of calcium channel blockers in high concentration, which also will produce a decrease in cardiac contractility (Grossman, 1993). Also systolic dysfunction can occur as result of an afterload mismatch, in which the heart increases wall stress by decreasing the extent of myocardial fiber shortening (Ross, 1976). Valvular heart diseases are also responsible for systolic dysfunction. These diseases can occur as a result of bacterial endocarditis, acute coronary occlusion or myocardial infarction (Grossman, 1993). Chronic valvular diseases lead to a hypertrophied ventricular chamber. Initially, adequate compensation occurs but with time, myocardial contractility becomes depressed and systolic heart failure develops (Grossman, 1993).

Diastolic dysfunction is an increase of resistance to filling pressure, which can occur because of increased resistance to ventricular inflow or impaired myocardial relaxation. Cardiomyopathies, constrictive pericarditis, and other
structural abnormalities are associated with increased resistance to flow. The increase in resistance can occur because of an increase in wall thickness and myocardial fibrosis. Ischemic heart disease, volume overload and the hypertrophic heart disease, aortic stenosis, are associated with impaired myocardial relaxation. Impaired relaxation results from sustained partial myocardial contraction after systole which may further impede diastolic filling (Mancini et al., 1986). Diastolic dysfunction can also precipitate due to impaired calcium homeostasis (Grossman, 1993). Decreased gene expression of calcium ATPase and phospholamban and depressed function of sarcoplasmic reticular calcium uptake will produce an impaired calcium cycling (Grossman, 1993). Patients with advanced CHF demonstrate the disappearance of calcium from the cytosol of the myocytes (Gwathmey et al., 1987).

The various cardiovascular disorders mentioned above will produce myocardial cell loss. To compensate, hypertrophy will occur in the remaining cells to sustain the higher load per cell leading to hypertrophy. The hypertrophy produces impaired contractility and change in ventricular compliance. The myocardial failure leads to pump failure and a decreased cardiac output (Mancini et al., 1986).
HYPERTENSION AND CHF

Hypertension plays an important part in elucidating CHF. Hypertension is a primary contributing factor in CHF in the elderly, women and African Americans (Vasan AND Levy, 1996). Hypertension can induce left ventricular hypertrophy (LVH) and myocardial infarction which can lead to a decrease in cardiac output. Hypertensive patients have a chance of developing LVH 2-3 times more than normotensive patients (Kannel et al., 1987). There is a "dose" response relation of systolic blood pressure to left ventricle mass (Levy et al., 1988). The increase in left ventricular mass leads to impaired diastolic ventricular filling with an enhancement of later diastolic filling indicating abnormal left ventricular relaxation (Devereux, 1989). Myocardial stiffness, myocardial fibrosis and altered chamber geometry are possible mechanisms of diastolic dysfunction in hypertensive patients (Hess et al., 1981).

Hypertension can cause myocardial infarction (Kannel et al., 1987). Myocardial infarction causes a reduction in contractile function, and resultant left ventricular remodeling (Pfeffer AND Braunwald, 1990). The left ventricular remodeling or the myocardial scarring will cause a systolic and diastolic dysfunction leading to CHF. Hypertension does not directly elucidate CHF; however through LVH or myocardial infarction the normal heart will deteriorate, losing its pumping capacity.
PATHOPHYSIOLOGY

When the heart is failing, the body is trying to compensate for the decreased cardiac function that is taking place. Many physiological mechanisms will be activated to compensate for the failing heart. The compensatory mechanisms include an increase in sympathetic activity, cardiac remodeling and increased levels of neurohormonal factors. Eventually the compensatory mechanism will cause excessive cardiac stimulation which will add to the overall burden and will cause further decline in function (Barnett et al., 1993). Decompensation will result in poor organ perfusion and death.

Myocardial Hypertrophy

When the heart is confronted with an increased workload over a prolonged period of time, it usually will compensate by increasing its muscle mass leading to an increase in size, i.e. cardiac hypertrophy (Dhalla et al., 1987). Pressure or volume overload can produce cardiac hypertrophy (Alpert AND Hamrell, 1976). This initial increase in heart size helps the heart to adapt to the elevated hemodynamic load. However, with time, the walls of the heart become thin and there is a necrosis of myocytes, stimulation of fibroblast growth and proliferation and replacement of myocardial cells with connective tissue (Ferandeze-Alfonzo et al., 1992). The increase in myocardial size
occurs because the cardiac myocyte is increasing in size not in number (Julian et al., 1981). The increase in size is mainly triggered by extensive stretching of the muscle fiber due to an increase in pressure or volume overload (Wikman-Coffelt et al., 1979). Cardiac myocytes increase protein synthesis (Schreiber et al., 1966) and the β fetal form of myosin isozyme is reexpressed (Mercadier et al., 1981). Biochemical changes also take place during cardiac hypertrophy. The sarcoplasm reticulum has a decrease in rate and capacity to accumulate calcium (Limas AND Cohn, 1977), the sarcolemma ATPase activity changes leading to abnormality of calcium movement in and out of the cell (Dhalla, 1976), and a decrease in ATP production by the mitochondria occurs in the hypertrophied heart (Katz, 1977). Fibrotic tissue deposition will occur in the hypertrophied heart. An increased in collagen concentration in the endocardium leads to decreased diastolic compliance during cardiac hypertrophy (Dodge et al., 1962). The increase in collagen synthesis is believed to be triggered by myocardial hypoxia (Honig AND Bourdeau-Martini, 1974). All of these physiological changes will remodel the heart, producing a heart of increased size which is not pumping efficiently.
Sympathetic Nervous System and Adrenoceptors Function

The sympathetic nervous system provides beneficial and detrimental effects on the circulation and the myocardium. As a result of a decrease in cardiac output, the sympathetic nervous system (SNS) is activated. Plasma catecholamine levels are elevated in CHF, even in asymptomatic patients. This indicates that the SNS plays an important role in the compensatory response (Barnett, 1993). The useful actions of the SNS include increased contractile force, diastolic relaxation and venous return. The detrimental actions include increased myocardial oxygen consumption, calcium overload, tachycardia, arrhythmias, and increased vasoconstriction which cause increases in peripheral vascular resistance leading to a loss of the ability of the circulation to respond to changing tissue requirements (Barnett, 1993).

The SNS also interacts with the renin-angiotensin system, causing an increase in renin release from the kidney. The release of renin will stimulate angiotensin II production which leads to potentiation of the SNS at various central and peripheral sites (Mancia et al., 1988). Because the SNS is not deactivated in CHF, an uncontrolled increase in sympathetic activity proceeds as the heart failure worsens.

Stimulation of β-receptors in the heart causes inotropic, chronotropic and arrhythmogenic effects. Stimulation of myocardial β-receptors leads to increased intracellular cyclic AMP production which activates protein kinases
causing phosphorylation of a number of proteins influencing calcium transport and binding. Increased intracellular calcium via voltage dependent calcium channels and enhanced release of calcium from the sarcoplasm reticulum lead to an increase in inotropic response (Barnett, 1993). In CHF, a 50% decrease in myocardial β1-receptors density occurs (Bristow et al., 1982). This decrease in receptor density contributes to the decrease in cardiac contractility in CHF. β1-receptors are stimulated by neuronally released norepinephrine, and the increase in SNS activity during compensatory stages of CHF can lead to their down regulation (Barnett, 1993). However there is the possibility that local tissue factors are responsible for the β-receptor down regulation (Bristow et al., 1982). Guanine nucleotide regulatory (G) proteins are able to transduce the signal of receptor stimulation to adenyl cyclase. Inhibitory G proteins (Gi) units are 37% elevated in patients with dilated cardiomyopathies (Böhm et al., 1990). The myocardial G proteins can play a role in the decreased contractile response of the heart CHF. The adrenergic system is believed to play a role in the progression into failure: however, it still remains to be determined whether the decreased receptor density is a compensatory response or a result of failure.
Neurohormonal factors

One of the compensatory responses in CHF is activation of neurohormonal systems. The renin-angiotensin system (RAS) is activated during the compensatory stages of CHF. RAS produces primarily vasoconstriction and increased aldosterone release which will cause sodium and water retention. Stimulation of renin release is elicited by decreased renal perfusion and sodium delivery to the macula densa, enhanced sympathetic activity and reduction of intracellular calcium (Cody AND Laragh, 1988). RAS is also associated with the pathophysiology and maintenance of cardiac remodeling or cardiac hypertrophy. Angiotensin II is capable of initiating and maintaining myocardial cell growth. It can initiate protein synthesis without changing total RNA content (Aceto AND Baker, 1990). In addition to the trophic effect on cardiac myocytes, angiotensin II is a growth promoting factor of fibroblasts (Schelling et al., 1978). It is not clear if the angiotensin II that contributes to cardiac hypertrophy originates from local or circulating RAS.

Atrial natriuretic peptide (ANP) is a hormone that can produce diuresis natriuresis and vasodilation as well as inhibit aldosterone, renin and vasopressin release (Cody et al., 1986). Plasma ANP levels rise during CHF, and ANP thought to counteract the activities of vasoconstrictor substances and maintain homeostasis. Also ventricular muscle ANP levels are
increased in cardiac hypertrophy in humans with cardiomyopathies (Yasue et al., 1989). The reason for the reexpression of ANP by the ventricles in CHF could be due to an increase in cardiac pressure overload and stretch (Roman et al., 1989).

Arginine vasopressin (AVP) is a hormone with vasoconstrictor properties. The levels of plasma AVP are elevated in severe CHF patients (Goldsmith et al., 1983), and lowering AVP levels in CHF patients leads to many hemodynamic improvements. The increase in AVP release from the posterior pituitary is due to nonosmotic stimulation by angiotensin II. The physiological effects of AVP are mediated through V1 and V2 receptors (Cowley et al., 1980).

Endothelium derived relaxation factor (EDRF) and endothelin (ET-1) have been showed to have effects on vasomotor tone. EDRF mediates vasodilation by stimulating cGMP. AVP, acetylcholine and changes in blood flow can stimulate EDRF release (McDonald AND Francis, 1993). In CHF the effect of EDRF is attenuated, suggesting a defect in endothelial mediated vasodilation (Kubo et al., 1991). Circulating and tissue levels of endothelin-1, a 21 amino acid peptide, are elevated in CHF, and a correlation between the severity of CHF and endothelin-1 circulating levels has been established (Pacher et al., 1996). ET-1 is inversely correlated with cardiac index and survival (Pacher et
Endothelin-1 is primarily synthesized by the lungs in CHF (Tsutamoto et al., 1994) and mediates vasoconstriction (Kiowski et al., 1995). Angiotensin II, TGF-β endotoxin, IL-1, hypoxia, and shear stress stimulate endothelin-1 gene expression (Kaddoura AND Poole-Wilson, 1996).

Neuropeptide Y is a peptide that is secreted with norepinephrine from neurons (Tatemoto, 1982). Neuropeptide Y levels are elevated in CHF (Maisel et al., 1989), and it has negative inotropic and chronotropic effects on the heart. Neuropeptide Y could contribute to the deterioration in ventricular function by inhibiting cAMP (Kassis et al., 1987).

The neurohormonal systems which are activated during CHF are part of a compensatory response of the body to the decreased cardiac output and performance. These neurohormonal systems produce a beneficial effect originally, but eventually with time, their effects get out of control, leading to even further decreases in myocardial function.
PHARMACOLOGICAL TREATMENT OF CHF

Cardiac Glycosides

These drugs are positive inotropic agents used to improve cardiac contractility and raise cardiac output (Applefeld AND Roffman, 1986). Cardiac glycosides inhibit the sodium potassium-adenosine triphosphate (ATPase) pump, producing complete inhibition of sodium and potassium transport. The increase in the intracellular sodium will activate the sodium calcium exchanger which will increase intracellular calcium (Barry et al., 1985). Calcium will cause an increase in contractility of the heart (Wier AND Hess, 1984) because the interaction of calcium with troponin C is responsible for activation of the cross bridge interaction between actin filaments and myosin cross bridges which will result in sarcomere shortening. However digoxin treatment has not been shown to prolong life span, and recent study suggests (Smith et al., 1992) the discontinuation of its use in a large portion of CHF patients.

Diuretics

These drugs are used to alleviate symptoms and improve prognosis of CHF. Diuretics can decrease edema either in the lung or in the peripheral circulation (Poole-Wilson, 1993). Loop and thiazide diuretics are used in acute and chronic CHF. Loop diuretics inhibit the sodium-potassium-chloride
symporter found in the ascending limb of loop of Henle, whereas thiazide diuretics inhibit the sodium-chloride cotransporter in the distal convoluted tubule. Combination of loop and thiazide diuretics produces natriuresis larger than if the drugs were given individually (Kelly AND Smith, 1995). Resistance to diuretics develops in CHF because of an excess dietary sodium intake, and/or decreased renal perfusion and glomerular filtration rate due to a further decline of cardiac output. (Kelly AND Smith, 1995). Combination therapy of digoxin, ACE inhibitors and nitrates are used with various diuretics to treat different stages of CHF; However diuretics have not been shown to prolong life span.

**Vasodilators**

These drugs are used in CHF in order to reduce high peripheral vascular resistance and to improve exercise capacity and survival. Insufficient cardiac pump function results in activation of baroreceptors, and in dilatation of the left ventricle. Eventually this leads to the activation of sympathetic nervous system causing an even further decrease in cardiac compliance (Silk, 1993). Vasodilators decrease the impedance, improving cardiac stroke volume in CHF. Some agents act as preload reduction agents by changing end diastolic volume and pressure, whereas other agents produce afterload reduction by affecting the resistance vessels.
Postsynaptic $\alpha_1$ antagonists such as prazosin and terazosin, produce vasodilation without increasing heart rate and myocardial oxygen wasting. $\alpha_1$ antagonists produce negative chronotropic effects, dilate arterioles and veins, and it also decrease filling pressure. Tolerance can be developed and limits long term effectiveness in CHF. Hydralazine is an orally effective vasodilator whose action influences left ventricular afterload (Packer et al., 1980). It decreases pulmonary, renal and systemic vascular resistance (Kelly AND Smith, 1995). The possibility of sustained efficacy and reflex tachycardia might limit therapy.

Nitrates relax vascular smooth muscle, and they can dilate both arteries and veins. However at low doses, they selectively cause venodilation. The reduction in venous return reduces cardiac preload and wall tension (Williams et al., 1975). The hemodynamic effects of nitrates occur because the nitrate molecule is converted to nitric oxide or S-nitrosothiol which will interact with guanylate cyclase leading to the formation of cyclic GMP producing vasodilation (Reithmann et al., 1991a). Some nitrates like nitroprusside are effective in reducing preload and afterload. The reduction of ventricular filling pressure results in redistribution of the blood from central to peripheral veins. The reduction in preload and afterload causes a decrease in wall stress leading to better energy consumption (Kelly AND Smith, 1995). After
prolonged use of nitrates, tolerance can develop primarily because of depletion of sulfhydryl groups (Silk, 1993). However combination therapy of nitrates + hydralazine has been shown to increase survival in CHF (Cohn et al., 1986).

Potassium channel openers such as hydralazine, are agents which produce vasodilation by opening potassium channels causing hyperpolarization of the resting membrane potential (Hamilton et al., 1986). These drugs do not affect veins. However they produce an effect on coronary, renal, and systemic arteries. In CHF patients, they cause a 30% increase in cardiac output and a fall in vascular resistance and systemic blood pressure. Left ventricular contractility and stroke volume are not changed (Silk, 1993).

Calcium channel blockers decrease the amount of calcium entering the myocardial and vascular smooth muscle cells. The decrease in calcium results in decreased contraction demonstrated by negative inotropic effect in the myocardium and vasodilation in coronary and smooth muscle. Vascular cells appear to be more sensitive to the effects of these drugs than the myocardial cells. Coronary arteries are more affected then the peripheral arteries (Baugman et al., 1986). There are three groups of calcium channel blockers, all of which are coronary vasodilators. Nifedipine is the most potent peripheral vasodilator. It produces a decrease in arterial pressure and a
decrease in ventricular stroke index. This cardiac depression can be a problem when this agent is used in CHF (Pouler, 1993). To compensate, for the vasodilation induced by the calcium antagonists, the sympathetic nervous system and renin-angiotensin system are activated (Francis, 1991). Initially they can mask the depression of contractility but in chronic treatment they can be detrimental (Packer, 1989). In pressure overload cardiac hypertrophy and idiopathic cardiomyopathy, a high level of cytoplasmic calcium causes a delayed relaxation of the heart. Calcium channel blockers can also play a role in preventing left ventricular enlargement. Many stimuli, including systolic wall stress, diastolic wall stress, angiotensin II and norepinephrine cause myocardial hypertrophy. Calcium is involved as a second messenger in many of the pathways activated by the above stimuli. Also calcium overload can be related to fibrosis, impaired relaxation, and microvascular spasm. Calcium channel blockers might reduce the rate of progression of ventricular remodeling by reducing wall stress, and preventing vascular spasm. Although these agents were used for treatment of CHF in early 1990s, as of today, only a small subpopulation of patients benefit from calcium channel blockers. These patients have normal systolic function and impaired diastolic function. In other forms of CHF calcium channel blockers produce detrimental effects rather than clinical benefits (Cohn, 1993).
Angiotensin converting enzyme (ACE) inhibitors are agents which produce a decrease in plasma norepinephrine, angiotensin II, aldosterone and ANP, which is related to improvement in symptoms and in survival (Held AND Swedberg, 1993). Vasodilatation, arterial pressure and left ventricular filling pressure are also reduced by these drugs, while cardiac output is increased. Some trials with captopril (Bussman et al., 1987) and enalapril (Sharpe et al., 1984) demonstrate symptomatic improvement in CHF patients. Although this improvement is not immediate (Bussman et al., 1987). Also delayed improvement in exercise capacity (Creager et al., 1985) occurs with these agents. ACE inhibitors show an improvement in prognosis in CHF patients. Many clinical studies indicate a reduction in mortality with ACE inhibitor therapy. The CONSENSUS trial (Cooperative North Scandinavian Enalapril Survival Study) showed a 40% reduction in mortality in patients treated with enalapril in addition to digitalis and with or without diuretics (Furberg AND Yusuf, 1988). The SOLVD trial confirmed the previous studies and specified that ACE inhibitors will decreased mortality due to progressive heart failure but not deaths due to arrhythmias (SOLVD Investigators, 1991).
Dopamine Receptor Agonists

The effects of dopamine agonists in CHF involve reduction of afterload, without affecting the perfusion of heart, kidney and brain. These agonists improve renal function and perfusion, lower the diuretic dose used in CHF patient and inhibit many neurohumoral factors (Cassarande, 1993). These drugs act through DA1 and DA2 receptors. DA1 receptor is located on smooth muscle cells in renal, coronary, mesenteric, and cerebral arteries (Goldberg AND Kohli, 1983). Activation of DA1 receptors produces vasodilation and can also be involved in promoting sodium excretion. DA2 receptor subtypes are located in sympathetic fibers afferent to blood vessels, kidney and heart and in the sympathetic ganglia. The activation of DA2 receptors leads to a decrease in sympathetic activity and norepinephrine release (Langer, 1981). Also activation of DA2 receptors in the adrenal cortex is responsible for the inhibition of aldosterone secretion (Missale et al., 1986). Overall, dopamine agonists reduce afterload, leading to an increase in cardiac output. DA1 agonists, fenoldopam and dopexamine, are responsible for renal vasodilatation, increased cardiac output and natriuretic effects. Ibopamine is more active at DA1 and DA2 than dopamine and can also be administered orally.
β-adrenergic agonists

Selective β agonists can be beneficial in improving cardiac performance. By producing positive inotropic effects and inhibiting vasoconstriction. However excessive stimulation of β-receptors could lead to myocardial necrosis (Rona et al., 1959) or arrhythmias (Bigger, 1987). One of the problems in using β-receptor agonists for CHF is the reduced receptor density in the myocardium of these patients. The reduced number of receptors impairs the cardiac responsiveness to endogenous catecholamines and to exogenous drugs. However, for acute effects in CHF, these drugs might be the drugs of choice.

Dobutamine is a β1, β2 and α agonist. This drug produces inotropic effects and peripheral vasodilation (Waller, 1990). In chronic treatment the drug produces adrenoceptors down regulation and rapid tolerance (Unverferth et al., 1980). The impaired contraction to dobutamine is used to determine the amount of β-receptors down regulation occurring in heart failure (Hohm, 1988). The new drugs which are being investigated in this class are the β1 selective partial agonists. Xamoterol, a partial agonist, produces both agonistic and antagonistic effects on the β-receptor. This drug prevents the down regulation of β-adrenoceptors and produces improvements in systolic and diastolic function during prolonged use. Patients that have a moderate
CHF who take β1 partial agonists regain the ability to exercise more without experiencing fatigue and dyspnea. In severe heart failure the action of these drugs may not be as beneficial, but this will require further investigation (Waller AND Davis, 1993).

β -Adrenergic Blockers

About 20 years ago, a study was done in patients with cardiomyopathy which showed an improvement in the patients' condition after treatment with β-blockers (Waagstein et al., 1975). Many clinical studies investigating the effect of these drugs demonstrated the same results. The heart is innervated by sympathetic nerves which release norepinephrine (Feldman AND Bristow, 1990). In heart failure, an increase in sympathetic activity produces continuous stimulation of β1 receptors leading to receptor down regulation, and an increase in afterload leading to an increase in myocardial energy demand. β-blockers can increase receptor density after 6 months of treatment (Heilbrunn et al., 1989) and recouple β2 receptors to Gs proteins. These effects may increase inotropic responsiveness to stress or exercise (Bristow et al., 1989). β-receptor antagonists may inhibit the toxic effect of catecholamine (Haft, 1974), and angiotensin II (Tan et al., 1989), improve energy consumption by shifting substrate utilization from fatty acid to glucose, increase myocardial efficiency (Eichhorn, 1993), and reduce ventricular
arrhythmias (Chadda et al., 1986). Although improvement in hemodynamic parameters is observed during therapy, deterioration occurs after withdrawal of this drug (Leimbach et al., 1986). Carvedilol, a non-selective β-blocker has recently been tested for CHF treatment. Carvedilol produces reduction in blood pressure without adverse effect on renal or cerebral blood flow (Morgan et al., 1991). Carvedilol decreases the risk of death in CHF (Packer et al., 1996); however it was not approved by the FDA (Pfeffer AND Stevenson, 1996). It is still not understood when is the best time to initiate β-blocker therapy in CHF, how long to continue therapy, what drug combination to use and how to classify which patients with CHF will benefit from the therapy and which will not. Answering these questions will help promote the therapy of β-blockers in CHF.

Phosphodiesterase Inhibitors

These drugs are potent vasodilator and inotropic drugs (Fischer et al., 1992). The mechanism of action is thought to be via inhibition of phosphodiesterase (PDE) enzymes. PDEs are found in the cytosol, membrane bound, or both (Fischer et al., 1992). PDEs degrade cAMP or cGMP, and the inhibition of these enzymes will lead to increased steady state concentration of cAMP or cGMP. Four PDE subtypes have been found in the heart, among those the dominant types are type III and IV PDE. These two types degrade cAMP. In
the myocardial cell, cAMP plays an important role in the generation of myocardial force. cAMP activates protein kinases which will phosphorylate the sarcolemma calcium channel which in turn will promote influx of calcium. This will cause even more calcium to be released from the sarcoplasmic reticulum (Morgan et al., 1991). The increase in intracellular calcium will lead to the formation of cross links between actin and myosin filaments, shortening the contractile unit, and leading to contraction of the myocytes (Chapman, 1980). cAMP also causes the phosphorylation of proteins that help to produce relaxation. These proteins are phospholamban which resequesters calcium, and troponin I which looses the affinity to calcium (Morgan et al., 1991). In the vascular system, PDE inhibitors produce vasodilation in the venous and arterial beds. cAMP is responsible for the smooth muscle relaxation (Kukovetz et al., 1981).

A type III PDE inhibitor are the drug of choice in CHF. The inhibition of PDE III leads to inotropic, lusitropic and vasodilation effects in humans (Fischer et al., 1992). Amrinone, a type III PDE inhibitor, is administered orally or intravenously producing a beneficial effect in acute CHF. The overall amrinone improvement in left ventricular end - systolic volume and cardiac index is greater than with any vasodilator (Konstam et al., 1986a). At higher doses, amrinone produces an inotropic effect, whereas vasodilation occurs in
lower doses (Fischer et al., 1992). Milrinone is more potent and better tolerated drug than amrinone (Alousi AND Johnson, 1986). Milrinone increases cardiac index and decreases pulmonary capillary wedge pressure in patients with left ventricular dysfunction without major side effects (Anderson et al., 1987). Milrinone produces its beneficial effect due to both myocardial and vascular actions. After short term administration, milrinone increases exercise capacity (White et al., 1985). Myocardial oxygen consumption is increased in CHF patients due to the positive inotropic effect and afterload reduction produced by the drug. Enoximone is an imidazolone derivative that has similar effects as milrinone and amrinone in acute treatment of CHF (Fischer et al., 1992).

The chronic effect of amrinone, milrinone and enoximone is not as beneficial as the acute effect. Amrinone did not demonstrate improvement after long term treatment (DiBianco et al., 1984). Some side effects are present with long term amrinone therapy including gastrointestinal intolerance, reversible thrombocytopenia, liver function abnormalities and fever (Silverman et al., 1985). In a PROMISE study, milrinone treatment of severe CHF patients for 6 months increased mortality by 28% compared with placebo (Packer et al., 1991). One of the proposed mechanisms for the increased mortality could be the induction of ventricular arrhythmias (Packer et al., 1991). Lower doses of milrinone did not result in an increase in death in the PROMISE study.
Titrating the dose in order to find the minimum effective dose might be the next step in determining the utility of this drug in the chronic treatment of CHF patients (Andrews AND Cowley, 1993). Enoximone also did not produce any beneficial effect in chronic treatment, and an increase in mortality was also demonstrated in some patients (Uretsky et al., 1990).

Vesnarinone is one of the newest type III PDE inhibitors but also has other effects (Colucci AND Landzberg, 1993). Vesnarinone produces a beneficial effect in chronic CHF patients and reduced mortality over placebo when a low dose of the drug was used (Feldman et al., 1993).
ANIMAL MODEL: SHHF/Mcc-fap

The SHHF/Mcc-fap rat is a genetically induced animal model for heart failure. These animals are selectively bred for spontaneous hypertension and heart failure. The Mcc means that the model was developed by Dr. McCune, the fap means that the offspring of the strain carry the corpulent gene (cp). All rats develop hypertension and CHF. Only 25% are obese (McCune et al., 1988). The rats are from the 7th backcross of the SHR/N-cp strain onto an SHR background. The SHR/N-cp strain was developed by mating a Koletzky rat heterozygous to the cp gene to SHR/N rat. The CHF has been maintained through 26 generations (McCune et al., 1990).

The obese animals are insulin resistant and die of CHF at younger ages than lean animals. (McCune et al., 1993). The obese males show glomerular lesions, elevated serum cholesterol and triglyceride levels, glucosuria, glucose intolerance and proteinuria. Lean males go into heart failure at 15-20 months, the clinical onset lasting 5-14 days. Edema, dyspnea, cyanosis and orthopnea are some of the symptoms in these rats. These CHF symptoms are very similar to CHF symptoms in humans. After death examination revealed enlarged hearts (up to 4g), thickened left and right ventricular walls, left and right atrial dilatation, hepatomegaly and pulmonary edema with pleural effusion. In lean CHF animals, atrial thrombi are very common (McCune et al., 1990). The liver, kidney and lungs are congested. The renal
tubules are plugged with hyaline due to proteinuria, and the glomeruli are bloodless and lesioned. The hearts contain interstitial fibrosis, and the myofibers are enlarged, and the Z bands are stretched indicating hypertrophic cardiomyopathy. Some myocytes are degenerated (Hoel et al., 1993). The left ventricular myocyte volume increased because of myocytes lengthening, whereas, the right ventricular myocytes growth was proportional with 23% increase in myocyte length and 18% increase in myocyte width (Gerdes et al., 1996). The fetal forms of the heart proteins actin and myosin are expressed. Plasma ANP, renin, norepinephrine and angiotensin II levels are elevated (McCune et al., 1988).

The systolic blood pressure rises (190-210 mm Hg) and plateaus in male rats by 4-5 month of age. Also myocardial hypertrophy occurs at the same age. Exercise tolerance and myocardial functional capacity are reduced (McCune et al., 1988). Sodium retention in the blood and decreased excretion of sodium in the urine is also common in this model.

The SHHF/Mcc-fa<sup>op</sup> rat is an animal model which resembles many features that appear in CHF patients. This model is a genetic model for CHF and not a surgically induced model. Hypertension is a common etiology which causes CHF in humans (Kannel, 1989). The exact mechanism for CHF development is not clear because not all hypertensive patients develop CHF.
The role of hypertension in CHF can be investigated using this rat model. Cardiac hypertrophy, elevated levels of neurohormonal factors in the plasma, compensated left ventricular dysfunction and dilated end-stages of CHF are the characteristics of the disease in humans. The SHHF/Mcc-fa\textsuperscript{op} rats experience cardiac hypertrophy due to hypertension, compensated left ventricular dysfunction, biventricular hypertrophy and eventually dilated CHF. Elevated plasma levels of ANP, aldosterone, plasma renin activity and a decrease in sodium excretion are all taking place as the rat ages. Animals in decompensated CHF show adipose and muscle wasting, subcutaneous edema, tachypnea, orthopnea, cyanosis and apparent malaise. Because the HF condition is reproducible in this model and all rats die of CHF, there are some advantages compared to surgically induced models. In surgically induced models not all animals survive the surgery and not all develop CHF. The SHHF/Mcc-fa\textsuperscript{op} rat model could be used as a model to study future drug effects and new therapies because it responds to currently used drugs to treat CHF. This model can provide answers to some of the questions that are currently being asked in CHF research (McCune et al., 1993).
TUMOR NECROSIS FACTOR-α

Tumor necrosis factor-α (TNF) is a cytokine which at low doses produces antitumor activity. The polypeptide plays a role in inflammation and immune responses (Old, 1985). The cytokines are a large family of molecules which includes interferons, interleukins, and hematopoietic growth factors (Schütze AND Machleidt, 1992). TNF I uses the shock state in sepsis (Tracy et al., 1986), has antiviral activity (Jaattela, 1991), and produces protection in parasitic infections (Malik AND Balkwill, 1988). In cerebral malaria and in inflammation, TNF is over expressed, causing cell damage and biochemical changes (Grau et al., 1989). TNF produces many cellular effects after binding to high affinity membrane receptors initiating intracellular signaling cascades.

TNF, a 17 kd molecule, is biologically active as a trimer. The propeptide is 235 amino acids long, and the mature peptide is 157 amino acids (Kunkel et al., 1989). Monocytes and macrophages are the primary source for TNF, however the blood is not the only source of TNF. The renal glomerular cells cardiac myocytes, spleen, thymus, fibroblasts and central nervous system are each capable of producing TNF (Spriggs et al., 1992). Lipopolysaccharide (LPS), phorbol esters, interleukins and free oxygen radicals are able to
increase production of TNF. LPS, the most potent stimulus of TNF, induces TNF production at the transcriptional level. LPS promotes the binding of DNA binding proteins to the enhancer element on the TNF promoter. Activation of phospholipase A₂ (Mohri et al., 1990), phospholipase C and protein kinase C are some of the pathways mediating the LPS induction of TNF production from monocytes (Kovacs et al., 1988). The TNF gene is found on chromosome 6 within the major histocompatibility complex (Spies et al., 1989). The promoter region of the TNF gene contains a TATA box, a GC box/Sp -1 binding site, and kB enhancers. Also the 3' untranslated region in the mRNA bears an AU rich region which makes the mRNA very unstable and marks the mRNA for rapid degradation (Beutler et al., 1988).

Glucocorticoids inhibit TNF production on the transcriptional and the translational levels. Dexamethasone may be acting on genetic elements present in the TNF promoter region (Beutler et al., 1992). Prostaglandin E₂, cAMP and phosphodiesterase inhibitors also inhibit TNF production.

TNF actions are mediated through two TNF surface receptors, denoted the myeloid cell type receptor which is 55kd, and an epithelial cell type receptor which is 75kd (Hohmann et al., 1989). The 55 kd receptor is responsible for the tumor cytotoxic effects and the 75 kd is responsible for growth stimulatory
effects, dermal necrosis, tissue damage and fever (Bazzoni AND Beutler 1996) Soluble TNF receptors are able to bind TNF and neutralize its action. These are receptors which are shed from the cell membrane. The soluble receptors are found in the urine or serum (Seckinger et al., 1990). The binding of TNF to its cell membrane receptors initiates intracellular responses. Activation of phospholipase A2 results in the production of arachidonic acid, and the arachidonic acid metabolites induce the activation of c-fos (Jaattela, 1991). The other signal transduction pathway which TNF induces is the activation of phospholipase C leading to the production of diacylglycerol. Diacylglycerol activates acidic sphingomylinase (Heller AND Krönke, 1994). Sphingomylinase will cleave sphingomyelin to ceramide and phosphatidylcholine. Ceramide leads to the liberation of nuclear factor kB(NFkB) complex. The NFkB complex is translocated to the nucleus where it binds to a DNA recognition sequence of various genes (Heller AND Krönke, 1994). Protein kinase A and protein kinase C are also activated by TNF signal transduction mechanisms. Diacylglycerol is responsible for PKC activation (Heller AND Krönke, 1994), and adenylate cyclase is responsible for PKA activation (Zhang et al., 1988). Activation of protein kinases will cause the phosphorylation of protein substrates in the cytosol and induction of transcriptional factors, NFkB (Heller AND Krönke, 1994).

The third messengers in TNF signal transduction are transcription factor
genes, interferon regulatory factor 1 (IRF-1) (Fujita et al., 1989), AP-1 (Brenner et al., 1989) and NF-kB (Jaattela, 1991). Through the activation of transcription factors TNF regulates the expression of many genes.

The regulation of growth in fibroblasts is associated with phospholipase activation by TNF (Palombella AND Vilcek, 1989). The proinflammatory actions of TNF are attributed to generation of leukotrienes and prostaglandins as a result of phospholipase activation. The induction of c-Fos and c-Jun is responsible for the production of matrix metalloproteases which can precipitate inflammatory disease such as arthritis. Understanding these pathways will provide insight into designing antagonists for the detrimental activities of TNF.

There are many biological activities of TNF. TNF is able to produce cytotoxic activity on some tumor cells while not killing normal cells in vitro and in vivo (Carswell et al., 1975). TNF is able to produce cytotoxic effects at very low concentration. Inhibitors of RNA and protein synthesis enhance TNF cytotoxicity (Wallach, 1984). Cell to cell communication might be important in determining if the cells will be sensitive or resistant to the cytotoxic effect of TNF. Resistant cells are usually gap junction deficient, indicating that intracellular contacts are important for TNF cytotoxicity.
The cytotoxic effects in tumor cells are mediated by generation of free radicals which will damage the DNA (Larrick AND Wright, 1990) and by activation of lysosomal enzymes endonucleases and proteases. TNF induces catabolic effects on adipocytes by suppression of lipoprotein lipase (LPL) (Buetler et al., 1985). The suppression of this enzyme leads to a decreased uptake of exogenous lipids by adipocytes, induces the loss of triglycerides from adipose and delays the differentiation of adipocytes (Torti et al., 1985). TNF depletes lipids and causes a mobilization of stored fat, causing a turnover of glycogen and free fatty acids (Grunfeld AND Feingold, 1991). TNF also alters insulin action through the inhibition of protein kinase C which is an important step in the insulin signaling pathway (Feinstein et al., 1993). TNF was shown to suppress the glucose transporter (GLUT4) mRNA levels (Stephens AND Pekala, 1991) and stimulate lipolysis which increases free fatty acid concentration in the plasma (Grunfeld AND Feingold, 1991). All of these TNF actions could lead to impaired insulin action on glucose metabolism and clearing.

Cachexia is a wasting syndrome that was found to be induced by TNF. Protein loss and depletion of fat lead to a decrease in body mass, or cachexia. TNF was shown to produce catabolism, a decrease in the synthesis of skeletal muscle proteins (Flores et al., 1989) and suppress lipogenic enzymes in adipose tissue (Torti et al., 1985).
TNF promotes the growth of many cells by increasing the number of EGF receptors on cell membranes. This can be important in tissue repair after inflammation and wound healing (Palombella et al., 1987). In addition TNF promotes leukocyte adhesion to vascular endothelium by increasing the production of adhesion molecules. Leukocyte activation then increases the production of other cytokines (Cavender et al., 1987).

A role for TNF in various disease conditions has been suggested. In septic shock TNF is able to initiate fever, hypotension, metabolic acidosis, hyperglycemia, hyperkalemia, diffuse pulmonary inflammation, ischemic hemorrhage lesions and acute renal tubular necrosis (Jaattela, 1991). In cachexia or wasting syndromes seen in chronic infections, HIV or cancer, TNF plays a major role. It induces the metabolic changes leading to weight loss, anemia, protein and lipid depletion and anorexia (Tracy AND Cerami, 1993). Recently TNF involvement in non insulin dependent diabetes (NIDDM) was suggested because TNF might alter insulin action (Spiegelman AND Hotamisligil, 1993).
TNF and the Heart

TNF is a cardiac depressant substance. The negative inotropic effects of this cytokine are well established in vivo and in vitro. Cardiac abnormalities are evident in patients with septic shock (Parrillo, 1990). These abnormalities include depression of left and right ejection fraction, ventricular dilatation, and altered Frank-Starling and diastolic pressure volume relationships (Parker et al., 1989). The cardiac depression in septic shock is not caused by endotoxin but requires macrophages (Salari AND Walker, 1989). Also serum from septic patients inhibits contraction of rat cardiac myocytes (Parrillo, 1990), indicating that the depressant substance is a circulating factor. When TNF is administered to dogs, cardiac function decreases, and left ventricular ejection fraction and contractility diminish (Natanson et al., 1989b) indicating the role of TNF in the production of cardiac depression. When myocytes are incubated with lymphocyte conditioned media, isoproterenol- induced contraction is inhibited up to 65% of the maximum response (Gulic et al., 1989). Incubation of myocytes with TNF alone is able to inhibit spontaneous beating and isoproterenol-stimulated cAMP accumulation (Hollenberg et al., 1993). When an anti TNF antibody is administered to patients with septic shock, improvement in cardiovascular performance occurs (Vincet et al., 1992). The mechanisms by which TNF produces cardiac depression are not well
established. Some suggest that LPS conditioned media affects β-adrenergic receptor coupling to adenyl cyclase without changing the quantity of Gi, or the Bmax or Kd of β-receptors (Chung et al., 1990). Others suggest TNF decreases β-adrenergic receptor density and increases quantity of Gi subunits (Reithmann et al., 1991a). TNF also can produce its cardiovascular effect by the release of secondary mediators (Odeh, 1993). TNF increases calcium independent nitric oxide synthase (iNOS), leading to an increase in nitric oxide (NO) production (Schulz et al., 1992). The increased concentration of NO in myocytes leads to increased cGMP concentration which produces a decrease in cardiac contractility (Finkel et al., 1992). TNF also promotes the release of oxygen free radicals, which can depress cardiac function and contractility (Prasad et al., 1989). TNF can also make cells more sensitive to injury produced by the free radicals by interfering with oxidant buffering capacity (Zimmerman et al., 1989). Scavengers of free radicals might protect the heart from the deteriorating effect of TNF (Odeh, 1993). TNF induces abnormalities of calcium homeostasis in feline myocytes, leading to a decrease in cardiac contractility. These changes in calcium homeostasis results from induction of the sphingomyelinase pathway (Oral et al., 1996).
TNF in Cardiovascular Disorders

Severe myocardial infarction results in TNF release into the circulation, because levels are elevated in patients with complicated myocardial infarction, whereas in patients with moderate infarcts TNF levels are not altered (Maury and Teppo, 1989). TNF might play a role in the disorder by promoting oxygen free radicals, NO and neutrophil adherence to the endothelial cells, which increase phagocytosis, degranulation and cytotoxicity (Beutler and Cerami, 1987). Neutrophils play a role in myocardial injury. Neutrophil infiltration is demonstrated in stunned myocardium after myocardial infarction (Kloner, 1989). Neutrophil infiltration and activation in the previously ischemic region results in extension of myocardial injury (Simpson et al., 1980). TNF also stimulates gene expression and protein synthesis of cytokines and interleukins which can produce an effect on neutrophil chemotaxis and activation (Strieter et al., 1989). TNF is an important myocardial depressant substance in myocardial infarction or ischemia.

TNF and CHF

In CHF the role of TNF is not established. TNF was first demonstrated in serum of CHF patients about a half decade ago (Levine et al., 1990). The amount of TNF is the highest in CHF patients with cachexia and increased
plasma renin activity (Levine et al., 1990). The role of TNF in cardiac cachexia was also suggested by McMurray (McMurray et al., 1991). This study confirmed that patients with cardiac cachexia and CHF have elevated TNF levels compared to non cachectic patients (McMurray et al., 1991). Elevated levels of TNF were detected in 35% of patients with dilated cardiomyopathies and in 20% of patients with hypertrophic cardiomyopathies (Matsumori et al., 1993). In a recent study, the levels of TNF in patients with left ventricular dysfunction increased as the heart failure symptoms worsened, and a possible correlation exists between survival and levels of TNF (Oral et al., 1995). Although the relationship of TNF plasma levels and survival did not show statistical significance the trend is apparent (Oral et al., 1995). The functional role of TNF in heart failure is suggested to involve NO synthesis. Elevated TNF and neopterin levels in CHF patients confirmed TNF involvement in NO synthesis (Wiedermann et al., 1993). Also elevated levels of TNF correlated to forearm blood flow response to acetylcholine and nitroglycerin (inducers of NO) in CHF patients (Katz et al., 1994).

The other potential effects of TNF in CHF include the production of pulmonary edema, cardiomyopathies, left ventricular dysfunction, cachexia, and β-receptors uncoupling from adenylyl cyclase and G proteins (Oral et al., 1995). TNF might play a role in left ventricular remodeling. When TNF is infused into dogs, significant LV dilatation occurs (Natanson et al., 1989b). TNF alters
diastolic elastic properties of the ventricle, resulting in an increase in LV chamber distention and a resultant decrease in LV chamber compliance (Pagani et al., 1992). On the biochemical levels, TNF produces stimulation of extracellular matrix proteins such as collagen, fibronectin and collagenase (Owens and Grimes, 1993). TNF effects on cardiac metabolism involve an increased expression of lipoprotein lipase in animal models (Semb et al., 1987). This might lead to an increase in triglyceride derived free fatty acid availability and utilization leading to an increase in myocardial oxygen demand (Oral et al., 1995).

Both types of TNF receptors are found in cardiac tissue (Torre-Amione et al., 1995). The extracellular domains of both of the receptors are shed from the cell surface of cardiac and non-cardiac tissues and are found as the soluble forms in the urine and serum (Ferrari et al., 1995). In CHF patients, soluble TNF receptor levels are elevated in CHF patients with NYHA class IV. The soluble TNF receptors are a stronger predictor of short term prognosis than the level of TNF itself or take levels of other cytokines (Bachetti et al., 1995). These receptors can bind circulating TNF and prevent it from binding to membrane receptors. Alternatively, the binding of TNF to soluble receptors leads to a decreased dissociation of TNF to inactive monomers. The study of the soluble TNF receptors and TNF levels can provide a better understanding of the activation of this cytokine in the CHF patients (Ferrari et al., 1995).
Although the increased level of TNF in CHF is well established, the role that TNF might play in CHF is not clear. TNF might be an indicator of severity of the disease, or the role of TNF might be of pathogenic nature. Many aspects of the disease can be attributed to TNF actions, but evidence that explains its role is limited (Oral et al., 1995). It is possible that TNF is secreted as a result of elevated levels of neurohormones during the compensatory state, leading to tissue injury resulting in activation of mononuclear cells which will increase expression of the cytokine TNF (Mann AND Young, 1994). Understanding the role of TNF in CHF can provide a better future treatment of CHF. The inhibition of cytokine production may lead to improvement of this complicated disease.
CHAPTER 2

PHARMACOLOGICAL MODULATION OF MYOCARDIAL
TUMOR NECROSIS FACTOR α PRODUCTION BY
PHOSPHODIESTERASE INHIBITORS IN SD RATS

INTRODUCTION

Congestive heart failure (CHF) is a disease that affects nearly 4 million Americans, with 400,000 new cases diagnosed each year (Ho et al., 1993). The treatment of heart failure has undergone considerable change in recent years, with many new drugs being investigated and used in its treatment (Garg et al., 1993). The main goal of the treatment is to increase cardiac contractility and to reduce left ventricular filling pressure. Traditional therapy of congestive heart failure includes digitalis glycosides, diuretics and sodium restriction along with addition of vasodilators (Rocci, Jr. AND Wilson, 1987). Because the digitalis glycosides have a very low therapeutic index and high potential for toxicity (Colucci et al., 1986a), a search for non-glycoside positive inotropic agents has been going on for the past two decades.
One of the therapeutic approaches for the treatment of CHF is the use of phosphodiesterase (PDE) inhibitors. PDE inhibitors have been recognized as potent inotropic and vasodilating drugs (Fischer et al., 1992). These potent inotropic agents are thought to augment cardiac contractility by increasing the concentration of intracellular cyclic AMP (cAMP) in the failing heart, which in turn enhances the entry of calcium into cells by activating membrane bound calcium channels (Packer, 1993). In humans, type III PDE inhibitors amrinone and milrinone induce improvement in left ventricular end-systolic volume and cardiac index after acute administration to patients (Konstam et al., 1986b; Baim et al., 1983). Despite these hemodynamic changes there is usually no significant increase in heart rate or decrease in mean arterial blood pressure (Ward et al., 1983). However, because some PDE inhibitors are also beneficial to the patient at lower doses that do not produce direct inotropic effects, it has been proposed that other mechanisms of action may be involved in producing the beneficial effects of PDE inhibitors (Matsumori et al., 1993).

Tumor necrosis factor-α (TNF) is a pleiotropic cytokine that produces cardiac depression in septic shock (Tracey AND Cerami, 1994). Recent studies in humans show an increase in circulating level of TNF in patients with heart failure (Levine et al., 1990). This provides a possibility that TNF may contribute to exercise intolerance and the impairment of left ventricle function.
The failing heart itself may produce and release TNF, which might act locally. When TNF acts on the cardiac receptors it causes an increase of inducible nitric oxide synthase (Nakayama et al., 1994). The resulting increase in nitric oxide within the heart not only may inhibit the contractility of myocardial cells (Finkel et al., 1992) but also may be cytotoxic because it can trigger apoptosis (Pinsky et al., 1995). In rat cardiac myocytes TNF decreases β-adrenergic receptor density and increases the quantity of inhibitory G-protein (Reithmann et al., 1991b).

PDE inhibitors have been demonstrated to block TNF gene transcription and consequently protein production (Molnar-Kimber et al., 1993). Amrinone and pentoxifylline (PTX) decrease serum levels of TNF and improve survival in endotoxin challenged rodents (Noel et al., 1995; Giroir AND Bruce, 1992). The inhibition of TNF production by PDE inhibitors might play a role in the acute beneficial effect of some PDE inhibitors in congestive heart failure.

The majority of studies performed to date have concentrated on the effect of PDE inhibitors on TNF production in leukocytes from human or animal models (Molnar-Kimber et al., 1993; Semmler et al., 1993; Foster AND McCormic, 1995) The production of TNF by cardiac tissue and the effect of PDE inhibitors on TNF production from cardiac tissue have not been investigated. In this study we report the effect of PDE inhibitors on TNF secretion from rat left ventricle. Because rat cardiac tissue contains predominantly type III and
IV PDE (Weishaar et al., 1987; Shahid AND Nicholson, 1990) we decided to study the effect of type III and type IV PDE inhibitors on TNF secretion from rat left ventricle.
METHODS

Experimental animals. All animals used in the experiments were male Sprague Dawley (SD) rats weighing between 250 to 350 g and were purchased from Harlan, (Indianapolis, IN). The animals were maintained for 5 days before experimentation. The animals were allowed food and water ad libitum.

Left ventricle tissue preparation. Rats were anesthetized with sodium pentobarbital (100 mg/kg i.p.). The thoracic cavity was opened, and the heart was removed and perfused through a cannulated aorta with 50 ml sterile medium consisting of 50% Dulbecco's modified Eagle's medium (DMEM) and 50% F12, supplemented 2.45 g/L with sodium bicarbonate and antibiotics. The left ventricle (LV) was dissected, minced with a razor blade into 1mm X 1mm sections and rinsed thoroughly with DMEM media to remove any excess blood. Then the LV was weighed, divided into pieces of equal weight and incubated for 4 h at 37°C in a gassed incubator (5% CO₂ / 95 % air ), in 2 ml DF5 media (DMEM/F12 + 5% fetal bovine serum (FBS) + 1% penicillin/streptomycin) in the presence or absence of PDE inhibitors or LPS (1μg/ml). DF5 media was used in all the experiments. Then the incubation media was collected under sterile conditions and was frozen at -80°C before evaluation for TNF-α quantity.
Preparation of other tissues. Rats were anesthetized with sodium pentobarbital (100mg/kg i.p.). The thoracic and abdominal cavities were opened and the lungs, spleen, thymus and retroperitoneal adipose tissue were removed, minced and washed. Then the tissues were weighed and divided into two sections, one which was incubated with LPS (1µg/ml) and the other was incubated without LPS in DF5 media. After 4 hours the incubation media was collected and was frozen at -80°C before evaluation for TNF quantity.

TNF determination. Cytotoxicity assay was performed as previously described by Matthews and Neale (Matthews and Neale, 1987). Briefly, L929 cells were grown in RPMI medium with 5% FBS and antibiotics in 96 well culture plates. The cells were allowed to incubate at 37°C overnight. The next day actinomycin D (1µg/ml) was added to the wells, and conditioned media were applied. After another overnight incubation, media was decanted from the cells, the cells were fixed with 5% formaldehyde in phosphate buffered saline (PBS) for 5 min, and then stained with 0.5% crystal violet for 5 min. After washing and drying, the extent of cytotoxicity was determined using a SLC Spectra plate reader by measuring the absorbance at 580 nm after solubilizing cells in 150 µl of 33% glacial acetic acid.
**Antibody studies.** To determine the antibody titer, the L929 cells were incubated with various concentrations of rabbit anti-mouse antibody and 0.1 ng mouse TNF for 24 hours.

To determine the effect of the neutralizing antibody on TNF secretion from rat left ventricle, L929 cells were incubated with 75 µg/ml rabbit anti-mouse antibody and the media that contained unstimulated or LPS-induced TNF from the left ventricle tissue. The effect of the neutralizing rabbit anti-mouse antibody was determined after 24 hours.

**Polymyxin B studies.** Rat left ventricle tissue was incubated in DF5 media with polymyxin B (20 µg/ml) or media alone for 4 hours, then the media samples were collected and were frozen at -80°C. The amount of secreted TNF was determined by cytotoxicity assay.

**Myeloperoxidase Assay.** The assay was performed as described by Bradley (Bradley et al., 1982).

**Drug treatment.** Left ventricle tissue was incubated with the following drugs: etazolate (type IV PDE inhibitor), Ro-20,1724 (type IV inhibitor) amrinone (type III inhibitor) milrinone (type III inhibitor), pentoxifylline (non-selective PDE inhibitor), dexamethasone, isoproterenol and dibutyryl-cAMP. Concentrated stocks of these drugs were made as described below. Etazolate was dissolved in distilled water. Ro-20,1724 was dissolved in 4.5% (w/v) aqueous 2-hydroxypropyl-β-cyclodextrin and diluted in DF5 media, the
final concentration of 2-hydroxypropyl-β-cyclodextrin in media was 4.5% or lower. In some experiments the solvent used to dissolve RO-20,1724 was ethanol, which was also diluted with DF5 media to final concentration of 10% or less. Amrinone was supplied as an injectable lactate salt which was dissolved in water supplemented with metabisulfite preservative (25 mg/ml). Milrinone was supplied as a lactate salt which was dissolved in water containing dextrose. Pentoxifylline, dexamethasone, isoproterenol and dibutyryl-cAMP were dissolved in water. Stock solutions were diluted in DF5 and sterilized by passing through a 0.2 micron filter prior to addition to L929 cells.

**Materials.** FBS, DMEM, F-12, PBS, mouse recombinant TNF, penicillin/streptomycin and RPMI media, were obtained from Gibco-BRL (Grand Island, NY). Actinomycin D, LPS, dexamethasone, isoproterenol and dibutyryl-cAMP were obtained from Sigma Chemicals Co. (St. Louis, MO). L929 cells were obtained from ATCC (Rockville, MD). Etazolate and RO-20,1724. were obtained from RBI (Natick, MA). Amrinone and milrinone were obtained from Sanofi-Winthrop (New York, NY). Pentoxifylline was obtained from Hoechst-Roussel Pharmaceuticals (Somerville, NJ). Rabbit Anti-Mouse TNF antibody was obtained from Calbiochem (San Diego, CA).
**Statistical analysis.** A one-way analysis of variance followed by Dunnett’s test was used to analyze the amount of TNF secretion at different time points (Fig. 2). Paired Student’s *t* test was used to compare the amount of unstimulated vs LPS-induced TNF release from individual tissues (Fig. 6). To compare the unstimulated and LPS-induced TNF release various tissues, a one way ANOVA followed by Newman-Keuls multiple comparison test was used (Fig. 10). Differences at *P*<0.05 were considered significant. IC\textsubscript{50} values were obtained from dose-response curves, utilizing non-linear curve fitting programs of Graph Pad software (San Diego, CA). Data are expressed as mean ± SEM.
RESULTS

The results demonstrated in figure 1 represent a typical mouse TNF standard curve obtained from a cytotoxicity assay. The optical density (OD) was measured after 24 hr incubation of L929 cells with various concentration of mouse TNF. The highest concentrations of mouse TNF (4x10^2 ng) produced a total death of L929 cells corresponding to a very low OD (0.026). The lowest concentration (2x10^5 ng) of mouse TNF produced a minimal cell death, corresponding to a high OD (0.695) which was not different from a sample without mouse TNF (0.701). Accurate measurements of TNF ranged from 0.1-10 pg of mouse TNF/ well. Although L929 cells sensitivity to rat TNF may differ from mouse TNF, all subsequent rat samples were calculated in mouse TNF values. (Note: At the time these studies were initiated, rat TNF standard was not available). The results demonstrated in figure 2 represent the amount of TNF secreted from rat left ventricular tissue at different time points. TNF secretion was measured at one hour intervals for 6 hours. Statistically significant amounts of TNF were secreted after 4 hours of incubation. All subsequent studies were performed at that time point. Prolonged incubation revealed that TNF secretion plateaus at 8-12 hours (data not shown). To
confirm that ventricular tissue was in fact secreting TNF and that conditioned media was not inducing cytotoxicity by a non-TNF mediated mechanism, antibody neutralization experiments were performed. Figure 3 represents the effect of rabbit anti-mouse antibody on cytotoxicity induced by exogenously administered mouse TNF. The cytotoxic effect of 0.1 ng of mouse TNF was neutralized in a concentration dependent manner by TNF antibody. A concentration of 6.25 μg/ml of antibody inhibited TNF induced cytotoxicity 66%, and total neutralization of TNF cytotoxicity occurred at 50 μg/ml of antibody. A non-immune rabbit IgG (6.25 μg/ml) which was used to determine whether neutralization of TNF was antibody specific, did not inhibit TNF induced death (data not shown), suggesting neutralization is TNF antibody specific.

The antibody was then coincubated with conditioned media from unstimulated and LPS stimulated rat left ventricle, resulting in inhibition of conditioned media induced cytotoxicity (figure 4). Cytotoxicity induced by conditioned media from unstimulated LV was inhibited by the antibody by 77.5±3.3%; inhibition which is consistent with manufacturers specifications of 85% inhibition of rat TNF. LPS-induced cytotoxicity was also inhibited by 70%.

To determine whether unstimulated TNF release from the left ventricular tissue occurred due to possible endotoxin contamination of the incubation media, we used polymyxin-B, an endotoxin inhibitor (20 μg/ml), in our
incubation media. TNF secretion from LV tissue that was incubated in polymyxin-B containing media for 4 hours was not reduced, suggesting there was no endotoxin contamination (polymyxin-B = 0.0381±0.0108 ng TNF; control 0.0106±0.004 ng TNF.) (figure 5).

To assure that TNF was secreted from cardiac cells and not from inflammatory cells, myeloperoxidase assay was performed. The tissue did not contain detectable amounts of myeloperoxidase (figure 6).

In figure 7, the inhibition of unstimulated TNF secretion from heart tissue was evaluated, using different pharmacological manipulations. Dose response curves were generated using type III and type IV PDE inhibitors. Ro-20,1724, a type IV PDE inhibitor was the most potent inhibitor of unstimulated TNF secretion (IC_{50}=1.87x10^{-6} M), but it only produced a maximum of 60% inhibition of basal TNF secretion. Amrinone, a type III PDE inhibitor, was less potent (IC_{50}= 1.39x10^{-5} M) but was more effective, producing an 80% inhibition of basal TNF secretion. Milrinone was the least potent of the three drugs (IC_{50}=1.53x10^{-4} M). Table 1 represents IC_{50} values of five PDE inhibitors tested. The 50% inhibition concentrations were obtained for unstimulated TNF secretion.

Figure 8 represents the effect of three PDE inhibitors on LPS-induced TNF secretion. The three drugs elicited a maximum of 80% inhibition of LPS-induced TNF secretion. Amrinone was the most potent inhibitor
Milrinone (IC₅₀ = 8.16 x 10⁻⁵ M) proved to be less potent than amrinone but more potent than pentoxifylline (IC₅₀ = 7.48 x 10⁻⁴ M), a non-selective PDE inhibitor. Table 2 represents IC₅₀ values obtained for LPS-induced TNF secretion for five PDE inhibitors tested. Type IV PDE inhibitors were the most potent inhibitors of unstimulated TNF release, while type IV phosphodiesterase inhibitors had no effect on LPS-induced TNF secretion in these studies.

In addition, 3 drugs, not of the PDE inhibitor class were tested for their ability to block unstimulated TNF secretion (figure 9). Dexamethasone, a corticosteroid, is a known inhibitor of TNF gene transcription and translation (Han et al., 1990). At a concentration of 10⁻⁶ M, it produced 95 ± 2% inhibition of unstimulated TNF secretion from the left ventricular tissue. Isoproterenol, a non selective β-adrenoceptor agonist, at 10⁻⁶ M produced 65 ± 5% inhibition of basal TNF secretion from left ventricular tissue. Dibutyryl-cAMP, a non-hydrolyzable cAMP analog, at 10⁻⁶ M produced 70 ± 8% inhibition of unstimulated TNF secretion from left ventricular tissue.

In order to compare the amount of TNF secretion from left ventricular tissue to other rat tissues, we isolated a number of other tissues, incubated them for 4 hours with or without LPS stimulation and then determined the amount of TNF secretion. Figure 10 demonstrates the amount of TNF secreted from tissues of six SD rats. The lung secreted the largest amount of TNF. If lung
data were removed from ANOVA due to large variability in data, then the LV secreted significantly more unstimulated TNF than adipose and thymus and approximately equal amounts as the spleen when normalized to tissue weight.

LPS induced a significant TNF secretion in all tissues except spleen.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Type</th>
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</thead>
<tbody>
<tr>
<td>Ro-20,1724</td>
<td>IV</td>
<td>1.87 ± 1.19*</td>
</tr>
<tr>
<td>Etazolate</td>
<td>IV</td>
<td>2.07 ± 1.55</td>
</tr>
<tr>
<td>Amrinone</td>
<td>III</td>
<td>13.95 ± 4.45</td>
</tr>
<tr>
<td>Milrinone</td>
<td>III</td>
<td>153.90 ± 47.73</td>
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<tr>
<td>PTX</td>
<td>non</td>
<td>201.40 ± 93.92</td>
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</table>

* Mean ± SEM


<table>
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<th>Type</th>
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<tr>
<td>Ro-20,1724</td>
<td>IV</td>
<td>&gt; 1000</td>
<td></td>
</tr>
<tr>
<td>Etazolite</td>
<td>IV</td>
<td>&gt;1000</td>
<td></td>
</tr>
<tr>
<td>Amrinone</td>
<td>III</td>
<td>14.86 ± 2.96&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Milrinone</td>
<td>III</td>
<td>81.65 ± 24.21</td>
<td></td>
</tr>
<tr>
<td>PTX</td>
<td>non-selective</td>
<td>748.83± 248.00</td>
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</tr>
</tbody>
</table>

<sup>a</sup> Mean ± SEM
Figure 1. A typical standard curve obtained from the cytotoxicity assay. This standard curve is performed on each plate of the cytotoxicity assay. Mouse TNF is applied to L929 cells at various concentrations. After 24 hours the cells are fixed and stained with crystal violet. The stained plate is read on the plate reader at 595 nm. The optical density is used to measure the amount of cell viability. Higher optical density values correspond to greater cell staining, indicating less TNF induced cell death.
Figure 2. Unstimulated TNF production by rat left ventricular tissue. Rat left ventricular tissue was minced then incubated for various time points in media. The conditioned media was collected at indicated time points and assayed for TNF by cytotoxicity assay. The data represent the mean ±SEM of four experiments. * Time points were significantly different from one hour time point P ≤ .01.
Figure 3. A titer curve for TNF antibody (Ab). Mouse TNF was neutralized by various concentrations of rabbit antimouse antibody. The antibody was applied for 24 hours to L929 cells containing 0.1 ng of mouse TNF. The neutralizing effect of the antibody was determined by comparing the OD to control sample without TNF.
Figure 4. The neutralizing effects of rabbit anti-mouse antibody on TNF secretion from rat left ventricle. The left ventricular tissue was incubated alone or with LPS for 4 hours. The conditioned media was collected and incubated with L929 cells in the presence or absence of antibody (75μg/ml), and the amount of secreted TNF was determined by cytotoxicity assay. Basal (unstimulated TNF release), LPS (treated with 1μg/ml LPS). The responses are represented as percent of control. The data represents the mean±SEM of four experiments.
Figure 5. Myocardial TNF production after polymyxin-B treatment. LV tissue was incubated for four hours in media containing polymyxin-B (20μg/ml). TNF content was determined and compared to control. The data represent the mean ±SEM of four experiments.
Figure 6. Myeloperoxidase assay. The amount of myeloperoxidase activity was determined in the LV tissue. $10^6$ white blood cells were used as a positive control. The data represent mean ± SEM of six experiments.
Figure. 7. Effect of PDE inhibitors on unstimulated TNF secretion. TNF secretion from left ventricular tissue was dose dependently inhibited after incubation with Ro-20,1724, amrinone and milrinone. The ventricular tissue was incubated for 4 hr in DF5 media at 37°C with different concentration of drugs. The data points represent the mean ±SEM of four, six and five experiments for Ro-20,1724, amrinone and milrinone, respectively.
Figure 8. Effect of PDE inhibitors on lipopolysaccharide (LPS)-induced TNF secretion. Amrinone, milrinone and pentoxifylline produced a dose dependent inhibition of LPS-induced TNF production from SD rat left ventricular tissue. The ventricular tissue was incubated for 4 hours in DF5 media at 37°C with LPS (1 µg/ml) and various concentration of drugs. The data points represent the mean ± SEM of six, five and three experiments for amrinone, milrinone and pentoxifylline, respectively.
Figure 9. Inhibition of unstimulated TNF secretion by dexamethasone (DEX), isoproterenol (ISO) and dibutyryl-cAMP (Dibut-cAMP). Rat left ventricular tissue was incubated for 4 hours with DEX, ISO or Dibut-cAMP each at $10^{-6}$ M in DF5 media at 37°C. Responses were calculated as percent inhibition. The data points represent the mean ± SEM of five experiments.
Figure 10. TNF secretion from various tissues of Sprague Dawley rat. Various rat tissues were minced and incubated with or without lipopolysaccharide (LPS) (1 μg/ml), for 4 hours at 37°C in DMEM media. The conditioned media was collected and assayed for TNF using the cytotoxicity assay. The data points represent mean±SEM of six experiments. * Indicates LPS-induced TNF release is significantly different than unstimulated TNF release from a given tissue (P<.05), † Indicates unstimulated TNF release is significantly different from lung (P< .05).

LV=Left ventricle
Cytokines participate in many important immunological pathways in response to pathogens or endogenous stimuli. Understanding how to pharmacologically modulate cytokine production or secretion may prove to be a beneficial therapeutic approach in some disease states. TNF is a cytokine that can cause many biological responses, including a decrease in myocardial contractility. Studies show TNF serum levels to be elevated in some patients with heart failure (Levine et al., 1990; McMurray et al., 1991; Dutka et al., 1993). The role that TNF might play in this condition is not clear, but drugs that inhibit TNF secretion, like the type III PDE inhibitors, amrinone, milrinone and vesnarinone, might be of beneficial effect in heart failure conditions (Matsumori et al., 1993).

In order to understand the role that TNF might play in pathological conditions, we decided to study first TNF secretion by non-pathologic left ventricle and the effect of different pharmacological manipulations on the secretion of that cytokine. In the present study, we demonstrated that rat left ventricle could secrete TNF under unstimulated conditions and after LPS stimulation. Initially, we had not anticipated TNF secretion from non-LPS stimulated
myocardium, because it was previously reported that minced myocardium from mice did not produce a spontaneous release of TNF (Giroir et al., 1992). However antibody neutralization experiments support the fact that the cytotoxic substance released by the rat myocardium is, in fact, TNF. The unstimulated TNF could be secreted because of species difference in response to stress, hypoxia, injury or a combination of all three factors induced by tissue mincing. Indeed a recent report (Herskowitz et al., 1995) demonstrates TNF gene expression in rat myocardial vascular endothelium following 15 minutes of experimentally induced ischemia, suggesting ischemia may be a mechanism of TNF induction.

Because myeloperoxidase activity was not detected in the minced LV tissue this supports the hypothesis that TNF was secreted from the myocardial tissue and not adherent inflammatory cells.

Although PDE inhibitors have been reported to decrease LPS-induced TNF release from leukocytes in vitro (Molnar-Kimber et al., 1993; Prabhakar et al., 1994) and following LPS administration in vivo (Chalkiadakis et al., 1985), the ability of PDE inhibitors to alter cardiac TNF production (either unstimulated or LPS stimulated) has not been previously reported. In order to minimize the release of TNF from adherent leukocytes (which contain primarily type IV PDE), and measure TNF primarily of myocardial cell origin, the left ventricular tissue was perfused and washed thoroughly. Rat cardiac tissue contains type
III PDE (Weishaar et al., 1987), type IV PDE, some type I and type II PDE (Shahid et al., 1989) but only the combined inhibition of type III and IV PDEs results in an inotropic effect (Nicholson et al., 1991). Therefore, both the type III and IV PDEs appear to play an important role in cardiac function (Shahid AND Nicholson, 1990). In our study we decided to test the effect of type III and IV PDE inhibitors on TNF secretion from rat left ventricle.

The mechanism of action of PDE inhibitors in the heart is not clear with respect to inhibition of TNF secretion. In mononuclear cells, inhibition of PDE (measured by ability to hydrolyze cAMP) correlates to a high degree with suppression of TNF secretion by PDE inhibitors (Semmler et al., 1993). Supernatant fractions from homogenized rat ventricles showed an inhibition (IC₅₀ = 1.256X10⁻⁶ M) of PDE IV by rolipram, a type IV PDE inhibitor (Shahid AND Nicholson, 1990). This IC₅₀ value was very similar to the IC₅₀ values that were determined in our studies using the type IV PDE inhibitors, etazolate and Ro-20,1724, for inhibition of unstimulated TNF secretion. Even in human cardiac ventricles, the inhibition of PDE type IV (IC₅₀ = 3.8X10⁻⁶ M) by Ro-20,1724 (Reeves et al., 1987) was very similar to the IC₅₀ that we determined in rat heart for unstimulated TNF secretion by Ro-20,1724. This supports the possibility that the mechanism by which type IV PDE inhibitors inhibit TNF release in the heart is through inhibition of PDE and subsequent increase in cAMP. The IC₅₀ value (30μM) for inhibition of PDE type III by amrinone in
bovine cardiac muscle (Harrison et al., 1987) was similar to the IC\textsubscript{50} value (12µM) of amrinone for inhibition of unstimulated TNF secretion from rat heart in our studies. An additional study in dog heart showed an inhibition of type III PDE by amrinone to have a very similar IC\textsubscript{50} (23µM) to the IC\textsubscript{50} value of amrinone from our studies. The IC\textsubscript{50} values for milrinone values in bovine heart (Harrison et al., 1987), dog aorta (Pagani et al., 1995) and rat ventricular myocardium (Shahid AND Nicholson, 1990) were much lower for cAMP generation than the IC\textsubscript{50} value determined by our study for unstimulated TNF secretion. Future studies will be necessary to explain this difference.

Pentoxifylline, a non selective PDE inhibitor, produced a significant increase in cAMP level in isolated rat heart (Vittone et al., 1980). The IC\textsubscript{50} (10^{-4}M) to increase cAMP in this study was similar to the IC\textsubscript{50} (2X10^{-4} M) values we obtained for PTX inhibition of unstimulated TNF secretion. Again this supports the hypothesis that the mechanism of action of PTX in the heart with respect to TNF production could be through PDE inhibition and an increase in intracellular cAMP. cAMP depresses the accumulation of TNF mRNA by inhibiting the transcription processes. Some studies show that in order for cAMP to depress TNF mRNA accumulation the promoter and 3'-untranslated region (3'-UTR) are required to be present (Beutler et al., 1992). We were able to demonstrate that, in the rat heart, a cAMP analog, dibutyryl cAMP, and a nonselective β-adrenoceptor agonist, isoproterenol, were able to inhibit
TNF secretion from the left ventricle. Dexamethasone was used as a positive control, because it is a known inhibitor of TNF production via a non-cAMP pathway. Because we were able to show that drugs which activate cAMP by mechanisms other than PDE inhibition can produce an inhibition of TNF secretion, these results support the possibility that inhibition of TNF secretion by PDE inhibitors in the heart might be mediated through a cAMP pathway. Further studies that directly measure the level of cAMP in the myocardium when PDE inhibitors are applied are necessary to determine whether cAMP plays a role in the inhibition of TNF secretion from rat heart.

Furthermore this study demonstrates that TNF secretion from the left ventricular tissue was differentially inhibited by PDE inhibitors. Type IV phosphodiesterase inhibitors Ro-20,1724 and etazolate most potently inhibited unstimulated TNF secretion, whereas the type III PDE inhibitors, amrinone and milrinone, had a lower potency compared to the type IV PDE inhibitors. This could be because in rat heart, both type III PDE and type IV PDE are found (Karia AND Dage, 1988), and PDE IV may be playing a more important role compared to type III PDE in the hydrolysis of cAMP during basal conditions. The distribution of the type III and IV PDEs in rat heart, and consequently, the control of local cAMP concentration, might be such that type IV inhibitors produce an efficient inhibition of TNF formation at low
concentrations of the drugs. Also, with respect to TNF inhibition, there is an apparent difference in potency of the two type III PDE inhibitors, with amrinone showing greater potency than milrinone. This is particularly interesting due to the fact that milrinone is reported to be more potent at inhibiting the enzymatic activity of PDE than amrinone in other animal models (Harrison et al., 1987).

The type IV PDE inhibitors produced a different inhibition pattern on LPS-induced secretion of TNF from the left ventricle. PDE type IV inhibitors were the most potent in blocking unstimulated TNF secretion from left ventricular tissue, but these drugs were unable to inhibit LPS-induced TNF secretion from left ventricular tissue. This is in marked contrast to effects of type IV inhibitors in the inhibition of LPS-induced TNF release from white blood cells as demonstrated by Prabhakar (Prabhakar et al., 1994). Amrinone was less potent at inhibiting unstimulated TNF secretion than the type IV inhibitors, but it was the most potent inhibitor of LPS-induced TNF secretion from left ventricle. The reasons for the different inhibition profiles are unknown.

Unstimulated secretion of TNF could be due to the surgical procedure that is used to remove and mince the heart, whereas LPS-induced TNF secretion could occur by an additional mechanism. For example, in human monocytes, LPS-induced TNF secretion occurs through the activation of protein kinase C.
and protein tyrosine kinase upstream to activation of the TNF gene transcription (Shapira et al., 1994). Alternatively LPS could indirectly activate type III PDE or inactivate type IV PDE in the heart, resulting in a new equilibrium among PDEs contributing to intracellular cAMP content. LPS has been shown both to increase and decrease PDE activity in murine macrophages (Okada et al., 1995; Okonogi et al., 1991). This could explain why type IV inhibitors did not inhibit LPS-induced TNF secretion but inhibited unstimulated TNF secretion and type III inhibitors inhibited both unstimulated and LPS-induced TNF secretion. Future studies of the different types of PDE that are present in the rat heart during unstimulated conditions versus LPS stimulation condition are necessary.

The IC_{50} values for PDE inhibitors in the LPS-induced TNF secretion studies were different from the values reported in previous studies. Although IC_{50} values have not been reported for cardiac TNF secretion, amrinone was shown to inhibit LPS-induced TNF secretion from murine macrophages with IC_{50} =2.67 \times 10^{-4} \text{ M} (Giroir AND Bruce, 1992). The IC_{50} value for amrinone in our study was 18 times more potent for rat ventricular tissue. When PTX was used to inhibit TNF secretion from LPS-stimulated human mononuclear cells it produced 50% inhibition at 113 \mu \text{M} concentration (Semmler et al., 1993). In rat blood, the IC_{50} (20.6 \mu \text{M}) was even lower (Foster AND McCormic, 1995). In our study rat left ventricle required 208 \mu \text{M} to produce 50%
inhibition. This could be because of different intracellular localization of the diverse PDEs in left ventricle vs white blood cells, or due to the presence of both type III and IV in heart while white blood cells contain primarily type IV PDE. Also comparison of IC\textsubscript{50} values among different species makes interpretation of results difficult because multiple factors need to be considered, including PDE isoenzyme content and distribution in the tissues studied.

Though other studies were not able to show unstimulated cardiac TNF secretion (Giroir \textit{et al.,} 1992), we were able to measure and compare the amount of unstimulated TNF secreted from LV to amounts secreted by other tissues. The amount of TNF secreted from the myocardium was higher than the amount secreted from both adipose and thymus tissues and was comparable to that secreted from the spleen. This result indicates that the amount of TNF secreted from the left ventricle was not minute as compared to that secreted from other organs and may be contributing to ambient concentration of TNF in the heart, perhaps resulting in alteration of cardiac function. The LPS stimulation of TNF secretion from the left ventricle was not as high as the adipose tissue or the lung but it was still higher than the unstimulated TNF secretion.
The capacity of the lung to secrete relatively large amounts of TNF may also have important implications on LV function due to the close proximity of the lung to the LV with regard to blood flow.

This study provides evidence for the release of TNF from left ventricular tissue, and that PDE inhibitors inhibit the release of this cytokine from the myocardium. TNF release may be an important factor in producing myocardial depression seen in heart failure. It has also been postulated that some PDE inhibitors like vesnarinone at very low doses inhibit TNF release (Matsumori et al., 1993) and this may contribute to the effectiveness of this drug in heart failure. It is important to understand the ventricular tissue distribution of PDEs in healthy animals because some disease states such as heart failure alter the PDE distribution in the heart. It was previously demonstrated in dogs with heart failure, that a change from type III PDE to type IV PDE occurs in the heart tissue (Smith et al., 1994). Understanding the changes in PDE isoenzyme distribution in the heart could open an additional door to the mystery of the pathophysiology of heart failure, and thus provide an explanation about why some PDE inhibitors are more beneficial than others in the treatment of heart failure. Future studies are needed using these drugs in heart failure models and investigating the effect of TNF production in heart failure.
In summary, these experiments have demonstrated that TNF was secreted from rat left ventricle after four hours *in vitro* incubation. There was unstimulated and LPS-induced secretion. Different pharmacological manipulations were able to inhibit the TNF secretion from left ventricle. Etazolate and Ro-20,1724 (type IV PDE inhibitors) were the most potent inhibitors of unstimulated TNF secretion, whereas amrinone (a type III PDE inhibitor) was the most potent inhibitor of LPS-induced TNF secretion. The mechanism of action of these inhibitors could involve cAMP. Additional studies are necessary to determine the causes of the differential activity of these PDE inhibitors and their exact mechanisms of action. However these initial pharmacological results may be an important tool for further investigation into conditions where TNF has been reported to be elevated, for example, in myocardial depression during septic shock or congestive heart failure.
CHAPTER 3

TNF SECRETION FROM RAT CARDIAC MYOCYTES

INTRODUCTION

TNF, the pro-inflammatory cytokine, is able to produce negative inotropic effects on the heart (Yokoyama et al., 1993; Oral et al., 1995). In septic shock TNF induces cardiac depression associated with hypotension and acidosis (Natanson et al., 1989b). In dogs recombinant human TNF infusion produces a decrease in LV performance which persists for 24-48 hours (Pagani et al., 1992). Rats treated with TNF one hour prior to sacrifice show a decrease of isoprenaline induced contraction in the isolated atria (Foulkes AND Shaw, 1992). Isolated cardiac myocytes treated with TNF produced 20-30% less cell shortening than untreated myocytes which is thought to be due to a direct result of alteration in intracellular calcium homeostasis (Yokoyama et al., 1993). In rat cardiac myocytes, nitric oxide synthase (iNOS) message and protein levels were elevated after treatment with TNF indicating that the negative inotropic effects of TNF may be mediated by increased nitric oxide.
production in the heart (Pinsky et al., 1995). TNF is able to up regulate inhibitory G protein subunits and decrease β-receptor density leading to a decrease in adenylyl cyclase responsiveness (Reithmann et al., 1991a). However some believe that the above findings are controversial (Chung et al., 1990).

In order to understand TNF effects on the heart, investigating the tissue and the cellular site of TNF production is of importance. TNF mRNA and protein were found in the heart of patients with end stages dilated cardiomyopathy and patients with ischemic heart diseases (Torre-Amione et al., 1995). In previous studies, TNF was not found constitutively expressed in the heart (Giroir et al., 1992; Oral et al., 1995). However, our lab demonstrated that TNF is secreted from the LV tissue under unstimulated conditions in SD rats. This findings could be due to differences in species or the experimental procedure used. To investigate the cellular site of production of unstimulated myocardial TNF, rat ventricular cardiac myocytes were isolated and the amount of TNF secretion was measured using the cytotoxicity assay.
METHODS

Animals. All animals used in the experiments were Sprague Dawley (SD) rats weighing between 250-300 g and were purchased from Harlan, (Indianapolis IN). The animals were maintained in the OSU vivarium for 5 days before experimentation. The animals were allowed food and water ad libitum.

Cardiac Myocyte Isolation. Cardiac myocytes were isolated using the method described by Wimstatt (Wimisatt et al., 1990). Briefly, rats were injected i.p with 0.8 ml of pentobarbital (100mg/kg), and the heart and lungs were removed quickly and placed in a cold incubation buffer (1X Krebs+ Minimum essential medium (MEM) amino acids solution+ basal medium eagle (BME) vitamin solution +pen/strep + 1μM insulin + 11mM dextrose + 25mM HEPES + 5mM pyruvic acid) without any bubbles. The aorta was cannulated and tied firmly with 4-0 silk suture thread, then the heart was flushed with 1 ml cold incubation buffer. The heart was placed on a Langendorff chamber and perfused with perfusion buffer (1x Krebs + BME vitamin solution 10x + BME and MEM amino acids solution 10x + 11 mM dextrose + 25mM NaHCO₃+ 5 mM pyruvic acid+ 1 mM CaCl₂ ) in a non recirculating mode. The pH was adjusted to 7.3-7.35 and maintained in this range by continuously gassing the medium with 95%O₂ -5% CO₂ at 37°C at a flow rate of 10ml per minute. After 50ml buffer was collected, the heart was
moved to a second chamber containing the perfusion medium without CaCl\textsubscript{2} which was supplemented with 0.02 mM EGTA. The heart was perfused for 5 min in a nonrecirculating manner before the addition of collagenase. At the end of the five minutes, the collagenase (50mg type II) and BSA (50mg) were filtered through a 0.8 µ syringe filter and added to the perfusion buffer. The perfusion was performed in the recirculating mode until the end. After 20 minutes, calcium was added back to a final concentration of 2 mM to the 50ml of perfusate. After all the calcium was added, the pump flow was increased by 1-2 ml every 4-5 minutes up to a rate of 16ml/min. When the fluid levels dropped in the reservoir chamber due to a decrease in back pressure, the heart was removed from the Lagendorff chamber. The ventricles were placed in 5 ml perfusion buffer containing 5 mg/kg collagenase. The heart was minced into small pieces and incubated at 37°C in a shaking water bath with an oxygen hood for 10-15 minutes. Then the cells were repetitively drawn up through a plastic pipet to disperse chunks. Later the cells were poured through cheesecloth and rinsed with 0.5 % BSA incubation buffer twice. The cells were centrifuged for 1 min (400-750 rpm), and the supernatant was discarded after each wash. To the washed cells 4% BSA buffer was added, and cells were centrifuged for 1 min. The supernatant was discarded, and the cells were suspended in plain incubation buffer. Cells obtained were 90% rod shaped and 80% viable after 4 hours incubation.
60,000 cells were incubated in a 95% air/5% CO₂ incubator at 37°C for 4 hours in DF5 media, media with LPS (1μg/ml) or media with dexamethasone (1μM). Then the conditioned media was collected and frozen at -80°C before evaluation for TNF content by cytotoxicity assay.

**TNF determination.** Cytotoxicity assay was performed as previously described by Matthews and Neale. (Matthews and Neale, 1987). Briefly, L929 cells were grown in RPMI medium with 5% FBS and antibiotics in 96 well culture plates. The cells were allowed to incubate at 37°C overnight. The next day actinomycin D (1μg/ml) was added to the wells, and conditioned media were applied. After another overnight incubation, media was decanted from the cells, the cells were fixed with 5% formaldehyde in phosphate buffered saline (PBS) for 5 min, and then stained with 0.5% crystal violet for 5 min. After washing and drying the extent of cytotoxicity was determined using a SLC Spectra plate reader by measuring the absorbance at 580 nm after solubilizing cells in 150 μl of 33% of glacial acetic acid.

**Drugs.** Concentrated stocks Ro-20,1724 (a type IV inhibitor) and amrinone (a type III inhibitor) were made as described below. Ro-20,1724 was dissolved in 4.5% (w/v) aqueous 2-hydroxypropyl-β-cyclodextrin and diluted in DF5 media, the final concentration of 2-hydroxypropyl-β-cyclodextrin in media was 4.5% or lower. Amrinone was supplied as an injectable lactate salt which was dissolved in water supplemented with metabisulfite preservative (25 mg/ml).
Data Analysis. Data were expressed as mean± SEM. The IC$_{50}$ values were obtained from dose-response curves, utilizing non-linear curve fitting programs of Graph Pad software (San Diego, CA). ANOVA following by Newman-Keuls was performed using the Number Cruncher Statistical System (NCSS Jerry Hintze, Kaysville UT).
Because we were able to show in our previous studies that the left ventricle tissue was able to secrete TNF, we decided to determine the exact cellular site of TNF secretion. The cardiac myocytes were isolated from the ventricles of young (3 months old) Sprague Dawley rats. The myocytes secreted bioactive TNF which was quantified using the cytotoxicity assay. LPS was able to increase TNF secretion by 60% compared to control after four hours of incubation, and dexamethasone was able to inhibit TNF secretion by 61% compared to control after four hours of incubation (figure 11). ANOVA did not show significance due to variability within the groups.

In order to rule out endotoxin contamination, polymyxin (20µg/ml) was used. Cells were incubated for five hours with media containing polymyxin, then the cells were washed with PBS, and incubated in new media without polymyxin for 4 hours. Polymyxin treatment abolished LPS stimulation of myocardial TNF secretion, but it did not affect the secretion of TNF from non stimulated myocytes nor the dexamethasone inhibition of myocardial TNF secretion (figure 12).
The phosphodiesterase inhibitor, amrinone, inhibited TNF secretion from the isolated myocytes in a dose dependent fashion (Figure 13). Amrinone potency (8.2x10^{-9} M) was similar to the concentration needed to inhibit TNF from minced LV (1.3x10^{-5} M) (see chapter 2). Ro-20,1724, a type IV phosphodiesterase inhibitor also inhibited TNF secretion from cardiac myocytes. Ro-20,1724 (figure 14) was a more potent inhibitor (1.2x10^{-6} M) of TNF secretion than amrinone, but its efficacy tended to be lower than amrinone (Table 3).
Table 3. Potency and efficacy of phosphodiesterase inhibitors.

<table>
<thead>
<tr>
<th></th>
<th>Amrinone</th>
<th>Ro-20,1724</th>
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<tbody>
<tr>
<td>-logIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>4.08±0.013</td>
<td>5.9±0.49 *</td>
</tr>
<tr>
<td>Max Inhib.</td>
<td>84.5±4.6</td>
<td>61.8±9.3</td>
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* significantly different than amrinone P<0.05
Figure 11. Myocardial TNF production by isolated rat cardiac myocytes. Rat left ventricle myocytes were isolated and incubated for 4 hours in media, media with LPS (1 µg/ml) and media with dexamethasone (1 µM). The media was collected and assayed for TNF by cytotoxicity assay. The results represent the mean ± SEM of five experiments.
Figure 12. TNF secretion from cardiac myocytes treated with polymyxin-B. The isolated cardiac myocytes were treated with polymyxin for five hours. After the polymyxin treatment the cells were incubated for 4 hr without polymyxin. The media was collected and assayed for TNF by cytotoxicity assay. The results represent the mean ± SEM of four experiments.
Figure 13. Amrinone inhibition of TNF secretion from cardiac myocytes. Amrinone at various doses was incubated for 4 hours with cardiac myocytes. The media was collected and assayed for TNF by cytotoxicity assay. The results represent the mean ± SEM of five experiments.
Figure 14. Ro-20,1724 inhibition of myocardial TNF secretion from cardiac myocytes. Ro-20,1724 was incubated at various doses with cardiac myocytes for 4 hours. Then the media was collected and assayed for TNF by cytotoxicity assay. The results represent the mean ± SEM of four experiments.
DISCUSSION

TNF is a cytokine that produces cardiac depression in septic shock (Tracy et al., 1986). Several studies support the involvement of TNF in sepsis. First TNF is detected in the serum of LPS treated animals and humans (Michie et al., 1988). Secondly, administration of recombinant TNF produces symptoms similar to septic shock. Thirdly, passive immunization against TNF reduces the lethality produced by endotoxin in sepsis (Tracy et al., 1987). The serum levels of TNF are elevated in some CHF patients (Levine et al., 1990; McMurray et al., 1991; Dutka et al., 1993). Recently TNF mRNA and protein were found in myocardial tissues from humans with dilated cardiomyopathies and ischemic heart diseases (Terre-Amione et al 1995). The cellular site of TNF secretion in SD rat hearts has not been determined. Ventricular cardiac myocytes are the contractility unit of the heart. These cells contain myofibrils and microtubules which are the molecular tools for producing contraction. Because these cells play an important role in normal cardiac function, the determination of the site of secretion of basal TNF could be of benefit for future determination of pharmacological approaches in cardiac disorders in which TNF levels are elevated.
In the heart, TNF is one of the inflammatory cytokines that increases nitric oxide production (Schulz et al., 1992) leading to an increase in cGMP which is a cyclic nucleotide that decreases myocyte contraction. TNF is also associated with a decrease in cardiac β-receptor density and an increase in inhibitory G protein subunits which leads to a decrease in the myocytes responsiveness to norepinephrine, leading to a decrease in cardiac contractility (Reithmann et al., 1991b). The factors that stimulate TNF secretion and the site of production in cardiac disorders is not well understood.

Our previous studies were performed in LV sections. To decrease the ischemic condition, we decided to isolate cardiac myocytes. We obtained > 95% rod shaped cells that were > 80% viable at 4 hours as measured by trypan blue. We were able to measure TNF secretion from the isolated cardiac cells. Because the results from our minced tissue studies were very similar to the results from the isolated non ischemic cells, we feel that TNF secretion from minced myocardium is a valid model to be used, and that TNF was not simply being secreted from dying hypoxic myocardium.

An additional reason for studying a more clean and pure system as the cardiac myocyte is that there is no cross contamination of other nonmyocardial cells and no diffusional barriers to oxygen or drugs that exist
when the whole heart tissue is studied (Severs, 1995). Determining the site of production will provide supportive evidence that the TNF protein is secreted from normal cardiac myocytes and it acts in an autocrine fashion in the heart.

The isolated cardiac cells secreted detectable bioactive TNF which tended to be stimulated by LPS and inhibited by dexamethasone. The reasons for TNF secretion could be the surgical procedure or stress that the isolation procedure produces on the cells leading to TNF secretion.

The effect of amrinone and Ro-20-1724 was also tested on the isolated cells. The potency of the two drugs was similar to the potency of the drugs in the LV tissue system. This indicates that the system used, either isolated cells from the LV or tissue preparation of the left ventricle, did not modify the drug action. Ro-20,1724 was a more potent drug to inhibit myocardial TNF secretion from myocytes than amrinone, but the efficacy of Ro-20,1724 was approximately 20% lower. The mechanism of action of these drugs could be through cAMP elevation; however, using the isolated cells for cAMP determination will need to be performed before drawing any conclusions.
The results from this study showed that basal TNF was secreted from isolated myocytes from SD rats after four hours of incubation. The drugs amrinone and Ro-20,1724 are able to block this secretion in a dose response manner. Because TNF was secreted from SD myocytes, further studies are needed to evaluated and compare the amount of TNF secreted from HF rat myocytes. The isolated preparation of myocytes provides a more pure in vitro system to study TNF secretion and function.
CHAPTER 4

EFFECT OF AGE AND PHOSPHODIESTERASE INHIBITORS ON MYOCARDIAL TUMOR NECROSIS FACTOR α PRODUCTION IN HYPERTENSIVE AND HEART FAILURE PRONE RATS

INTRODUCTION

Congestive heart failure (CHF) is a disease that affects nearly 4 million Americans with 400,000 new cases diagnosed each year (Ho et al., 1993). Prognosis of patients with CHF is poor, even with available pharmacological treatment. Therefore emphasis in CHF research has shifted towards the development of pharmacological tools that will prevent or delay the onset of later stages of CHF. This emphasis necessitates identification of physiologically relevant mediators of progressive deterioration of cardiac function and use of appropriate models to study their pathophysiological effect. One family of mediators that gained attention in the past few years is the cytokines, and their role in heart failure has been suggested by many
research groups (Levine et al., 1990; McMurray et al., 1991; Terre-Amino et al., 1995; Katz et al., 1994). The cytokine that our study concentrates on is tumor necrosis factor α (TNF).

The cytokine TNF is a physiologically important depressant of cardiac function during septic shock (Tracey AND Cerami, 1994). Studies in humans revealed that plasma TNF is elevated in CHF patients (Levine et al., 1990; McMurray et al., 1991; Dutka et al., 1993). Because acute increases in TNF decrease cardiac function in septic shock, it is hypothesized that chronic elevation of TNF in cardiac tissue (autocrine/paracrine or circulating origin) during states of onset of CHF and progression to decompensated CHF, could mediate prolonged depression of cardiac function. The primary site of TNF production, the factors stimulating TNF production, and the contribution of TNF to deterioration of heart function in CHF are unknown.

One of the therapeutic approaches for the treatment of CHF is the use of phosphodiesterase (PDE) inhibitors. PDE inhibitors have been recognized as potent inotropic and vasodilating drugs (Fischer et al., 1992). The PDE isozymes which are present in the heart are predominantly type III and IV and are responsible for breaking down cyclic AMP (cAMP). PDE inhibitors are thought to augment cardiac contractility by increasing the concentration of
intracellular cAMP in the failing heart, which in turn enhances the entry of calcium into cells by activating membrane bound calcium channels (Packer, 1993). In humans, type III PDE inhibitors, amrinone and milrinone, induce improvements in left ventricular end systolic volume and cardiac index after acute administration to patients. However, because some PDE inhibitors are also beneficial when administered chronically to the patient at lower doses that do not produce direct inotropic effects, it has been proposed that other mechanisms of action may be involved in producing the beneficial effect of PDE inhibitors (Matsumori et al., 1993).

Type III and IV PDE inhibitors have been shown to block TNF gene transcription and consequently protein production in white blood cells (Molnar-Kimber et al., 1993). Amrinone and pentoxifylline decrease serum levels of TNF and improve survival in endotoxin challenged rodents (Noel et al., 1995). The inhibition of TNF by PDE inhibitors might play a role in the acute or chronic beneficial effects of some PDE inhibitors in CHF. We have previously demonstrated myocardial TNF production and inhibition by PDE inhibitors in hearts taken from young Sprague Dawely rats (SD) (Bergman AND Holycross, 1996). The goals of the present study were to determine whether there was an effect of age, rat strain and disease state on myocardial TNF secretion and its modulation by PDE inhibitors. We hypothesized that
if TNF contributes to HF, then rats genetically prone to HF would have increased myocardial release of TNF compared to normotensive rats or spontaneous hypertensive rats which do not routinely succumb to failure. To attain these goals, 6,12 and 18 month old rats of SD, SHR and SHHF/Mcc-fa<sup>op</sup> (HF) strains were used. The PDE inhibitors amrinone and Ro-20,1724 were chosen for the present work to determine whether there was a differential ability of PDE inhibitors to block TNF release with respect to age or disease state.
METHODS

Experimental animals. The animals used in the experiments were male Sprague Dawley (SD), SHR and HF rats. HF rats were from the SHHF/Mcc-fa² rat colony maintained by Dr. Sylvia McCune at The Ohio State University. HF rats originated from a mating between the koletsky rat and an inbred SHR from the Okamoto strain. Longstanding hypertension elicits cardiac hypertrophy and progressive fibrosis. Plasma ANP, aldosterone and PRA gradually increase as the animals age, defining the onset and extent of compensatory left ventricular dysfunction. Animals in decompensated CHF typically present with adipose and skeletal muscle wasting, subcutaneous edema, dyspnea, cyanosis, and malaise (Gerdes et al., 1996).

Rats were sacrificed at 6, 12 and 18 month old. SD and SHR were obtained from Harlan (Indianapolis, IN) at 5 months of age and maintained in the OSU animal facility until appropriate age for experiments. The animals were allowed food and water ad libitum.

Blood Pressure Measurements. Systolic blood pressure was measured using an IITC tail cuff pump (Woodland Hills CA) attached to a Gilson Duograph (Gilson Medical Electronics, Middle town WI) immediately after
light anesthesia with intraperitoneal ketamine/xylazine (10 mg/kg and 50 mg/kg respectively). These measurements were done three days prior to sacrifice.

**Left ventricle tissue preparation.** Rats were weighed and sacrificed between 9 and 12 a.m. To anesthetize the rats, sodium pentobarbital (100 mg/kg) was injected i.p. The thoracic cavity was opened, and the heart was removed and perfused through a cannulated aorta with 50 ml of sterile 50% Dulbecco's modified Eagle's medium (DMEM) + 50% F12 supplemented with 2.45 g/liter sodium bicarbonate and 1% penicillin/streptomycin. The heart was weighed then dissected into the four chambers (right atrium, left atrium, right ventricle, and left ventricle including septum). These chambers were weighed individually. A portion of left ventricle was dissected, minced with a razor blade into 1 mm X 1 mm sections and rinsed thoroughly with DMEM media to remove any excess blood. Then the left ventricle was weighed, divided into 12 pieces of equal weight and incubated for 4 h at 37°C in a gassed incubator (5% CO₂ / 95% air), in 2 ml DMEM + 5% fetal bovine serum (FBS) + 1% penicillin/streptomycin (DF5). Then the incubation media was collected under sterile conditions and was frozen at -80°C before evaluation for TNF quantity.

**Drugs.** Left ventricular tissues were incubated with amrinone (type III PDE inhibitor), or Ro-20,1724 (type IV PDE inhibitor). Amrinone was supplied as
an injectable lactate salt which was dissolved in water supplemented with metabisulfite preservative (25mg/kg) and diluted with DF5 media. Ro-20,1724 was dissolved in 4.5% (w/v) aqueous 2-hydroxypropyl-β-cyclodextrin and diluted in DF5 media.

**TNF determination.** Cytotoxicity assay was performed as previously described by Matthews and Neale (Matthews and Neale, 1987). Briefly, L929 cells were grown in RPMI medium with 5% FBS and antibiotics in 96 well culture plates. The cells were allowed to incubate at 37°C overnight. The next day actinomycin D (1μg/ml) was added to the wells, and conditioned media was applied. After another overnight incubation, media was decanted from the cells, then the cells were fixed with 5% formaldehyde in phosphate buffered saline (PBS) for 5 min, and then stained with 0.5% crystal violet for 5 min. After washing and drying the extent of cytotoxicity was determined using the SLC Spectra plate reader by measuring the absorbance at 580 nm after solubilizing cells in 150 μl of 33% of glacial acetic acid.

**Statistical analysis.** Statistical differences among groups with respect to BP heart weight, TNF secretion and IC\(_{50}\) values were evaluated using 2 factor ANOVA followed by Newman Keuls post-hoc multiple comparison tests using the Number Cruncher Statistical System (NCSS, Jerry Hintze, Kaysville, UT).
IC$_{50}$ values were calculated from dose response curves obtained from individual rats, utilizing the Graph Pad statistical package. Significance level was set at $P < 0.05$. All results are expressed as mean ± SEM. Correlation coefficients were determined using least squares multiple regression analysis.
RESULTS

At 6 and 12 months of age, none of the HF rats exhibited any overt signs of CHF. At 18 months, one of the HF rats showed signs of labored breathing, piloerection and cachexia. Upon sacrifice, fluid was found in the chest cavity and a large thrombus was observed in the left atrium. Another animal from the same group had to be sacrificed at 17 months of age because of severe peripheral edema, and a large left atrial thrombus was observed at sacrifice. The other animals within this group showed no external signs of heart failure before sacrifice. At all age groups, SD and SHR showed no external signs of heart failure. However, at autopsy one of the 12 month old SHR had evidence of an apical infarction, with a significant increase in right ventricle weight. One SD rat assigned to the 18 mo group died of unknown causes prior to age of sacrifice; therefore n=5 in this age group.

Systolic blood pressure did not change with age in SD rats and was significantly elevated in SHR and HF compared to SD at all ages (Figure 15). Blood pressure was modestly, but significantly lower in the 12 month old SHR group than the 6 month old group, but blood pressure in the 18 month old SHR was not significantly different from either of the younger age groups. However, blood pressure in the HF rats significantly decreased with age. The elevated blood pressure in the SHR and HF animals was reflected in the left
ventricle/body weight (LV/BW) ratios depicted in Figure 16. LV/BW ratio did not change in the SD rats with age, but was significantly elevated in all ages of SHR and HF rats compared to SD. LV/BW ratio was significantly greater in 12 month old SHR than 6 month old SHR or 12 month old HF. However, by 18 months of age, the HF rats had significantly increased LV mass compared to 12 month old HF rats, and were not different than the 18 month old SHR.

Left ventricle taken from 6 month old SHR and HF rats secreted approximately 2 fold the amount of TNF secreted from 6 month old normotensive controls (Figure 17). At 12 months of age, the amount of TNF secreted from SD left ventricle was not significantly different from the amount secreted from SD rats at 6 months of age. However, SHR and HF secreted significantly less TNF at 12 months of age than at 6 months of age, and neither hypertensive group secreted amounts different than 12 month old SD. At 18 months of age, the amounts of TNF secreted from the left ventricle of SD, SHR and HF were significantly less than the amounts secreted from 6 month old rats of the same strains. However, 18 month old HF secreted significantly more TNF than age matched SHR and SD rats.

Amrinone exerted a dose-dependent inhibition of TNF secretion from left ventricle taken from all groups of rats (Figures 18A, 18B, and 18C). The
maximal effect of amrinone ranged from 80-100% inhibition of TNF secretion, and did not differ among the rat strains or ages. However, the potency of amrinone (determined by its IC_{50}) was significantly less in SD rats as they aged from 6 months to 12 months, and remained at the 12 month sensitivity in 18 month old rats (Table 4). There was no effect of age on amrinone IC_{50} in the SHR or HF rats; however, the inhibition curve for the 6 month old HF did not approach zero at the lower end of amrinone concentrations used, which may have shifted to the right the calculation of the IC_{50} value for this group of animals.

We had shown previously that Type IV PDE inhibitors such as RO-20,1724 had similar efficacy but were more potent than amrinone at inhibiting TNF secretion in 3 month old SD. In 6 month old SD, the efficacy of RO-20,1724 was similar to that of amrinone (approximately 80% inhibition of TNF release), and was slightly more potent (Table 5). Although RO-20,1724 remained more potent than amrinone at all other age groups of SD and HF rats, the maximal inhibitory effect of RO-12,1724 was less than that observed for amrinone; (RO-20,1724 inhibition of TNF secretion ranged from 44-68% for SD and HF rats). Most striking was the lack of effect of RO-20,1724 in SHR, where inhibition of TNF secretion ranged from 30-50%. IC_{50} values for RO-20,1724 were not calculated for SHR because of this marginal effect.
In order to determine whether a relationship existed between 1) ambient blood pressure and TNF release from the left ventricle, 2) left ventricular size and TNF release from the left ventricle, and 3) amount of TNF secreted and the inhibitory effect of amrinone, we performed correlation analysis of these parameters for all rats. Global regression analysis revealed no correlation among these parameters. However, subgroup analysis, performed for groups classified by age and strain revealed several significant correlations. At 6 months of age, blood pressure was linearly correlated with TNF secretion ($r=0.68$, $p<0.01$) (Figure 19). At 12 and 18 months of age, this relationship did not exist. Also, when all ages of HF rats were analyzed, there was a significant correlation between blood pressure and TNF secretion ($r=0.64$, $p<0.01$) (Figure 20). There was no correlation between blood pressure and TNF release in SD or SHR.

At 6 months of age, there was a highly significant linear correlation between an increase in left ventricular size and TNF secretion ($r=0.757$, $p<0.001$)(Figure 21), that like the correlation seen with blood pressure, disappeared with age. There were no correlations observed between left ventricular size and TNF secretion when SD or HF rats were analyzed, but in SHR, as left ventricular size continued to increase with age, TNF secretion decreased ($r=-0.66$, $p<0.01$) (Figure 22).

To determine whether the potency of amrinone was altered by the basal ability of the left ventricle to secrete TNF, we correlated TNF secretion to $-\log IC_{50}$.
values. In SD rats, left ventricles that secreted a greater amount of TNF also appeared to be more sensitive to inhibition by amrinone ($r=0.78$, $p<0.01$) (Figure 23). This relationship was not observed in SHR or HF rats.
Figure 15. Comparison of systolic blood pressure (SBP) in Sprague Dawley (SD), Spontaneously Hypertensive (SHR), and Heart Failure (HF) rats of various ages. The data points represent mean ± SEM of six animals per group, with the exception of 18 mo SD (n=5). * Indicates values are significantly different from values obtained from SD rats of the same age. (P <0.05).
Figure 16. Left ventricular weight/body weight (LV/BW) of Sprague Dawley (SD), Spontaneously Hypertensive (SHR), and Heart Failure (HF) rats of various ages. The data points represent mean ± SEM of six animals per group, with the exception of 18 mo SD (n=5). * Indicates values are significantly different than values obtained from SD rats of the same age. (P <0.05). ** Indicates value is significantly different than values obtained from SD and HF rats of the same age. (P <0.05)
Figure 17. Comparison of myocardial TNF secretion in Sprague Dawley (SD), Spontaneously Hypertensive (SHR) and Heart Failure (HF) rats of various ages. Myocardial TNF secretion was measured in conditioned media after 4 hours of incubation with minced left ventricle, and ng of TNF detected were normalized to 100 mg of tissue incubated. Cytotoxicity assay was used to evaluate the amount of TNF secreted. The data represent mean ± SEM of six animals per group, with the exception of 18 mo SD (n=5). * Indicates values are significantly different than values obtained from rats of the same strain at 12 and 18 months of age. (P <0.05). # Indicates value is greater than value obtained from 18 mo SD rats (P <0.05).
Figure 18. Dose dependent effect of amrinone on myocardial tumor necrosis factor α (TNF) secretion from 6, 12 and 18 mo Sprague Dawley (SD) (Panel A), Spontaneously Hypertensive (SHR) (Panel B), and Heart Failure (HF) (Panel C) rats. Minced left ventricular tissue was incubated for 4 hr in media at 37°C with increasing concentrations of amrinone, and TNF secretion was measured using a cytotoxicity assay. The data represent the mean ± SEM of 4-5 experiments.
Figure 19. Correlation analysis of systolic blood pressure (SBP) and tumor necrosis factor α (TNF) secretion from left ventricle of 6 month old Sprague Dawley (SD), Spontaneously Hypertensive (SHR) and Heart Failure (HF) rats. At 6 months of age there was a linear correlation between SBP and TNF. However, this correlation did not exist at older ages.
Figure 20. Correlation analysis of systolic blood pressure (SBP) and tumor necrosis factor α (TNF) secretion from left ventricle of 6, 12 and 18 month Heart Failure (HF) rats. In HF rats, there was a linear correlation between SBP and TNF. However, this correlation did not exist in the other 2 rat strains.
Figure 21. Correlation analysis of left ventricle/body weight ratio (LV/BW) and tumor necrosis factor α (TNF) secretion from left ventricle of 6 month old Sprague Dawley (SD), Spontaneously Hypertensive (SHR) and Heart Failure (HF) rats. At 6 months of age there was a linear correlation between LV/BW and TNF. However, this correlation did not exist at older ages.
Figure 22. Correlation analysis of left ventricle/body weight ratio (LV/BW) and tumor necrosis factor α (TNF) secretion from left ventricle of 6, 12, and 18 month old Spontaneously Hypertensive rats (SHR). In SHR, there was an inverse correlation between LV/BW and TNF secretion. However, this correlation did not exist in rats from the other 2 strains analyzed.
Figure 23. Correlation analysis of left ventricular secretion of tumor necrosis factor $\alpha$ (TNF) to the concentration of amrinone required to inhibit secretion by 50% ($IC_{50}$) in 6, 12 and 18 month old Sprague Dawley (SD) rats. With the exception of one outlier from the 12 month old group, there was a linear correlation between TNF secretion and $IC_{50}$ values in SD rats. However, this correlation did not exist in rats from the other 2 strains analyzed.
TABLE 4. Amrinone -log IC₅₀ and IC₅₀ values in 6, 12 and 18 month old SD SHR and HF rats.

<table>
<thead>
<tr>
<th>Strain</th>
<th>6 month old</th>
<th>12 month old</th>
<th>18 month old</th>
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<td></td>
<td>-log IC₅₀</td>
<td>IC₅₀ (µM)</td>
<td>-log IC₅₀</td>
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<td>SD</td>
<td>6.2 ± 0.5</td>
<td>0.67</td>
<td>4.5 ± 0.3**</td>
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<td>SHR</td>
<td>5.4 ± 0.4</td>
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<td>5.2 ± 0.3</td>
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<td>HF</td>
<td>4.5 ± 0.3*</td>
<td>31.6</td>
<td>5.0 ± 0.2</td>
</tr>
</tbody>
</table>

* = Significantly less than age matched SD rats

** = Significantly less than 6 mo SD rats
TABLE 5. Ro-20,1724 -log IC$_{50}$ values and maximal inhibition in 6, 12 and 18 mo SD, SHR and HF rats.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Age</th>
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<th>12 month old</th>
<th>18 month old</th>
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<td></td>
<td>-log IC$_{50}$</td>
<td>Max Inh.</td>
<td>-log IC$_{50}$</td>
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<td>SD</td>
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</tr>
<tr>
<td>HF</td>
<td></td>
<td>6.7 ± .4</td>
<td>47.6 ± 12</td>
<td>6.9 ± .4</td>
</tr>
</tbody>
</table>

ND=not able to determine
DISCUSSION

TNF has been implicated in the pathogenesis of heart failure. We have shown previously that TNF can be secreted from left ventricle of normal rats and the amount of bioactive TNF secreted can be modulated by phosphodiesterase inhibition (Bergman AND Holycross, 1996). It was the goal of these studies to investigate whether TNF secretion was altered during the progression of heart failure and whether there was a difference in the ability of phosphodiesterase inhibitors to inhibit TNF secretion during this progression of heart failure. Although there are many causative factors of heart failure, we chose a rat genetic model of heart failure, the SHHF/Mcc-fa® rat, which demonstrates a natural progression from cardiac hypertrophy due to hypertension, to a stage of compensated left ventricular dysfunction characterized by increased circulating factors such as ANP, PRA, and aldosterone, to overt decompensated heart failure characterized by fluid accumulation, dyspnea, and decreased dP/dT (McCune et al., 1990). SHR were used as a control for the effect of hypertension on TNF production and to delineate whether TNF secretion could be an indicator for heart failure in animals destined to succumb to failure.
The hypothesis that the amount of TNF secreted from the LV ventricle would be elevated as the animal progresses to end stages of HF was not supported by the findings in the present study. Instead, this study showed that myocardial TNF secretion was significantly elevated in HF rats at 6 months of age compared to SD rats, and went down significantly later when the rats aged. This could indicate that TNF was stimulated at early stages of CHF and might be playing an active role in producing various detrimental effects on the heart leading to the vicious cycle of decreased cardiac function or a passive role as a marker that is secreted when the heart begins to fail.

The correlation between hypertension and TNF secretion exists early in life and the elevated blood pressure could be one reason for the increased TNF secretion in 6 month old HF rats. The HF group was the only group to show a significant correlation (although not very strong) between BP and TNF secretion at all ages. In both SHR and HF, blood pressure remained elevated throughout 18 months, but TNF levels decreased dramatically from 6 months to 12 months, indicating that the early influence of blood pressure on TNF secretion was not maintained throughout life span. Preliminary evidence (Oral et al., 1995) suggested that increase LV stretch is correlated to increase TNF release from working feline heart, and this may be the reason for TNF secretion in young SHR and HF rats.
HF rats appear to be able to recover the ability to secrete more TNF when approaching end stages of failure. At the end stages of the disease a decrease in β-adrenoreceptors occurs (Jones et al., 1989), leading to a decrease in cAMP production (Feldman et al., 1987). The lower levels of cAMP might be responsible for the increase in myocardial TNF in 18 mo HF rats compared to the other groups.

TNF secretion correlated to left ventricle size in younger animals. However as the animals aged the increased TNF secretion was not maintained with increased LV size. This indicates that some feedback mechanism or some unknown factor may be preventing TNF secretion in aged animals, especially in SHR. SHRs had a negative correlation between LV size and TNF secretion suggesting that in aged animals, TNF secretion is controlled by mechanisms that are different from those in younger animals.

Amrinone inhibited myocardial TNF secretion with the same efficacy (90% inhibition) in the SHR, HF and control groups at various ages. Amrinone's potency decreased with age in SD rats, but was not affected by age in HF or SHR. The potency of amrinone correlated to the amount of TNF secreted in SD rats, indicating that when more TNF was secreted the heart was more sensitive to the inhibition by amrinone. The amrinone IC_{50} values for inhibiting
myocardial TNF production in this study correlated to amrinone IC\textsubscript{50} values for increase cAMP levels in the heart as reported in the literature (Harrison et al., 1987). Amrinone may block TNF production through the inhibition of type III PDE leading to an increase in cyclic AMP(cAMP). cAMP inhibits TNF mRNA by blocking the transcription process leading to a decrease in TNF production. There was a decrease in potency of amrinone with age in the SD group, indicating PDE type III activity or cellular distribution of PDE might be altered when the animal aged. It has been suggested that PDE activity is increased with age (Canepari et al., 1994) and the cellular distribution is also altered with age (Picq et al., 1995). There was no difference in amrinone potency among strains; therefore the presence of failure should not alter the effect of amrinone in vivo, unless the presence of failure alters distribution or elimination of the drug. The reason for the low potency of amrinone in 6 mo HF rats is due to the fact that the low doses of the drug was still able to inhibit TNF secretion by 40%. Expanding the concentration range in HF rats may be necessary to determine accurate IC\textsubscript{50} values.

Ro-20,1724, a type IV PDE inhibitor, was more potent drug than amrinone in inhibiting myocardial TNF production in our study. However, the maximal efficacy of Ro-20,1724 was lower compared to amrinone in all rat groups. It was not possible to determine IC\textsubscript{50} for the SHR group, due to a very low
maximal inhibition. This effect of Ro-20,1724 observed in SHR indicates that the drug efficacy and potency are influenced by rat strain. The efficacy of Ro-20,1724 in the HF group was lower compared to control. The potency was the same in the HF group and the control. Although, the normal non-failing heart contains predominantly type III and IV PDE, type IV PDE might play a smaller role as compared to type III PDE in the hydrolysis of cAMP in the failing heart. It was previously demonstrated in dogs with heart failure that a change in PDE isozyme levels occurs in the heart tissue (Smith et al., 1994). Understanding the changes in PDE isozyme distribution in the heart could provide additional information into the pathophysiology of heart failure and potential drug treatment. Future studies will need to evaluate the content and mRNA levels of PDE isozymes in hearts from HF and control rats.

In summary, TNF secretion was correlated to BP in HF rats and was significantly elevated in younger HF rats compared to age matched control. As the HF rats aged and progressed toward failure, the amount of TNF secreted was lower than the 6 months old HF rats, but it was higher than age matched controls. Ro-20,1724, a type IV PDE inhibitor, is a more potent drug at decreasing TNF secretion than a type III PDE inhibitor, amrinone, in all rat groups, However, amrinone efficacy was higher than Ro-20,1724. The potency of amrinone was decreased with age in SD but was not altered with
age in HF rat, which indicates that amrinone potency is not affected by the CHF state. In order to understand the mechanism of action of PDE inhibitors in CHF, other effects of these drugs such as sensitization of contractile protein to calcium (Colucci et al., 1986b), depression of myocardial ATPase activity and interaction with adenosine receptors still remain to be tested in HF. Inhibition of myocardial TNF production may be one of the beneficial actions of these drugs in heart failure.
CHAPTER 5

THE EFFECTS OF AMRINONE AFTER IN VIVO ADMINISTRATION ON MYOCARDIAL TUMOR NECROSIS FACTOR α PRODUCTION IN HEART FAILURE PRONE RATS

INTRODUCTION

Amrinone, a bipyridine, is a phosphodiesterase inhibitor that has both the ability to produce positive inotropic effects and vasodilation (Levy AND Bailey, 1989). Amrinone produces a significant increase in cardiac output, a decrease in right atrial filling pressure and a decrease in systemic and pulmonary vascular resistance in HF. These improvements occur without changes in heart rate, systemic blood pressure and myocardial oxygen consumption (Goenen, 1989). Amrinone half life is 2.6 hours and is eliminated by renal excretion and hepatic acetylation (Benotti et al., 1978).

Amrinone is used in the treatment of acute HF, impaired myocardial function after myocardial infarction, (Verma et al., 1985) in patients waiting for cardiac
transplant (Bolling et al., 1988) and in septic shock to improve survival (Vincet et al., 1988). In addition to its hemodynamic effects, amrinone prevents muscle protein wasting in sepsis (Juransinski et al., 1995), and inhibits TNF production from macrophages in vitro (Giroir AND Bruce, 1992). However the in vivo effect of amrinone on myocardial TNF secretion in HF has not been investigated. In order to evaluate the effect of amrinone in vivo, HF rats were treated with amrinone for seven days, then the animals were sacrificed and myocardial TNF levels were measured.
METHODS

Experimental animals. Male heart failure (HF) rats 15.5 month of age, from the SHHF/Mcc-fa\textsuperscript{op} colony were purchased from GMI (Chicago, IL). The rats were divided into three groups (n=4): a control (vehicle injected only), a low dose drug (5mg/kg) and high dose drug (15mg/kg). The rats were injected with amrinone (AMR) i.p. daily for 7 days. During that period the rats were allowed water and food \textit{ad libitum} and their weight was monitored and recorded daily. On the last day of the treatment the rats were sacrificed and various organs and blood were collected and frozen at -80\degree C for further evaluation.

Amrinone preparation. Amrinone powder was received from Sanofi Winthrop. The drug was dissolved in DMSO at the concentration of 30mg/ml. Dilutions were made in DMSO in order to inject 5mg/kg or 15 mg/kg to rats. Control rats received 1 ml of DMSO (i.p).

Plasma serum preparations. Rats were anesthetized with sodium pentobarbital (100 mg/kg i.p.). The thoracic cavity was opened, cardiac puncture was performed, and blood was withdrawn into a 10cc syringe. The blood was divided into various tubes containing 25\mu l 10\% EDTA, 25\mu l heparin(100U/ml), Heparin (100U/ml) + aprotinin (10\mu g/\mu l). Serum, plasma and packed cells samples were collected in the above tubes for further analysis. Many other organs were frozen back for future use. However for this study, only the LV was utilized.
Left ventricle preparation. The heart was removed and perfused through a cannulated aorta with 50 ml sterile medium consisting of 50% Dulbecco's modified Eagle's medium (DMEM) and 50% F12, supplemented with 2.45 g/L sodium bicarbonate and antibiotics. The left ventricle (LV) was dissected, weighed, and divided to three pieces. One piece was used for TNF determination in the LV without mincing or incubating. The tissue was weighed, rapidly homogenized in 2 mM PMSF in PBS, centrifuged and the supernatant fraction was collected and stored at -80°C until measurement of TNF content by ELISA. Another section of LV was immediately frozen in liquid nitrogen then stored at -80°C for future mRNA determination. The third piece was used for measurements of inactive and bioactive TNF secretion. This LV piece was minced with a razor blade into two 1mm X 1mm sections and rinsed thoroughly with DMEM media to remove any excess blood, and incubated for 4 h at 37°C in a gassed incubator (5% CO₂ / 95% air), in 2 ml DF5 media (DMEM/F12 + 5% fetal bovine serum (FBS) + 1% penicillin/streptomycin. Then the incubation media was collected under sterile conditions and was frozen at -80°C before evaluation for TNF quantity. The remaining heart chambers were frozen back for future analysis.

TNF determination. Cytotoxicity assay was performed as previously described by Matthews and Neale (Matthews and Neale 1987). Briefly, L929 cells were grown in RPMI medium with 5% FBS and antibiotics in 96 well culture plates. The cells were allowed to incubate at 37°C overnight. The next
day actinomycin D (1 μg/ml) was added to the wells, and conditioned media were applied. After another overnight incubation, media was decanted from the cells, the cells were fixed with 5% formaldehyde in phosphate buffered saline (PBS) for 5 min, and then stained with 0.5% crystal violet for 5 min. After washing and drying the extent of cytotoxicity was determined using a SLC Spectra plate reader by measuring the absorbance at 580 nm after solubilizing cells in 150 μl of 33% of glacial acetic acid.

Rat TNF ELISA assay. The supernatant from the PMSF homogenization, and conditioned media samples were analyzed using a rat TNF ELISA kit from Biosource International. The samples were applied to a precoated TNF-antibody plate. After three hours a biotinylated antibody was added to the TNF-antibody complex. Then streptavidine peroxidase was added to the three layer antibody-TNF antibody complex and later the substrate was added. The amount of color produced was proportional to the amount of TNF in the sample.

Plasma Renin Activity. Rat plasma was assayed for renin activity using an INCSTAR radioimmunoassay kit. Plasma renin activity was determined by the amount of angiotensin I generated at 4°C and 37°C for 90 min in the presence of protease inhibitors (10mM phenylmethylsulfonyl fluoride, and 5mM EDTA 1 mM hydroxyquinoline) The 4°C value is subtracted from the 37°C value as a background level. Angiotensin I levels are measured by direct radioimmunoassay using 125I- Angiotensin I.
mRNA Isolation. The kidney and the heart tissues taken from the various groups and were placed in liquid nitrogen and stored at -80°C until processed. Total RNA from tissues was isolated by the method of Chirgwin (Chirgwin et al 1979). Total RNA was analyzed for specific mRNA expression using Northern blot analysis.

The northern blots were hybridized with renin cDNA. Renin cDNA was full length 1400 bp rat cDNA obtained from K. Lynch (Burham et al., 1987). The prehybridization and hybridation procedures used were by Church and Gilbert (Church AND Gilbert 1984). The prehybridization lasted for one hour at 65°C, and the hybridization took place over night at 65°C. After the hybridization procedure was completed, the blots were washed using the Church and Gilbert methods for washing. The phosphorimager was used to visualize and quantify the ³²P labeled blots.

Data analysis. The values are represented as mean ± SEM. Due to the small number of animals used per group the values were not statistically significant.
In the present study the SHHF/Mcc-fa<sup>®</sup> heart failure (HF) rats were treated with amrinone (AMR) for one week. The weight of the rats was monitored daily over the 7-day period of the drug treatment and absolute weights are demonstrated in figure 24. There were no significant differences in baseline body weight in the 3 HF groups (control = 493±14.9g, AMR 5mg/kg=514±9.5, AMR 15mg/kg=484±4.3) nor in percent of body weight lost over the treatment period (control 1.3±0.7%, AMR 5mg/kg= 2.6±0.5%, AMR 15mg/kg= 3.2±1.1%). Age matched SD rats treated with DMSO lost 4.6% ± 0.3% of body weight (data not shown).

Table 6 contains absolute weight and weight/body weight ratio values for right atria, left atria, right ventricle, left ventricle+septum, and total heart. There was no affect of amrinone treatment on any of these parameters, although there was a nearly significant dose dependent increase in heart/body weight ratio (P=0.055) (Figure 25). Left ventricle / body weight and heart/body weight ratios were significantly greater than age matched SD rats (LV/BW=2.22±0.05; Heart /BW 3.02 ± 0.08).

The total amount of TNF in the LV was measured by ELISA (Figure 26). Both the low and high doses of AMR resulted in a decreased TNF content in the
LV. However when ELISA was performed on conditioned media to determine the amount of TNF secreted from the LV in 4 hours, there were no significant effects of AMR treatment (figure 27). Because ELISA detects both inactive and bioactive TNF, we also performed a cytotoxicity assay on the conditioned media to determine whether the secreted TNF was bioactive. The results of this assay demonstrated that the high doses of AMR tended to decreased bioactive TNF release, whereas the low dose of AMR did not (figure 28). However there was no significant effect of AMR at either dose.

The amount of inactive TNF in the conditioned media was much higher than the amount of bioactive TNF. However there was no significant effect of AMR on the percent of total TNF secreted that was bioactive (Figure 29).

The level of renal renin gene expression in DMSO treated HF rats was elevated compared to both of the AMR treated groups (Figure 30). However, both the low and the high doses of AMR resulted in elevated plasma renin activity compared to control (Figure 31). There was no difference in activity level of renin between the two amrinone treated groups. The AMR treated groups PRA (5mg/kg group=9.4±1.4ng/ml/min, 15mg/kg=10±1.2 ng/ml/min) were 40% higher than control (5.7±1.4 ng/ml/min). There were no significant changes in myocardial ANP expression (Table 7).
Figure 24. Rat weights. The rat weights were measured daily for the 7 days of treatment. The weights for individual groups were averaged and were reported as means ± SEM.
Figure 25. Heart weight/ body weight ratios. Rat heart weights were normalized per body weight. The results are reported as mean ± SEM for each group. * indicates value is almost significantly different than control. (P<0.055)
Figure 26. TNF content in the heart. The amount of TNF in the LV was determined using ELISA. The LV tissue was weighed and homogenized in PMSF solution. The supernatant fraction was assayed for TNF. The values for each group were mean ± SEM.
Figure 27. TNF secretion from the myocardium. The amount of TNF in the LV was determined using the ELISA assay. The LV was incubated for 4 hours and the amount of TNF secreted into the media was determined using the ELISA assay. The values for each group were mean ± SEM.
Figure 28. Bioactive TNF secretion from myocardium. The amount of bioactive TNF was determined in the LV by the cytotoxicity assay. The LV was incubated for 4 hours, and the amount of TNF secreted was measured. The reported values are mean ± SEM.
Figure 29. Percent of bioactive TNF. The amount of bioactive TNF compared to the total amount secreted from LV was expressed as % bioactive (Bioactive /total × 100). The values are the mean ± SEM.
Figure 30. Renal renin gene expression. Kidney mRNA was isolated, and the levels of renin gene expression were determined and compared between the three groups. Densitometry values are reported as mean ± SEM. * indicates value is significantly different than control (P<0.05)
Figure 31. Plasma Renin Activity (PRA). Plasma renin activity was determined using a commercially available RIA kit. Rat blood was collected into prechilled tubes containing EDTA. The PRA was measured by determining the difference in ANG I generated in plasma at 4 and 37°C. The reported values are mean ±SEM.
Table 6. Weights of the four heart chamber.

<table>
<thead>
<tr>
<th></th>
<th>RA (g)</th>
<th>RA/BW (g/kg)</th>
<th>LA (g)</th>
<th>LA/BW (g/kg)</th>
<th>RV (g)</th>
<th>RV/BW (g/kg)</th>
<th>LV (g)</th>
<th>LV/BW (g/kg)</th>
<th>HW/BW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>.043 ± 0.007</td>
<td>0.066 ± 0.012</td>
<td>0.04 ± 0.003</td>
<td>0.078 ± 0.007</td>
<td>0.28 ± 0.03</td>
<td>0.58 ± 0.03</td>
<td>1.44 ± 0.07</td>
<td>2.98 ± 0.07</td>
<td>3.53 ± 0.06</td>
</tr>
<tr>
<td>AMR 5 (mg/kg)</td>
<td>.045 ± 0.007</td>
<td>0.09 ± 0.011</td>
<td>0.06 ± 0.01</td>
<td>0.128 ± 0.004</td>
<td>0.29 ± 0.03</td>
<td>0.58 ± 0.05</td>
<td>1.5 ± 0.08</td>
<td>2.97 ± 0.10</td>
<td>3.74 ± 0.09</td>
</tr>
<tr>
<td>AMR 15 (mg/kg)</td>
<td>.056 ± 0.012</td>
<td>0.119 ± 0.03</td>
<td>0.06 ± 0.008</td>
<td>0.12 ± 0.014</td>
<td>0.3 ± 0.013</td>
<td>0.63 ± 0.04</td>
<td>1.41 ± 0.08</td>
<td>2.98 ± 0.12</td>
<td>3.82 ± 0.08</td>
</tr>
<tr>
<td>Statistical Analysis</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>P&lt;0.055</td>
</tr>
</tbody>
</table>

NS=not significant
Table 7. Densitometry of ANP gene expression (x10^3).

<table>
<thead>
<tr>
<th></th>
<th>Right Atria</th>
<th>Left Atria</th>
<th>Right Ventr</th>
<th>Left Ventr</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>1058±290</td>
<td>389±196</td>
<td>198±134</td>
<td>183±86</td>
</tr>
<tr>
<td>AMR 5mg/kg</td>
<td>1485±446</td>
<td>673±228</td>
<td>107±34</td>
<td>161±53</td>
</tr>
<tr>
<td>AMR 15mg/kg</td>
<td>1057±392</td>
<td>467±180</td>
<td>66±43</td>
<td>311±132</td>
</tr>
</tbody>
</table>
Amrinone (AMR) treatment of HF rats in vivo tended to demonstrate an inhibition of myocardial TNF content, but not a difference in TNF secretion. In humans, pharmacological effects of AMR have been attributed to its inotropic action and vasodilating properties. This study however, demonstrated an additional action of AMR on the heart with respect to cytokine inhibition. AMR is a type III phosphodiesterase inhibitor which can increase intracellular cAMP. cAMP can inhibit TNF gene expression leading to a decrease in TNF production and secretion (Beutler et al., 1992). The effect of amrinone on TNF has been evaluated in septic shock (Giroir AND Bruce, 1992), but in heart failure the effect of AMR on TNF has not been evaluated. Our study demonstrates another possible role that amrinone might play in heart failure beyond its inotropic actions. Indeed, in rats, AMR does not produce an inotropic effect (Weishaar et al., 1987). Therefore the use of the HF rat model allowed us to assess the effect of AMR on myocardial TNF secretion without confounding effects on cardiac contractile force.

The HF rats go into end stage ventricular dysfunction at 18-20 months. Prior to that they experience a compensatory stage which occurs as a result of the long standing hypertension. Due to the increased blood pressure, the heart becomes enlarged and is not able to pump blood efficiently. As a result of
this, the body is trying to compensate for the heart. During that period various mechanism are activated. Many hormones are secreted, and the body is doing whatever it can to postpone the end stage HF. In our study AMR was administered in two doses to rats 15-16 months old for seven days. This particular age was chosen because it was prior to the onset of decompensated failure and after the heart had hypertrophied due to chronically elevated circulating factors. This stage would be analogous to that seen in humans with compensated left ventricular dysfunction. During the compensatory stages the effect of the AMR had not been investigated. The hypothesis of our study was that AMR would decrease myocardial TNF production prior to end stage HF.

The change in weight over the seven day period was not significantly different between the control group and the AMR treated groups indicating that AMR did not affect weight loss or gain during the treatment. This could indirectly indicate no effect on appetite or food intake in the treated rats compared to control.

Because the drug was only soluble in DMSO, the rats in the control group were given 1 ml DMSO for seven days. Previous studies indicated that, DMSO does not produce any effect on TNF (Juransinski et al., 1995). Therefore was DMSO used for our study as a vehicle.
Heart weights per body weight were higher in the HF rats compared to age matched SD (data not shown). The hearts were slightly bigger in HF group treated with AMR compared to the HF rats treated with DMSO although the individual chambers were not different. The increase in heart weights in HF group compared to SD was expected because rats of the HF strain have long standing hypertension (SBP 180-200mmHg) and have elevated PRA (McCune et al., 1991).

The effect of AMR on myocardial TNF production in vivo has not been evaluated before in heart failure. This might be because the correlation between TNF and heart failure is not well understood. In this study the effect of AMR on myocardial TNF secretion in vivo was tested in HF rats. The cytotoxicity assay demonstrated that either the doses or the dosing schedule of amrinone was not sufficient to produce an inhibitory effect on myocardial secretion of bioactive TNF. When a rat TNF ELISA kit was used to determine the amount of TNF secreted from LV, quantitative but qualitatively different results were obtained compared to cytotoxicity assay. The amount of TNF secreted in all the groups was higher compared to the amount of bioactive TNF detected using cytotoxicity assay. The ability of the ELISA kit to detect active and non active TNF may explain why the amount of TNF in all groups was elevated compared to the amount detected on cytotoxicity assay which detects only bioactive TNF.
The role of inactive TNF has not been investigated before, and the evidence it is secreted from the LV should be investigated in future studies. Although some of the secreted TNF is cytotoxic and therefore would be physiologically active, it is still possible that inactive TNF may bind to soluble TNF receptors or cell surface receptors in vivo and affect the activity of secreted TNF that is bioactive. In these studies AMR was not able to alter the inactive TNF secretion.

The TNF content in the LV was measured in HF rats by ELISA. The AMR treated HF groups contained the least amount of TNF, indicating that AMR was able to inhibit some of the production of TNF leading to a decrease in content of TNF in the LV of the HF rats. During that procedure, the LV tissue was not minced, chopped or incubated for four hours, but instead the tissue was homogenized in PMSF immediately following weighing. By using this procedure the tissue was not exposed to the stress of mincing and the four hours incubation. Because the reason for secretion of myocardial TNF is not clear, eliminating procedures such as mincing and incubation could provide a better indication of the effect of AMR. Determination of tissue content provided an immediate evaluation of TNF in the LV. The content of TNF on mg tissue basis was approximately 30 fold more than secreted TNF (content=0.45ng/100mg vs secreted=0.015 ng/100mg vs 0.0015ng/mg bioactive).
When an in vivo study is performed, there are many parameters that may be affected by the drug treatment. These physiological factors may be able to modify indirectly the drug effect and the pathological condition. Because an in vivo system is a composite system, the drug effect on other organ systems capable of producing physiological mediators of CHF should be taken into account. In order to determine whether the effect of AMR was selective to cardiac TNF or perhaps was an indirect effect of AMR pharmacological action at other sites in the body, we measured cardiac ANP and renal renin gene expression and plasma renin activity. ANP and renin are important compensatory players in HF, and the beneficial effect of AMR could be partially due by alteration of these compensatory modulators.

Renal renin gene expression decreased in AMR treated rats. However plasma renin activity was elevated in the HF rats treated with low and high doses of AMR, indicating that AMR might have increased juxtaglomerular cells cAMP content, which increased renin release. Alternatively, AMR may have reduced blood pressure in these animals, plasma renin activity may have increased to compensate for any decrease in BP. Further studies measuring the blood pressure will need to be performed to test this hypothesis. The effect on BP may be of importance in TNF release from LV because data presented in Chapter 4 showed a significant correlation between BP and TNF at all ages of HF rats.
The results demonstrated that AMR at 5mg/kg and 15mg/kg tended to decrease myocardial TNF content. However the dose or dosage regimen selected did not alter the amount of TNF secreted from the LV. AMR increased PRA and had no effect on ANP gene expression in the heart. This study demonstrates a potential mechanism of action for AMR in HF, in addition to its inotropic effects.
CHAPTER 6

DISCUSSION

The correlation between TNF and congestive heart failure has recently been suggested. TNF, a pre-inflammatory cytokine mediates the cardiac depression in sepsis and has been initially found to be elevated in the plasma of some CHF patients with cachexia (Levine et al., 1990; McMurray et al., 1991; Dutka et al., 1993). In the past it has been demonstrated that TNF was not constitutively expressed in the normal heart (Giroir AND Bruce, 1992). Recently TNF mRNA and TNF protein were found to be elevated in the heart of patients with end stages of dilated cardiomyopathy and ischemic heart disease (Terre-Amione et al., 1995). This suggests that during disease conditions TNF gene expression and protein production are stimulated and their levels in the cardiac tissue rise.

Despite a variety of pharmacological tools available to treat CHF, few have shown promise in prolonging life span. Therefore a shift in focus in CHF research has occurred to identify and modulate endogenous substances which may contribute to the progression to end stage failure. TNF has been
identified as a cardiac suppressant which is elevated in CHF. However, the
time course of production and factors stimulating TNF production during the
progression to end stage CHF are unknown.

In this study the results obtained were slightly different than what we
hypothesized. First after 4 hours incubation, the LV tissue from Sprague
Dawley rats was able to secrete unstimulated TNF. With age, the amount of
TNF secreted from the myocardium in SD rats decreased significantly. We
were surprised to discover the unstimulated release because it has been
reported that TNF is not constitutively present in the normal hearts (Giroir
AND Bruce, 1992). Also in our laboratory preliminary studies showed no gene
expression in the young SD rats. There are a few explanations for the
unstimulated release of TNF from the heart: the surgical procedure could be
inducing TNF or the tissue might be hypoxic and hypoxia is known to induce
TNF (Herskowitz et al., 1995). The reason for mRNA not being detected in SD
rats could be that the gene expression and the protein production of TNF are
two separate events. Because the mRNA is not highly expressed in the tissue
does not mean the protein is absent.

Secondly we anticipated that LV of HF rats which progressed into failure
would secrete more TNF compared to control. However in our studies as the
HF rats aged, TNF levels went down. At 18 months HF rats secreted a
significantly higher amount than control, but the amount secreted by the older
rats was lower than the amount secreted in 6 months old HF rats. Although we
expected to see an increase in TNF secretion as the HF rats aged it is
possible that TNF might be playing a different role in HF than what we
anticipated. It has been shown that TNF secretion occurs in the later stages
of the disease. However maybe it is secreted in the early stages for a totally
different reason. The elevated TNF secretion early in life could be the cause
for the initial cardiac deterioration. Also the elevated blood pressure could be
producing some effect on the TNF secretion early in life. In our studies the
HF rats showed a correlation between BP and TNF, although with age the
elevated level of blood pressure was not able to maintain the high levels of
myocardial TNF in older animals.

The LV size could be one reason for the increase in TNF levels during HF,
because in our studies LV size correlated to TNF secretion in younger
animals. The increase in LV size was not able to maintain the increase in TNF
secretion as the animal aged, indicating that other factors are involved in
controlling TNF secretion from HF rat myocardium as the animals age and the
disease progresses.

Most current treatment regimens for CHF are not beneficial in prolonging life
span; people with heart failure have a survival rate of 3-5 years (Barnett et
al., 1993). Today research in CHF is aimed towards treatments which will
prevent or delay the onset of CHF. Phosphodiesterase inhibitors have some beneficial effects in acute treatment of CHF (Fischer et al., 1992). But because in some cases PDE inhibitors increased mortality, their use is questioned. However, when the dose of PDE inhibitors used was lowered, the drugs were able to benefit the patients. The use of phosphodiesterase inhibitors with respect to myocardial TNF was not investigated prior to this study. We hypothesized that the beneficial effect of these drugs in the lower doses could be in part due to inhibition of cytokine production. In our studies, PDE inhibitors amrinone and Ro-20,1724 were evaluated with respect to myocardial TNF production in vitro, and amrinone was used in vivo in HF rats. We were able to demonstrate an effect of these drugs on myocardial TNF production, indicating that these drugs might be useful in CHF except at lower doses and during the initial stages of the disease. Currently PDE inhibitors are used as inotropic and vasodilation agents for acute treatment. Maybe in the future using these drugs at lower doses at the initial stages of the disease might be even more beneficial.

If TNF is playing a role in initial stages of CHF, then inhibition of this cytokine might be beneficial in delaying or even preventing the onset of CHF. In order to test this hypothesis, TNF levels will need to be determined in humans in the early stages, then modulation of the myocardial TNF with drugs that inhibit TNF secretion or production might be beneficial.
We also demonstrated that isolated SD cardiac myocytes secreted unstimulated TNF. The isolated cardiac myocytes could be a good system to study TNF secretion in normal and diseased hearts. Because we were able to demonstrate that SD cardiac myocytes are able to secrete TNF, it will be interesting to isolate myocytes from HF rats and compare the amount of TNF secreted to control. The myocytes are isolated under non-hypoxic conditions and are a more pure system than the whole LV ventricle tissue preparation.

The exact role of TNF in CHF is not well established, but it was demonstrated that TNF is able to produce a negative inotropic effect on isolated cardiac myocytes (Oral et al., 1995; Chung et al., 1990) TNF can also produce left ventricular dysfunction in humans (Suffredini et al., 1989), cause pulmonary edema (Millar et al., 1989), promote left ventricular cardiac remodeling (Pagani et al., 1992) and uncouple beta-adrenoceptors from adenylyl cyclase (Chung et al., 1990). This data suggests that TNF might play an important role in the deterioration of the heart in HF. Because we demonstrated that TNF was secreted at high levels from LV of younger HF rats, this could indicate that TNF plays a role as a marker for cardiac deterioration in CHF.

In our studies, SHR also secreted higher level of TNF compared to control, but these animals did not die of CHF. The reason for the strain difference between SHR and HF rat will need to be investigated in the future.
Other mechanisms such as apoptosis, NO production, second messenger systems, and TNF receptors levels will need to be evaluated to explain why SHR rat secrete high levels of TNF but do not go into CHF.

Today a newer PDE inhibitor vesnarinone is being investigated for potential use in CHF. This drug is able to block TNF production and is found to be beneficial at lower doses in CHF indicating that inhibition of TNF might be important in the treatment of CHF, and that PDE inhibitors could be evaluated all over again in the treatment of CHF.
APPENDIX

COMPARISON OF MOUSE RAT AND HUMAN TNF STANDARD

INTRODUCTION

During the course of performing the cytotoxicity assay describe in this dissertation, the rat TNF standard became available. Because our initial studies measured TNF in mouse units of activity and we wondered whether this was an accurate reflection of the actual rat units of activity, we needed to compare cytotoxicity elicited by mouse vs. rat TNF.

TNF is a cytokine which is responsible for many inflammatory and immunological activities. In order to quantitate the amount of TNF in various biological fluids or tissues, many detection assays were developed (Jaattela, 1991). TNF exists in two forms, bioactive and inactive. Bioactive TNF is a trimer which is able to bind to TNF receptors and produce a response. Inactive TNF can be a monomer, a TNF trimer bound to a soluble receptor or other degraded forms of TNF trimer. To assay bioactive TNF, a cytotoxicity assay is used. The TNF trimer will bind to TNF receptors leading to cytotoxic response. Tumor cell are very sensitive to cytotoxicity of TNF and are used
frequently for cytotoxicity assays. The enzyme linked immuno sorbent assay (ELISA) is an immunoassay which detects bioactive and inactive TNF as long as the inactive form contains the antibody recognition site.

The cytotoxicity assay uses tumor cells which are sensitive to TNF. These cells are incubated with standard TNF and conditioned media containing unknown amounts of TNF. Death of tumor cells is proportional to the amount of TNF in the media. Various dyes, radiolabels or enzyme activities are used to determine cell viability versus cell death (Aggarawall et al., 1985; Mosmann, 1983; Beutler et al., 1985).

In order to determine the quantity of TNF in the conditioned media, a standard is used in various concentrations to produce a standard curve. The content of TNF in the conditioned media samples can be interpolated from this curve. The recombinant human and mouse TNF are commonly used as a TNF standard in cytotoxicity assays. Recently rat TNF became available by Biosource International (Camarillo, CA) and Genzyme (Cambridge, MA). The standard used for the cytotoxicity assay should be determined by the species source of the unknown TNF samples to be assayed. In the past samples from rat were assayed for TNF using mouse TNF as a standard (Monastra AND Secchi, 1993; Pizarro et al., 1993). Although the rat and mouse TNF are very homologous to each other, there still might be a difference in the amount of cytotoxicity produced by the preparations on certain cell types. The mouse lymphoma L929 cells are sensitive to TNF cytotoxic effects and are
commonly used for cytotoxicity assay. However, the cytotoxic effects of rat TNF on these cells have not been evaluated. Because many times a cytotoxicity assay (used to measure active TNF) is followed by an ELISA (used to measure total TNF bioactive and inactive), the use of the same standard for both assays is very important.

In order to compare the results from the rat ELISA to the results obtained from the cytotoxicity assay, knowing whether rat TNF gives similar cell death as mouse TNF could be beneficial. Evaluation of the three standards can provide useful information about the type of standards one should use in cytotoxic assays.
METHODS

The L929 cells. The mouse lymphoma cells L929 (ATCC CCL1: American Type Culture Collection, Rockville, MD) were grown in RPMI media supplemented with 5% fetal bovine serum (FBS) and antibiotics in T-75 culture flasks (Coming). The day prior to cytotoxicity assay, the cells were passage from the T-75 flasks to 96 well plates (Coming), and were plated at a density of 2.5x10^4 cells/well. After 20-24 hours, the cells were confluent and used for the cytotoxicity assay.

TNF Standards. The human and mouse TNF standard were purchased from Gibco-BRL in the lyophilized form. They were resuspended in PBS and aliquoted into working concentrations. The aliquotes were stored at -80°C until use. Rat TNF standard was purchased from Bio-Source International in lyophilized form, and resuspended in a buffer provided by the company, aliquotted to working concentration, and stored at -80°C until use.

TNF Determination. Cytotoxicity assay was performed as previously described by Matthews and Neale (Matthews and Neale 1987). Briefly, L929 cells were grown in RPMI medium with 5% FBS and antibiotics in 96 well culture plates. The cells were allowed to incubate at 37°C overnight. The next day actinomycin D (1 µg/ml) was added to the wells, and conditioned media were applied. After another overnight incubation, media was decanted from the cells, the cells were fixed with 5% formaldehyde in phosphate buffered
saline (PBS) for 5 min, and then stained with 0.5% crystal violet for 5 min. After washing and drying the extent of cytotoxicity was determined using a SLC Spectra plate reader by measuring the absorbance at 580 nm after solubilizing cells in 150 µl of 33% of glacial acetic acid.

**Data Analysis.** Data was expressed as mean ± SEM. The EC$_{50}$ were calculated using the Graph-Pad Inplot program. ANOVA following by Newman-Keuls was performed using the Number Cruncher Statistical System (NCSS Jerry Hintze Kaysville UT).
RESULTS

The cytotoxic effect of recombinant mouse and rat TNF were similar using the L929 cells. The EC$_{50}$ values of mouse and rat TNF were 5.13x10$^{-3}$ and 4.46x10$^{-3}$ ng respectively. The concentration of 0.04 ng/well mouse or rat TNF produced a 100% death (OD=.0628±0.03). The lowest concentration (1.95x10$^{-5}$ ng/well) produced low death rate of L929(OD =0.7±0.056).

Recombinant human TNF was less toxic to the cells compared to mouse or rat recombinant TNF. The EC$_{50}$ of human TNF was 1.14 ng. The high concentration (4ng/well) produced 84% cell death. At a concentration of 0.04 ng/well, human TNF produced very low levels of toxicity (OD=.571±.03) similar to a toxicity produced by the lowest concentration (1.95x10$^{-5}$ ng) of mouse or rat TNF. The potency of human TNF was significantly lower than mouse and rat TNF when the L929 cells were used.
Figure 32. Standard curve of rat, mouse and human TNF. L929 cells were incubated with the various species TNF for 18 hours. Then the cells were stained with crystal violet and the optical density was measured. The data represents mean ± SEM of nine experiments.
Figure 33. Human TNF standard. L929 cells were incubated with recombinant human TNF for 18 hours. Then the cells were stained with crystal violet and the optical density was measured. The data points represent the mean ± SEM of eight experiments.
DISCUSSION

The cytotoxic effect of TNF is mediated through oxygen free radical phospholipase activation and damage to nuclear DNA (Larrick AND Wright, 1990). TNF binding to its receptors and TNF internalization are important for the cytotoxic effect of this cytokine (Kull, Jr. AND Cuatrecasas, 1981). In the cell TNF activates G-proteins leading to activation of phospholipase, protein kinases and calcium mobilization, all resulting in the cytotoxic effect of TNF. The cytotoxic effect of TNF has been used in many assays to determine the quantity of TNF.

The sensitivity of the cytotoxicity assay can be increased by plating the cells at the correct density, and the use of transcription or protein synthesis inhibitors (Barrsch et al., 1991). The type of TNF standard used for the cytotoxicity assay is very important when unknown amounts of TNF are to be determined.

Recently ELISA assays have been developed which are very sensitive and very popular for quantification of the amount of TNF. However in order to detect bioactive TNF, cytotoxicity assays still need to be performed. To compare the amount of active to inactive TNF secreted, the use of the same type of TNF standard in both types of assays can be of importance. Rat ELISA kits are available currently. In order to compare the ELISA assay to a
cytotoxicity assay, the rat standard needed to be tested in the cytotoxicity assay system. Because the cytotoxic effect of rat TNF standard has not been evaluated we conducted the present study.

In the present study the effect of three recombinant TNF standards were compared. Mouse and rat were similar in their potency and efficacy. Human on the other hand had a lower potency when the L929 cells were used. Although the three recombinant TNF are very similar in structure (79% homology between human and mouse (Marmenout et al., 1985) there is a difference in their action. The L929 cells are murine cells, and it is possible that human TNF doesn't bind with the same affinity as the mouse and rat TNF, therefore, requiring a twenty times greater concentration to produce the same amount of cell death as the mouse or rat TNF standard. The rat and the mouse were very similar in their cytotoxicity on the L929, making the future comparisons between results obtained from cytotoxicity assay using the mouse standard and results obtained using a rat ELISA assay are justified.
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