SIGNALING PATHWAYS OF TUMOR NECROSIS FACTOR α IN VENTRICULAR MYOCYTES

BY SHAREEF MUSTAPHA

A thesis submitted to the Faculty of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

Department of Physiology
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ABSTRACT

Elevated levels of serum tumor necrosis factor alpha (TNFα) have been reported in patients with cardiac hypertrophy and heart failure. However, the role played by TNFα in the pathogenesis of cardiac disease remains unknown. TNFα may contribute to ventricular dysfunction through the modulation of cardiac muscle gene expression. To test this possibility we transfected neonatal ventricular myocytes with luciferase reporter constructs driven by the α–myosin heavy chain (αMHC), β–myosin heavy chain, α–cardiac actin, and α–skeletal actin genes. TNFα significantly repressed cardiac specific gene expression ($p<0.05$). The repression was judged not to be due to the cytotoxicity of TNFα as TNFα did not induce myocyte cell death. The nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), abrogated the repressive effects of TNFα. Furthermore, myocytes expressing an inhibitor of the transcription factor NFκB prevented TNFα-mediated repression of cardiac specific genes. Our data provide the first indication for the repression of cardiac specific gene expression by TNFα through an NFκB and NO dependent pathway. To further delineate the TNFα signal transduction pathway we tested the involvement of the TNFα Receptor Associated Factor 2 (TRAF2) for signaling of NFκB. The ring finger and zinc finger domains of TRAF2 are capable of transactivating NFκB and are thought to be indispensible for the induction of NFκB by TNFα in some cell types. We tested several structural mutations of TRAF2 in both myocytes and 293 cells. Our data suggests that TRAF2 is essential for TNFα signaling of NFκB in 293 cells but not in myocytes. Furthermore, we found that the ring finger domain of TRAF2 is not important for TRAF2 mediated NFκB activation in myocytes.

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TNFα – Tumor Necrosis Factor alpha

αMHC – alpha myosin heavy chain

βMHC – beta myosin heavy chain

αSkl – alpha skeletal muscle actin

αSml – alpha smooth muscle actin

αCA – alpha cardiac actin

CMV – cytomegalovirus promoter

β-gal – betal galactosidase

HSTK – herpes simplex thymidine kinase promoter

Luc – luciferase

L-NAME – N^G–nitro-L-arginine methyl ester

NFκB – nuclear factor kappa B

SE – standard error

IκBα – inhibitory kappa B alpha

TRAF2 – TNFα receptor associated factor 2

TRADD – TNFα receptor associated death domain

FADD – Fas associated death domain

NCM – neonatal cardiac myocytes

I. INTRODUCTION

Recent evidence suggests that cytokines are important mediators of heart disease. Cytokines are small proteins that act in an autocrine or paracrine manner and function by binding specific receptors on the cell membrane (18). Engaging the receptor sets off a cascade of effects including the induction or inhibition of a number of cytokine regulated genes.

The tumor necrosis factor alpha (TNFα), a pro-inflammatory cytokine, has a broad spectrum of diverse physiologic and pathophysiologic effects and has been shown to modulate the growth, differentiation and function of essentially every cell type investigated (42). In the heart, TNFα may activate numerous signaling pathways leading to alterations in cardiac gene expression, modulation of myocardial contractile function and the death of cardiomyocytes (38). Indeed, elevated levels of TNFα have been associated with adverse cytotoxic effects contributing in particular to congestive heart disease (88,89), hypertrophic cardiomyopathy (115), and severe chronic heart failure (46, 47). However, the precise role of TNFα in these conditions is not yet fully understood.

A characteristic feature of heart failure is the progressive deterioration of ventricular function. The mechanism(s) responsible for this decline in cardiac function is (are) not fully understood, however, they may be related to progressive and ongoing myocardial degeneration through the loss of viable cardiomyocytes. TNFα has been shown to promote cardiac remodeling in vivo (92), and induce apoptotic cell death of adult ventricular myocytes in primary culture (110).

Apoptosis, or programmed cell death, can be defined as a genetically regulated, energy requiring process of cell destruction. Apoptosis is morphologically characterized by cell shrinkage, chromatin condensation and cell fragmentation (into what are called 'apoptotic bodies') (143). In contrast to necrosis, apoptotic cell death does not provoke an inflammatory response. While, the molecular mechanisms of apoptosis in the heart are poorly understood, growing evidence suggests that cardiomyocyte cell loss by apoptosis may contribute to the progressive ventricular dysfunction seen in heart failure (117). Previous studies have shown that overexpression of TNFα in cardiac myocytes using transgenic mice showed severe impairment of cardiac function and severe ventricular dilatation (22). In a related study, continuous infusion of 100 U per mL of TNFα produced cardiac remodeling, progressive contractile dysfunction, myocyte hypertrophy and significant DNA damage (92). The concentration used in this study is similar to that seen in serum of patients with heart failure. Together, these findings demonstrated that TNFα overexpression in vivo was sufficient to mimic some of the characteristics seen in experimental and clinical models of heart failure.

TNFα has been associated with heart hypertrophy and end stage congestive heart failure. Cardiac hypertrophy entails mycocyte growth and characteristic changes in the expression of numerous cardiac specific genes (56). A hallmark of this regulatory event is selective activation of genes encoding isoforms ordinarily associated with the embryonic or fetal heart (62). For example, the atrial natriuretic factor, β-tropomyosin, and atrial myosin light chain are examples of genes that are reactivated during hypertrophy. The prototypes for this transition are thought to include the activation of β-myosin heavy chain (βMHC), α-skeletal actin (αSkl) and α-smooth muscle (αSml) genes

(55). In addition, a selective down regulation of αMHC mRNA and protein levels have been reported in human hypertrophied, failing ventricular myocardium (63). Although the precise mechanism of action of TNFα in disease states is unknown, TNFα has been shown to invoke remodeling and modulate cardiac muscle gene expression (56,87,90,91,92).

TNFα plays an important physiologic role in adaptive homeostatic responses and in the regulation of local host defenses (18 – 21). In the heart, Mann (1996) suggested the possibility that TNFα may act as an early stress response gene (38). When activated, TNFα may play an important role in regulating myocardial homeostasis in the adult human heart in response to certain enviromental stresses. One stress relavent to the heart is ischemia and TNFα has been shown to confer resistance to hypoxic injury in the adult mammalian cardiac myocyte (21). Furthermore, TNFα has been shown to activate the heat shock protein 72 (HSP72), thought to be protective against enviromental stresses (38). It is becoming apparent that TNFα may activate both detrimental and protective processes.

TNFα, like other cytokines, functions by binding to a specific receptor on the surface of the cell membrane. TNFα binds to two cell surface receptors, the TNF receptor 1 (TNFR1 or p55-R) and TNF receptor 2 (TNFR2 or p75-R) (103). Both TNFα receptors have been shown to interact with the TNF Receptor Associated Factor 2 or (TRAF2). TNFR1 is thought to act indirectly with TRAF2 through an adapter protein TRADD (TNFR1 associated Death Domain). TNFR2 can directly interact with TRAF2. TRAF2 is a 501 amino acid protein that is thought to be important for transducing extracellular signals from TNFα. It is composed of 4 distinct domains. The RING finger

domain is composed of amino acids 1 – 80 at the N-terminus. The ZINC finger domain is composed of amino acids 80 – 225. The RING finger and ZINC finger domains are required for NFκB activation in some cell types. At the C terminus, there are 2 domains called TRAF-N and TRAF-C (comprised of amino acids 225 – 501) which are thought to be important for interaction with the TNFα receptor. In cardiac myocytes, TNFα stimulation may ultimately activate detrimental pathways within the cell through the TNF receptors and TRAF2.

An important downstream target of TNFα stimulation is the activation of the ubiquitously expressed transcription factor NFκB (8, 32, 33, 37). NFκB belongs to a family of transcription factors that are involved in a myriad of important cellular processes. NFκB commonly exists as a heterodimeric protein consisting of p65 kilodalton (kDa) and p50 kDa subunits. The active subunit of the NFκB complex is p65 or RelA. In most cells NFκB is sequestered as an inactive complex in the cytoplasm by the 'Inhibitor of kappa B' (IkB) (104). A variety of extracellular signals, including TNFα, induce site specific phosphorylation on serine residues 32 and 36 of human IκBα, which leads to the subsequent degradation of IκBα by the proteasome allowing NFκB translocation to the nucleus. (51,52,53). Once in the nucleus, NFκB may activate a number of genes.

Nitric oxide (NO) is another important signaling molecule of TNFα (96,105). Nitric oxide can modulate the expression of genes in a number of different cell types (93,94). In myocytes, the primary source of NO is from the metabolism of L-arginine into nitric oxide and citrulline, which is catalyzed by the inducible nitric oxide synthase enzyme (iNOS). N^G-nitro-L-arginine methyl ester (L-NAME) is an analog of L-arginine

and acts as a competitive inhibitor of the inducible nitric oxide synthase (iNOS). Using L-NAME we could test the functional significance of TNFα mediated production of NO. Interestingly, multiple NFκB enhancer elements are present in the promotor of the human iNOS gene that confers inducibility to TNFα stimulation (96). *We hypothesized that TNFα mediated repression of cardiac muscle genes is via an NFκB and iNOS dependent pathway.*

TRAF2 is capable of transactivating the transcription factor NFκB and is required for induction of NFκB by TNFα in 293 cells and HeLa cells (25, 98 - 100). Takeuchi et al. (1996) demonstrated that the NFκB activating ability of TRAF2 resides within the N-terminal half, which comprises a RING finger domain in amino acids 1 – 98 and a zinc finger domain in amino acids 99 – 249 (97). In some cell types a TRAF2 mutant lacking its N-terminal RING finger domain acts as a dominant negative inhibitor of TNFα-mediated NFκB activation (97). In view of the fact that TRAF2 has been shown to be important for TNFα signaling, we tested whether TRAF2 is important for TNFα induced NFκB activation in cardiac myocytes.

The main goal of my research studies are (I) to determine the impact of TNFα on neonatal ventricular myocytes and (II) to identify the molecular mechanisms and signaling pathways which are important for TNFα to exert its effects. Thus, the specific aims of the study are to (1) determine the impact of TNFα stimulation of neonatal ventricular myocytes with respect to cell death; (2) determine the effect of TNFα stimulation on cardiac specific gene expression; (3) determine if NFκB, an important transcription factor known to be activated by TNFα, is activated in

ventricular myocytes by TNFα; (4) determine the functional significance of TNFα mediated NFκB activation; (5) determine the importance of the structural domains of the TRAF2 molecule with respect to TNFα induced NFκB activation.

II. REVIEW OF THE LITERATURE

A. General background

Clinically, heart failure is a syndrome that arises when the heart is unable to pump sufficient blood to meet the metabolic needs of the body. The basic mechanisms underlying heart failure continue to be a very active area of investigation and numerous potential mechanisms have been explored. The body responds to primary events such as acute myocardial infarction, pressure overload or volume overload by activating adaptive mechanisms intended to maintain homeostasis, such as compensatory hypertrophy, dilatation, and enhanced activity of the sympathetic nervous system and renin angiotensin system. However, these compensatory measures themselves may eventually play a role in the process of heart failure. In addition, many structural and biochemical alterations occur in the myocardium during the progression to heart failure. Defects in excitation contraction coupling (167), ATP synthesis (166), calcium handling (168) and oxidative stress (162) can contribute to increasing ventricular dysfunction. Several structural alterations of cardiac myocytes and cardiac interstitium also occur in heart failure. These alterations include, myocyte hypertrophy, changes in myocyte contractile protein composition, defects in mitochondrial function, and increased collagen accumulation (leading to interstitial fibrosis) (c.f. 137). Recent studies in experimental animals and in humans have shown that cardiac myocyte loss through programmed cell death occurs in cardiovascular disease. Importantly, significant loss of cardiac myocytes by apoptosis has been detected in patients with idiopathic cardiomyopathy, ischemic cardiomyopathy, and heart failure secondary to myocardial infarction (138,139). In view of the fact that adult cardiac myocytes have a very limited capacity for cell division, death of a

significant number of cardiac muscle cells can have devastating consequences. To date, little is known about the pathophysiological factors present in heart failure which trigger cardiomyocyte apoptosis.

Activation of proinflammatory cytokines such as the tumor necrosis factor alpha (TNFα) occurs in many cardiovascular diseases. Specifically, serum levels of TNFα are elevated in human cardiac conditions such as chronic heart failure, viral myocarditis and ischemic heart disease (38). The negative inotropic effects of TNFα are well documented (c.f. 140). Thus, TNFα may represent an additional factor that contributes to the progression of heart failure. The mechanisms of TNFα's effects, however, are not well defined.

B. Proinflammatory cytokines and the heart

B.1 Overview

There are many known cytokines including interleukins (1 to 18), tumour necrosis factors (alpha and beta), and interferons (alpha, beta and gamma). Tumor necrosis factor alpha (TNFα), interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β) and interleukin-6 (IL-6) have been classified as proinflammatory cytokines. These cytokines can initiate both primary host responses and tissue repair and can, by definition, increase their own production and the synthesis of other inflammatory mediators such as Platelet Activating Factor, ecosanoids, and oxygen radicals. Growing evidence suggests that proinflammatory cytokines play an important role in cardiovascular disease (87).

For many years, physicians and scientists have recognized that patients with heart failure share many of the clinical symptoms of those afflicted with chronic inflammatory

diseases (150). Indeed, patients with heart failure frequently develop "cachexia", which is characterized by a dramatic loss of body mass, anorexia, anemia, inflammation and other biochemical changes associated with severe malnutrition (150). An important discovery by Levine et al. (1990) identified the tumor necrosis factor as an important contributor to the pathogenesis of cachexia (46). This study examined the circulating levels of TNFα in patients with severe chronic heart failure and found that TNFα was elevated in patients with heart failure as compaired to healthy controls. Furthermore, heart failure patients with high levels of TNFα were more cachectic and had more advanced heart failure than those patients with lower levels of TNFα. These findings suggested a link between TNFα and cachectic patients with severe chronic heart failure. Tumor necrosis factor apha (TNFα) is a proinflammatory cytokine that influences growth, differentiation, and function of every cell type thus far investigated. In experimental animal models, long term administration of TNFα causes cachexia, anorexia and inflammation. In humans, elevated levels of circulating TNFα has also been associated with a variety of neoplastic, infectious and collagen vascular disorders characterized by muscle wasting and malnutrition (c.f. 91).

TNFα was first discovered by Carswell et al. (1975) when they noticed that the serum of mice injected with bacterial endotoxin lipopolysacharide (LPS) had anti-tumor activity (146). Subsequent studies by Matthews et al. (1978) and others revealed that this activity was due to a substance produced by macrophages (147). Due to its cytotoxicity to tumor cells the substance was named tumor necrosis factor. At around the same time, another substance was identified that was produced by lipopolysaccharide (LPS) stimulated macrophages that inhibited phospholipase activity. This factor could provoke

progressive wasting (cachexia) in rats and therefore was named 'cachectin'. Purification of the TNFα and cachectin proteins and analysis of their amino acid composition revealed that these substances were one protein (148). Molecular cloning and characterization of the human gene encoding TNFα revealed a 2767 base pair gene that is transcribed into a 1672 base pair cDNA. The cDNA codes for a 233 amino acid precursor that is processed into a 157 amino acid protein (c.f. 149). The cloning of TNFα made possible the production of recombinant TNFα. Due to TNFα's apparent anti-tumor activity and its involvement in cachexia, it was thought that both TNFα antagonists and agonists may have clinical application.

In the heart, TNFα may have both adaptive and maladaptive effects depending on the duration, intensity of exposure and context in which it is produced (38). Myocardial TNFα can be produced, by resident myocardial macrophages and cardiac myocytes themselves, in response to infection (endotoxemia) (39), ischemia reperfusion (40 - 42), burn trauma (49), myocardial infarction (42 - 44), and cardiopulmonary bypass (45). Although elevated levels of TNFα have been detected peripherally in cardiac diseases such as chronic heart failure (46 – 49), acute viral myocarditis (115), unstable angina (46), hypertrophic cardiomyopathy (115), and septic cardiomyopathy (165), the clinical and functional significance of this finding remains unknown (c.f.42). Given that TNFα is induced in response to a wide variety of adverse stimuli, it has been postulated that TNFα functions as a stress response gene in the heart. A number of observations have lent support for this idea. The first observation was that TNFα expression is not linked to a specific form of cardiac disease but rather it is observed in virtually all forms of cardiac injury (87). In patients, TNFα mRNA transcript and protein was detected in failing

hearts while it was undetectable in non stressed hearts (48). Similarly, in experimental models, TNFα mRNA and protein are rapidly synthesized by the heart in response to stressful stimuli and once the stimuli is removed, the TNFα mRNA is rapidly degraded (39). Interestingly, TNFα stimulation of adult cardiac myocytes has been shown to induce the expression of heat shock protein 72, which is thought to play a protective role against different forms of enviromental stress (109). Nakano et al. (1997) tested whether short exposures to TNFα would protect isolated cardiac myocytes against enviromental stress. To test this, isolated adult ventricular myocytes were pretreated with TNFα for 12 hours and then subjected to 12 hours of continuous hypoxic injury (21). Cell injury in these experiments was assessed by lactic dehydrogenase release, Ca2+ uptake and MTT metabolism. MTT is a tetrazolium salt that turns blue when reduced by the respiratory enzymes present in functioning mitochondria and it is an indicator of cell viability. TNFα pretreatment resulted in increased resistance to hypoxic stress. The mechanism of this effect remains unknown but lends support to the possibility that TNFα expression may be adaptive in some conditions. Generally, short term expression of TNFα within the heart is thought to be adaptive and beneficial for the heart (21,38). On the other hand, long term expression of TNFα may be detrimental and contribute to cardiac disease (163).

Importantly, over expression of TNFα has been associated with left ventricular dysfunction, LV remodelling, and hypertrophic cardiomyopathy. Recently, evidence has accumulated to support the 'cytokine hypothesis' for heart failure implicating TNFα as an important contributor to disease progression by virtue of its direct cytotoxic effects (87,90,91). Mann et al. (1997) showed that systemic administration of

pathophysiologically relavent concentrations of TNFα was sufficient to produce left ventricular dysfunction, remodeling and DNA damage (92). The origin of TNFα, under some conditions, is cardiac myocytes themselves (39,48). Production of TNFα, by cardiac myocytes isolated from transgenic mice that overexpress myocardial TNFα, was sufficient to cause severe impairment of cardiac function, marked dilatation, depressed ejection fractions, and myocarditis.

IL-1β, a proinflammatory cytokine, has been shown to modulate the growth and phenotype of neonatal rat cardiac myocytes. Thaik et al. (1995) discovered that IL-1b could increase protein synthesis and activate the transcription of the atrial natriuretic factor mRNA, and βMHC mRNA and repress transcription of the sarcoplasmic reticulum Calcium-ATPase, calcium release channels, and voltage dependent calcium channels (159). Cardiac gene modulation by IL-1β was thought to occur by a nitric oxide independent mechanism. IL-6 is another proinflammatory cytokine that has been associated with a number of cardiovascular disorders. Kinugawa et al. (1994) studied the effects of IL-6 on intracellular calcium concentration and cell contraction in isolated cardiac myocytes (93). They found that IL-6 acutely decreases intracellular calium concentration and depresses cell contractility by a nitric oxide – cGMP mediated pathway.

TNFα intitiates numerous signaling cascades after engaging its receptor. It is thought that myocardial TNFα impacts on cardiac function by both nitric oxide - independent (sphingosine dependent) and NO-dependent mechanisms (via NFκB and iNOS). Whether TNFα's function in these and other biological processes is adaptive or maladaptive is unclear.

B.2 TNFα signaling in the heart

How does TNFα signal its biological response in the heart? TNFα can bind to two cell surface receptors; the TNFR1 (55 kDa) and the TNFR2 (75 kDa) which are present on the surface of most cells including cells in the myocardium of normal and diseased human hearts. Interestingly, the intracellular domain of TNFR1 and TNFR2 share little homology suggesting that these receptors transduce distinct signals inside the cell (50). TNFR1 and TNFR2 knockout mice and experiments using receptor specific agonistic antibodies have confirmed this idea and demonstrated that the two TNF receptors generate independent intracellular signals. TNFR2 signaling occurs less extensively and seems to be confined to cells of the immune system. Some of the consequences of TNFR1 activation include cell death, gene modulation, antiviral activity, phospholipase C activation, phospholipase A activation, activation of acidic and neutral sphingomyelinases and activation of the transcription factor nuclear factor kappa B (NFκB).

TNFα engagement of TNFR1 leads to the recruitment of the receptor associated factor TRADD (TNFR2 associated Death Domain) and RIP (receptor interacting protein) (4). TRADD can interact with TRAF2 (TNFR-associated factor 2) and FADD (Fas-associated death domain protein) (99). There are many mediators of TNFα signaling and multiple intermediate steps in the TNFα signaling cascade. TNFα engagement of TNFR2 results in the recruitment of TRAF2 (25). The recruitment of TRAF2 and RIP may be important for the transduction of TNFα signaling of NFκB (100). The pathway from TRAF2 and RIP to NFκB activation may involve the activation of NIK (NFκB

inducing kinase) (4). NIK activates the Inhibitor kappa B kinase (IKK) which in turn phophorylates the inhibitory kappa B (IkBα) leading to IkBα degradation allowing NFκB to translocate to the nucleus and transactivate genes. Importantly, it has been hypothesized that NFκB, once activated, may turn on antiapoptotic genes in some cell types (25). However, the functional significance of TNFα induced NFκB activity in the heart is unknown and is an important component of my current studies. TRAF2 and RIP have also been implicated in the activation of the Janus N-terminal Kinase pathway (JNK/AP-1) (142). The death domain containing proteins such as TRADD and FADD, once recruited to the transmembrane domain of the TNFR1 may function to activate both pro-apoptotic and anti-apoptotic pathways. Although, the exact mechanisms of action of TNFα in the heart and the impact it has on cardiac function is unclear, NFκB and nitric oxide (NO) are important downstream targets that may play pivotal roles in TNFα signaling.

B.3 Role of nuclear factor kappa B in TNFα signaling

NFκB is the name given to a family of dimeric transcription factors that are involved in many important cellular processes. The family includes five members (c-Rel, Rel B, p50/p105, p52/p100, Rel A) that contain DNA binding motifs (54). NFκB can be activated in response to a variety of stimuli including ionizing radiation, hypoxia, phorbol esters, and pro-inflammatory cytokines such as the tumor necrosis factor – alpha (TNFα) and interleukin – 1 (IL-1) (32,33). The down stream gene targets of NFκB likely vary according to the cell type, the activating stimulus and the physiological state of the cell. NFκB has been shown to activate genes which encode cell adhesion molecules (14 - 16),

heat shock proteins (109), inducible nitric oxide synthase (96) and genes involved in the inflammatory response (11 – 13). More recently, it is apparent that NFκB may also be capable of activating the transcription of anti-apoptotic proteins such as the cellular Inhibitors of Apoptosis proteins (cIAP) (8,9), A20 (164) or IEX-1L (10). NFκB also influences genes that control cell proliferation, a cellular state that is thought to be incompatible with apoptosis.

In most cells NFκB is sequestered as a latent complex in the cytoplasm by IkBα. A variety of extracellular signals, including TNFα, induce site specific phosphorylation of the N-terminal serine residues 32 and 36 of IkBα. This leads to the ubiquitination of IkBα and its subsequent degradation by the proteasome allowing NFκB to translocate to the nucleus (51,52,53).

Much of the knowledge of NFκB function has come from p65 knock-out studies. P65 -/- mice, for example, suffer massive losses of liver cells due to apoptosis, suggesting a role for p65 containing NFκB dimers in protecting cells from apoptosis (17). Baltimore et al. (1996) demonstrated an essential role for NFκB in preventing TNFα-induced cell death (28). In a related paper, TNFα induced apoptosis of a tumor cell line was potentiated by the inhibition of NFκB (30). The anti-apoptotic action of NFκB was also demonstrated by Mayo et al. (1998). In this study, NFκB activation was required to suppress p53-independent apoptosis. These findings suggest NFκB activation is important to protect cells from pro-apoptotic stimuli (25 - 30).

Over the past few years, studies have shown that NFκB is activated in a number of cardiac disease conditions. An important study published by Wong et al. (1998) examined the presence and localization of NFκB in patients with end-stage heart failure

(141). They found that NFκB was activated in myocytes and inflammatory cells in the infarcted myocardium and the throughout the myocardium of septic hearts while there was no NFκB present in normal hearts. Both in vivo and in vitro studies show that NFκB activation occurs in ischemia reperfusion, myocardial ischemia, and congestive heart failure and recently has been found to be upregulated in aging. These findings emphasize the clinical importance of NFκB in cardiovascular disease.

B.4 Role of nitric oxide in TNFα signaling

Recent evidence suggests that inflammatory mediators such as cytokines and nitric oxide may play an important role in heart failure. Nitric oxide is synthesized by a family of nitric oxide synthase (NOS) enzymes including consitutive NOS, endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (c.f. 160). Cytokines such as TNFα and IL-1β can induce iNOS in a number of cell types including cardiac myocytes (111). Once activated, iNOS is capable of mediating the production of large amounts of nitric oxide. The iNOS enzyme is found in cardiac myocytes, vascular smooth muscle cells and inflammatory cells. Interestingly, increased iNOS levels have been found in patients with heart failure (160). The negative inotropic effects of TNFα have been well documented (c.f. 158). In view of the findings that TNFα is elevated in heart failure, it is possible that TNFα's negative inotropic effects are mediated by the production of nitric oxide. Finkel et al.(1992) demonstrated that TNFα inhibited the contractility of isolated papillary muscles in a concentration dependent manner and that this effect was abrogated by the nitric oxide synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) (161).

C. Programmed cell death

C.1 Definition

Apoptosis, also known as programmed cell death, is an important and widespread biological phenomenon that is a highly conserved and genetically regulated process of cell destruction (118). Once provoked, apoptosis follows a carefully orchestrated script that can be identified by characteristic morphological and biochemical alterations. Cell shrinkage, membrane blebbing, and condensation of nuclear chromatin are early morphological changes which can be visualized by light microscope (119). Cells undergoing apoptosis in vivo also show loss of membrane contact with neighbouring cells and disappearance of gap junctions (135). A hallmark of apoptosis is the activation of specific nucleases that degrade chromosomal DNA into small oligonucleosomal fragments that can be visualized by agarose gel electrophoresis (121). Finally, the last stage of apoptotis involves cellular fragmentation into membrane bound 'apoptotic bodies', which are then engulfed by neighboring cells. In this way, dead cells are rapidly removed preventing the leakage of noxious and potentially dangerous contents, thus avoiding an inflammatory response. In contrast to apoptosis, necrosis is characterized by cell swelling, loss of membrane integrity, random degradation of DNA and is often accompanied by an intense inflammatory response (120).

Apoptosis plays a prominent role in embryologic development (122). For example, the regression of tissue in the interdigital zone between fingers and toes occurs as a result of a genetically pre-determined program of apoptotic cell death. Apoptosis is also responsible for the positive and negative selection of T and B lymphocytes (129).

The immune system relies heavily on the ability to initiate apoptotic cell death as an important defence mechanism that removes unwanted and potentially dangerous cells (124 - 128). Tissue homeostasis and cell numbers are carefully maintained in a steady state through the processes of programmed cell death and cell division (123). Importantly, suppression of apoptosis is associated with cell growth, and the maintenance of genetic stability (126). Evidently the processes of cell death and cell division are intimately coupled and have a substantial impact on the development and overall well being of the organism.

The importance of apoptosis is realized when the balance of cell death and cell division is disrupted. Too much cell death can result in impaired development and degenerative diseases, while insufficient cell death can lead to cancer and persistent viral infection (129). Inappropriate cell death has been implicated in the pathogenesis of several neurodegenerative disorders such as Alzheimers (130), Parkinsons (131), and Huntington's diseases (132).

A well defined example of insufficient cell death contributing to disease involves a critical regulator of apoptosis, the anti-death B-cell lymphoma/leukemia-2 (Bcl-2) gene product (133). Bcl-2 is known for its involvement in B-cell lymophomas (134). In the vast majority of cases of B-cell lymphoma, a chromosomal translocation (t14:8) results in the deregulation of Bcl-2 transcription and overproduction of the Bcl-2 gene product, which produces malignancy. More recently, it has become apparent that apoptosis may play a critical role in the progression of cardiovascular disease. Increasingly, programmed cell death is being recognized as a contributing cause of myocyte loss with

ischemia reperfusion injury, myocardial infarction, end-stage heart failure, cardiac allograft rejection, and vascular wall remodeling (c.f. 71).

C.2 Mechanisms of programmed cell death in the heart

The precise mechanisms of apoptosis in the heart remain poorly understood. In general, the process of apoptosis can be divided into an activation phase, a decision phase, and the execution phase. Numerous factors, both internal and external can trigger the activation phase of apoptosis. Production of reactive oxygen species, for example, is an important mediator of cell damage in the heart and can also activate the apoptotic pathway (154). Another potential activator of apoptosis in some cell types is the proinflammatory cytokine TNFα, which has been implicated in numerous cardiac diseases. Once the apoptotic pathway is activated, a 'decision' is made to execute. The execution stage of the apoptotic pathway is highly conserved among different species and indeed, throughout evolution.

Numerous families of positive and negative regulators of apoptosis have been identified. As already mentioned, the Bcl-2 family of proteins are important regulators of apoptosis. This family includes both pro-apoptotic (Bak and Bax) and anti-apoptotic (Bcl-2, Bcl_{xL}) members (c.f. 71). Although the molecular mechanisms of myocardial apoptosis remain poorly defined recent reports have implicated the *Bcl-2* gene product as an important regulator of myocardial apoptosis. A seminal finding by our laboratory demonstrated that expression of Bcl-2 in ventricular myoocytes rendered myocytes more resistant to apoptotic stimuli (2). Another important molecule involved in the regulation of apoptosis is the tumor suppressor protein p53 (152). Recently, Kirshenbaum et al.

(1997) and others have identified a role for p53 in apoptosis of cardiac muscle cells (2,157).

The motivation for studying the molecular mechanisms of apoptosis is that it is involved in the pathogenesis of a number of diseases including heart disease (118). Apoptosis has been detected in patients and experimental models of cardiac remodeling, dilated cardiomyopathy, ischemic heart disease, myocardial infarction, and pressure overload hypertrophy (induced by aortic banding, or in spontaneously hypertensive rats) (c.f. 71). The pathophysiologically important triggers of apoptosis in these cardiovascular diseases may include oxidative stress (154), ischemia / hypoxia (156), stimulation by proapoptotic immunologic or neurohormonal factors (171) and calcium overload (c.f. 71).

C.3 Programmed cell death in heart failure

Heart failure is the leading cause of death in the Western world and it is estimated that over 3 million people in the United States are afflicted with the disease (139). Heart failure can be defined as the progressive deterioration of myocardial function and can result from a variety of conditions including hypertension, ischemic heart disease, coronary artery disease, valvular heart disease, inflammatory heart disease, and ischemic heart disease. Little is known about the mechanisms that contribute to the progressive cardiac dysfunction seen in heart failure. Recently, it has been hypothesized that ongoing myocyte cell death by apoptosis may contribute to the progressive deterioration of cardiac function. Adult, terminally differentiated myocytes have exited the cell cycle and no longer retain the capacity to divide or repair themselves. Therefore loss of viable

cardiomyoycte cells may be an important contributing factor in the progressive deterioration of ventricular performance with time. Numerous reports of apoptosis in heart disease lend support to this hypothesis (68 – 71). Narula et al. (1996), examined 7 explanted hearts from patients with idiopathic dilated cardiomyopathy and ischemic cardiomyopathy and found significant levels of apoptosis in the subendocardial and subepicardial regions of the heart by in situ DNA terminal deoxynucleotide nick-end labeling (TdT assay) and DNA laddering (139). Double staining for actin demonstrated that the apoptosis was predominantly confined to myocytes. In comparison, control hearts showed only rare isolated apoptotic myocytes. The high incidence of apoptosis reported in this study was not addressed but likely reflects the sensitivity of the TdT assay. In a more extensive study by the Anversa lab (1996), 36 patients with ischemic cardiomyopathy, idiopathic dilated cardiomyopathy and valvular heart disease were examined for signs of apoptosis using DNA ladering and a modified TdT assay with a fluorescence probe. In this study control hearts showed an incidence of apoptosis of approximately 10 per million (or 0.001 %). Diseased hearts exhibited an increase in DNA laddering and incidence of TdT positive nuclei (2400 per million or 0.24 %).

Given the limited potential for terminally differentiated adult myocytes to proliferate after birth, cardiac cell death may represent an important factor contributing to the progression of hypertrophy and end stage heart failure (73). With substantial evidence for the existence of apoptosis in cardiovascular disease scientists are actively investigating the mechanisms of apoptotic cell death in the heart.

D. Cardiac growth and hypertrophy

D.1 Definition

Cardiac hypertrophy is defined as a process wherein there is an increase in chamber mass produced largely by an increase in the size of terminally differentiated cardiomyocytes (57). Cardiac hypertrophy can be divided into two classes; (1) physiologic growth without clinical complications; and (2) pathophysiologic growth, which is accompanied by clinical signs and symptoms. The increase in heart size that evolves during senescence and that which occurs during physical conditioning in athletes is considered physiologic hypertrophy. It is a normal adaptive response to increased stress. Pathological hypertrophy occurs in response to abnormal global or regional increases in cardiac work. Initially, the increased mass serves to normalize wall stress, and permit normal cardiovascular function (at rest and during exercise). This is called compensated hypertrophy. If the stimulus for pathologic hypertrophy is sufficiently intense or prolonged, decompensated hypertrophy and heart failure ensue (58). A variety of pathologic stimuli such as myocardial infarction, hypertension, valvular diseases, viral myocarditis, dilated cardiomyopathy, and certain genetic diseases such as hypertrophic cardiomyopathy (HCM) can lead to an increase in work load resulting in hypertrophy. Cardiac hypertrophy can become progressively maladaptive and is often an indicator of poor prognosis for patients with cardiovascular disease (57).

While the cardiac hypertrophic growth response initially functions to counter act the increased work load, in many instances, the response becomes maladaptive and heart failure occurs. As hemodynamic overload persists, the stressed heart enters a critical transition from compensated hypertrophy to decompensated heart failure. The molecular

basis for this progression is not well understood. Changes in myocardial gene expression (ex. abnormal myosin expression) (64), activation of neurohormonal systems (ex. sympathetic activation) (151), activation of local tissue renin angiotensin aldosterone system (172), increased metalloproteinase activity (169,170), production of immunologic molecules such as TNFα (46), and myocyte cell death may all be involved in the development and progression of heart failure (c.f. 73). Common clinical and pathological features of end-stage failing hearts include chamber dilatation, excitation-contraction uncoupling, abnormal interstitial morphology, sarcomeric disorganization, altered energy metabolism, and the loss of viable myocytes (c.f 144, 145).

D.2 Molecular aspects of cardiac hypertrophy

An important property of the mammalian myocardium is the ability to adapt to an increased hemodynamic load. Numerous changes in myocardial gene expression occur during the adaptation of the heart to chronic stresses (ex. Pressure or volume overload) and the nature of these changes could influence the long term prognosis of patients with heart failure. A hallmark of this event in rodent models of cardiac hypertrophy, is the expression of numerous cardiac-specific genes encoding protein isoforms ordinarily associated with the embryonic or fetal heart (55). As yet, the molecular mechanisms that bring about the changes in cardiac gene expression and subsequent remodeling of the myocardium are not well defined.

In the hypertrophied myocardium, alterations exist in terms of vascular supply (60), composition of the myocyte cytoplasm (61), expression of fetal proteins (62), changes in myosin isozymes (63,64), and mechanical properties of the muscle (65).

Overloading of the adult heart initiates an immediate-early response which is characterized by re-activation of immediate early proto-oncogenes such as c-myc, c-fos, c-jun which are normally associated with cell proliferation and active protein synthesis (85). A hallmark of this transition involves the reactivation of the 'fetal' phenotype' that includes activation of the βMHC, atrial natriuretic protein (ANP) and α-skeletal actin genes. The precise mechanism by which hemodynamic overload induced hypertrophy transduces these changes in cardiac gene expression is not known. However, a number of transcription factors have been found to be important for the regulation of cardiac genes such as the βMHC and αMHC. Hasegawa et al. (1997) demonstrated that a GATA transcription factor that binds to a GATA element within the βMHC promotor plays a role in the regulation of βMHC expression during hemodynamic overload induced by aortic constriction in rats (178). Other transcription factors have been implicated as important regulators of cardiac gene expression such as TEF-1 (179) and MEF-2 (180).

Overall, studies in rodents have shown that surgically induced hemodynamic overload leads to an overall increase in the rate of protein synthesis. In specific, the atrial natriuretic factor, β-tropomyosin, atrial myosin light chain, β-myosin heavy chain (βMHC), α-skeletal muscle actin (αSkl) and α-smooth muscle actin all tend to be up-regulated during pressure overload. Of particular importance, is the myosin heavy chain (MHC) that is a major structural component of the sarcomere and the molecular motor of muscle.

Three isozymes of myosin exist that are denoted V1, V2 and V3. These isozymes consist of 2 heavy chains called alpha-MHC and beta-MHC. V1 is the αMHC/αMHC homodimer, V2 exists as an αMHC/βMHC heterodimer and V3 is the β–MHC/β–MHC

homodimer. Hemodynamic overload has been shown to result in an isoform transition from V1 to V3 in animal models (80,81). The adult rodent predominantly expresses the alpha-myosin heavy chain isoform (αMHC). The beta isoform is normally expressed during fetal development. Izumo et al. (1987) and others reported a rapid induction of mRNA levels of the fetal isoform βMHC, followed by an increase in βMHC protein in parallel with an increase in left ventricular weight in rats subjected to aortic coarctation (77,82). The changes in βMHC mRNA levels progressively increased during the first week of banding and persisted as long as the load was maintained. Theoretically the changes in gene expression observed may be due to several different mechanisms. Changes in transcriptional regulation, mRNA stability, preferential translation of particular mRNAs, and changes in protein stability are all factors which may contribute to changes in mRNA levels and/or protein levels. The observed close correlation between the relative levels of MHC mRNAs and the corresponding proteins suggested that the MHC isozyme transition during overload is due to a pre-translational mechanism.

The close correlation between the velocity of contraction and the specific activity of the myosin ATPase lead to the close investigation of the distinct myosin isoforms. V1, the a/a homodimer, has the highest ATPase activity, V3, the b/b homodimer, has the lowest ATPase activity, and V2, the a/b heterodimer, has an intermediate ATPase activity. In several models of cardiac hypertrophy, an increase in myocardial mass has been shown to be associated with a decrease in wall tension and a decrease in the velocity of shortening (78). A relationship between decreased mechanical performance and decreased myosin ATPase activity was reported in several animal species in various models of hemodynamic load (79). The alpha isoform of myosin possesses a higher

ATPase activity than the beta counter part and as a result the isozyme switch from alpha to beta leads to a slower and a more efficient contraction of the cardiac fiber.

Gwathmey et al. (1991) investigated whether cross bridge kinetics contributed to the contractile abnormalities observed in heart failure. The study compared cross-bridge cyyling rates in explanted myopathic hearts and control hearts and found a reduction in cycling rates in the failing hearts (84). They also observed that there was no change in maximal calcium activation between control and failing hearts. The authors concluded that the potential for force development is similar in control and myopathic hearts and therefore changes at the level of contractile proteins are important in reducing cross bridge cycling rate and lowered energy requirements.

Another major component of the sarcomere is actin. There are 3 sarcomeric actins that exist, alpha skeletal actin (αSkl), alpha smooth muscle actin (αSml) and alpha cardiac actin (αCA). The α-skeletal actin mRNA's and α-smooth muscle actins accumulate in hypetrophied adult rat hearts (76,55). The α-skeletal actins appear to peak by 2 – 4 days then decrease to low levels in rat models of hemodynamic overload.

Recent studies in humans, using quantitative reverse transcriptase polymerase chain reaction (RT-QPCR), have measured mRNA expression in biopsy sized samples from intact human hearts. Myosin mRNA, showed substantial differential expression in control and failing hearts using RT-QPCR. Bristow et al. examined endomyocardial biopsy sized specimans from the right and left ventricles of intact human hearts and measured changes in mRNA levels of β–adrenergic receptors (β1 and β2), αMHC, ANP, βMHC, sarcoplasmic reticulum calcium ATPase (63). Patients studied had primary pulmonary hypertension or idiopathic dilated cardiomyopathy. Adult αMHC mRNA

content, as a percentage of total mRNA, was decreased from 28% to 6.1% in the left ventricle as determined by quantitative RT-PCR on biopsy sized samples from the intact adult heart (63). Similar reductions in αMHC mRNA was seen in samples taken from the right ventricular free wall, and RV endomyocardium. Data indicates that αMHC mRNA in non-failing ventricular myocardium comprises approximately 23 – 34 percent of total myosin RNAand is profoundly decreased in heart failure. Down regulation in αMHC was coupled with a reciprocal upregulation of βMHC. Given the differential ATPase activity in αMHC and βMHC isoforms, the down regulation of αMHC could theoretically have an effect on the velocity of contraction in humans. In support of this, hearts expressing more αMHC have a more rapid contractile velocity. Hearts with more βMHC allow for a slower contraction and a greater economy in force generation. The observed αMHC to βMHC transition corresponds to the multiple reports of decreased ATPase activity in human heart failure (86). These findings are corroborated by another study by Nakao et al. (1997) that quantitated the relative amounts of α and β MHC mRNAs in the left ventricular free walls of 14 heart donor candidates and 19 patients with chronic end stage heart failure. Two techniques were utilized to quantitate mRNA expression levels, a quantitative RT-PCR assay and a ribonuclease protection assay (64). Approximately 33 $\pm$ 19 percent of the total myosin mRNA (that is, αMHC and βMHC) in the left ventricle was identified as the αMHC isoform. Failing hearts showed significantly lower αMHC mRNA (2.2 $\pm$ 3.5 %, $p<.0001$) in the left ventricles compared to controls. One question arises from the αMHC gene expression data in humans and animal models. That is, what is the mechanism by which cardiac muscle gene expression is modulated?

E. Gene transfer into cardiac muscle cells

Gene transfer, in vitro, has been accomplished using microinjection, projectile bombardment (gene gun), electroporation and by direct injection (c.f. 113). A variety of chemical methods have also been employed including calcium phosphate, DEAE dextran, and lipofection. Microinjection, the introduction of naked plasmid DNA into an individual cell, has its uses, but is impractical for large scale transfections. Direct injection, which is the gross injection of naked plasmid DNA into a tissue, is impractical because it results in a high degree of trauma and poor efficiency and uniformity of gene transfer. Chemical methods have varied results, depending on the chemical used and the cell type being transfected. Lipofection for example, can transfect some cell types with good uniformity and efficiency and has a low toxicity. Cardiac muscle cells, however, are difficult to transfect. Only recently have scientists devised methodologies for highly efficient and uniform gene transfer in mammalian cells (5).

Several viruses have been studied and used as potential gene transfer systems, however the most notable are the retrovirus and adenovirus. The recombinant adenovirus, in particular, has been extensively studied for the transfer of genes into mammalian cells in vitro and in vivo with great success. Of particular importance to the current studies is the seminal finding by Kirshenbaum et al. (1993) which demonstrated the feasibility of using recombinant adenovirus to transfer genes into cardiac muscle (3). Several reviews dealing with the potential and limitations of the various techniques for gene transfer into cardiomycocytes and application of gene therapy in different animal models are available including a review by Kirshenbaum (1997) (5). The technology of gene transfer has opened a world of possibilities for research in biology. It has

contributed greatly to our knowledge of the signaling pathways and key players that seem to be important in the pathogenesis of cardiac disease. My current studies rely heavily on the technology of gene transfer, using both chemical methods and recombinant adenoviruses.

While cardiovascular disease is the leading cause of death in western populations and significant progress has been made in the prevention and management of cardiovascular diseases there is as yet no universal cure. Molecular cardiology is a rapidly growing field that is at the forefront of understanding the underlying molecular mechanisms of cardiovascular disease. Progress in our understanding of specific pathways and mediators that play critical roles in the pathogenesis of cardiovascular disease (and in normal cell homeostasis) has provided many potential targets for therapeutic intervention. Furthermore, understanding how genes regulate cell processes such as cell proliferation, cell repair and apoptosis reveals another potential site of therapeutic intervention - at the level of cardiac gene expression. The transfer of DNA into cells of patients to interfere with pathogenic processes may prove to be a novel approach for treatment and prevention of cardiac disease. Already, recombinant adenovirus-mediated gene transfer technology has even been applied to the correction of the cystic fibrosis gene defect in humans (112).

III. MATERIALS AND METHODS

ISOLATION OF NEONATAL CARDIOMYOCYTES

Neonatal ventricular myocytes were isolated from 2 day old Sprague-Dawley rat hearts, and submitted to primary culture in DF 10 as previously described (1). DF 10 contains Dulbecco's modified Eagle's medium (DMEM)/ Ham's nutrient mixture F – 12, (DF) 1:1, which is supplemented with 17 mM HEPES, 3mM NaHCO3, 2 mM L – glutamine, 50 μg/mL gentamicin, and 10 % fetal bovine serum (FBS). Neonatal rats were sacrificed by cervical dislocation and immediately placed in 70 % ethanol. Hearts were then excised from the rats and placed in chilled Phosphate Buffered Saline (PBS). Hearts were minced and rinsed in cold PBS to remove red blood elements and cellular debris. The minced heart tissue was then placed in 8.5 mL PBS to which 740 U collegenase, 370 U trypsin and 2880 U DNase is added. The solution was agitated gently for 10 minutes allowing the digestion of heart tissue. The enzymatic digestion was inactivated by adding 20 mL of DF 20. In total, 6 digestions were performed, each time pooling the cells. Cells were then centrifuged at 115 x g for 5 minutes and the supernatant discarded. Neonatal ventricular myocytes were isolated using percoll gradient purification. The percoll gradient solutions consisted of varying densities; 1.050 g, 1.060 g and 1.082 g per mL in Ads buffer 10X (1.16 M NaCl, 55 mM dextrose, 109 mM NaH2PO4, 54 mM KCl, 4.1 mM MgSO4, 200 mM HEPES with and without 0.6 mM Phenol red). After percoll purification, 4 distinct bands appear, consisting of myocytes, fibroblasts, red blood cells and debris, and a 'red phase'. Myocytes were plated at a density of $1*10^6$ cells per 35 mm dish. After overnight incubation in DF 10 cells were transferred to serum free media and utilized for experimental protocols.

PLASMID CONSTRUCTS

To analyze cardiac gene expression we utilized plasmids containing promotor elements of the βMHC, αMHC, αCA and αSk genes in front of the luciferase gene. The luciferase reporter constructs were transfected into neonatal ventricular myocytes. After twenty-four hours cells were treated with 10 ng / mL of TNFα for 24 hours and analyzed for luciferase activity. To measure transcriptional activity of NFκB we used a luciferase reporter construct containing 3 NFκB 'cis acting' elements, denoted 3XkB luc. A plasmid construct encoding the human IκBα wild type, driven by the cytomegalovirus promotor (CMV), was provided by Dean Ballard (Vanderbilt University) (108). The flag tagged CMV IκBα (S32/36A) construct denotes the human IκBα protein with serine to alanine mutations at positions 32 and 36. These alterations prevents IκBα from being targeted for ubiquitination and degradation by the proteasome and as a result, it remains constitutively active and bound to NFκB.

Experiments involving the "TNFα Associated Factor 2" (TRAF2), various structural mutants were utilized. The flag tagged TRAF2 (80 – 501) structural mutant was provided by David Goeddel (Tularik Inc.) (25). The TRAF2 wild type designated pC FLAG – TRAF2 (1 – 501) and other structural mutants designated pC FLAG – TRAF2D (225 – 501), and pC FLAG – TRAF2 RING (1 – 224) were provided by David Wallach (Weizman Institute) (4). The various TRAF2 structural mutants are illustrated in the appendix.

To control for variability in transfection efficiency between conditions, luciferase activity was normalized to βgalactosidase (CMV βgal) activity and expressed as relative

light units.

CELL TRANSFECTIONS

For transfection of neonatal myocytes, cells were incubated in DMEM containing DEAE-dextran, 5 μg of luciferase reporter plasmid DNA, 3 μg of CMVβgal, and 2 μg of plasmid DNA in 2.5 % calf serum for 40 minutes. Subsequent to the transfection, myocytes were stimulated with 10 ng / mL of human recombinant TNFα (R&D Systems) or 1 mM N^G-nitro-L-arginine methyl ester (L-NAME) in serum free conditions for 24 hours (Sigma Chemical Company). TNFα has an 'Effective Dose' or ED_{50} of .02 - .05 ng / mL in murine L-929 cells. L-NAME is an analog of L-arginine and acts as a competitive inhibitor of inducible nitric oxide synthase (iNOS).

RECOMBINANT ADENOVIRUSES

Adenoviruses were propagated, harvested, titered, and purified as previously reported (2). Replication defective adenorvirus was propagated in human embryonic kidney 293 cells that contain and express the genes necessary for adenoviral replication. IκBα (S32/36A) denotes the recombinant adenovirus that encodes a human IκBα protein with serine to alanine mutations at residues 32 and 36. The dominant negative IκBα S32/36A was engineered into recombinant adenovirus by methodology described by Kirshenbaum (5). The 'AdCMV IκBα (S32/36A)' or 'AdCMV IκBα mutant' was engineered in our lab by first inserting the IkB mutant gene into the PCA3 plasmid using HindIII/XbaI restriction enzyme sites. The transgene was rescued into virus by cotransfecting the IkB (S32/36A) PCA3 construct with the PJM17 plasmid consisting of

the viral genome in 293 cells. To confirm the presence of the IkB mutant gene in the engineered adenovirus, viral DNA was isolated and PCR primers specific for the IkB gene was used to detect the presence of the transgene. AdCMV IkB wild type, generously provided by Fritz Bach (107), denotes a recombinant adenovirus that encodes the wild type human IkB alpha protein driven by the human cytomegalovirus immediate early enhancer (CMV). To control for the effects of viral infection alone, we used the adenovirus designated AdCMV that contains the CMV enhancer-promotor with an empty expression cassette.

ADENOVIRUS INFECTIONS

Myocyte cultures were infected for four hours with 10 - 200 plaque forming units per cell of recombinant adenoviruses that encode either IkB wild type, IkB (S32A/S36A), AdCMV. Viral stocks were diluted to 2 10^8 pfu per mL in serum free for 4 hours. The infectious material was removed and replaced with fresh serum free media for an additional 20 hours. Using these conditions, greater than 95 % of neonatal ventricular myocytes express the transgene (2,3). Following infection, media was replaced with DFSF for 20 hours and supplemented for 24 hours with 10 ng per mL of recombinant human TNFα (R & D Systems) then harvested for protein or nuclear extract or fixed for immunocytochemistry.

LIVE / DEAD ANALYSIS

The effects of TNFα on cell viability was determined by vital staining of ventricular myocytes in the presence and absence of TNFα. Cells were labeled with 2

uM of calcein acetoxymethyl ester and 2 uM of ethidium homodimer-1 (Molecular Probes). This allows us to distinguish the live cells from dead cells by epifluoresence microscopy; green cells are alive; red cells are dead. The relative number of green versus red cells were taken to indicate cell viability (1).

ELECTROMOBILITY GEL SHIFT ASSAY

Nuclear extracts of neonatal ventricular myocytes were prepared by scraping cells in 200 uL of lysis buffer (10mM Tris pH 8.0, 60 mM KCl, 1mM EDTA, 0.3 % NP40, 1 mM DTT and protease inhibitors) as described previously (31). The cells were then centrifuged and resuspended in a buffer containing 20mM hepes pH 7.9, 0.4 M NaCl, 1mM EDTA, 1 mM EGTA, 1mM DTT and 1 mM PMSF. A ^{32}P radiolabelled oligonucleotide probe 5'AGTTGAGGGGACTTTCGCAGGC'3 was used as a template for the gel shift experiments. DNA binding reaction mixtures contained 5 μg nuclear extract in 20 mM HEPES, 5 % glycerol, 1mM EDTA, 5 mM dithiothretol. DNA-protein complexes were resolved on a native 5 % polyacrylamide gel in 1X Tris Borate - EDTA pH 8.0.

WESTERN BLOT ANALYSIS

For detection of proteins in cardiac myocytes, cells were harvested in lysis buffer (1% Triton X, 0.1% sodium dodecyl sulfate, 140 mM NaCl, 10 mM Tris, and 1% deoxycholate) containing protease inhibitors. Cell lystates were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel at 140 volts for 4 hr and electrophoretically transferred to a polyvinylidene difluoride (PVDF) (Roche Diagnostics) membrane at 30

volts. Membranes were blocked for 1 hour in 5 % powdered milk in TBS-TWEEN (150 mM NaCl, 50 mM Tris-HCl, 0.3 % Tween-20, pH 7.4) and incubated with primary IgG antibodies at a 1:1000 dilution overnight at 4 C directed toward a given protein of interest. For detection of IkB, the PVDF membrane was incubated with a rabbit polyclonal antibody directed toward amino acids 297 – 317 from the carboxy terminus of human IκBα (Santa Cruz Biotechnology). Bound proteins were detected by a chemiluminescence reaction using horseradish peroxidase conjugated antibody with ECL reagents (Amersham).

STATISTICAL ANALYSIS

Statistical analysis was determined by comparing the relative light units from control versus treated conditions for individual experiments using the student t-test to compare the means, assuming equal variance. Experiments conducted multiple times were analyzed individually for statistical significance with each independent experiment having a sample size of n=3. A p value less than 0.05 for each experiment was considered statistically significant. Data is presented as the average fold increase or percent change with respect to control.

IV. RESULTS

Effects of stimulation of neonatal ventricular myocytes with TNFα

Cultured myocytes were exposed to 10 ng / mL of human recombinant TNFα (R & D Systems). This concentration is comparable to that used in similar studies and corresponds to approximately 200 - 500 Units per mL (110). As the duration of exposure to TNFα has a significant effect on its functionality, we tested various exposure times to test for potential cytotoxicity. Live dead analysis determined that stimulation with TNFα did not provoke cardiac cell death at any of the exposure times tested (24, 48 and 72 hours). There was no statistical difference between the number of dead cells between control and TNFα stimulated cells ($p<.05$). (Figure 1) This indicated that TNFα, at the concentration used here is not cytotoxic to neonatal ventricular myocytes. Our finding was corroborated by recent reports in the literature documenting that TNFα does not induce cell death of neonatal ventricular myocytes (110,111).

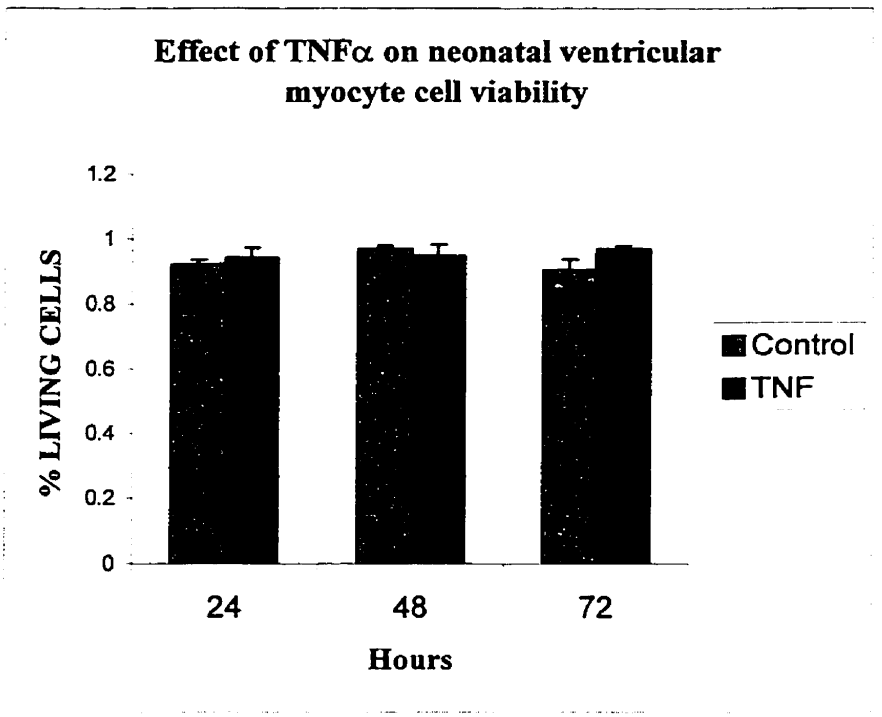


FIGURE 1. Effect of TNFα on neonatal ventricular myocyte cell viability. After overnight incubation in DF, media was replaced with DF serum free media with and without 10 ng / mL of TNFα. Cell viability was determined using calcein acetoxymethyl ester (green) versus ethidium homodimer –1 (red) which distinguish live and dead cells respectively. Nine random fields containing approximately 25 cells per field were examined (n=9). No significant changes in cell death was seen at any of the time points tested ($p<.05$).

TNFα represses cardiac specific gene expression

TNFα has been shown to promote cardiac remodeling and contractile dysfunction in vivo (56,87,90,91,92). Numerous physiological changes occur during cardiac remodeling, including cardiomyocyte cell loss and modulation of cardiac gene expression. To determine whether TNFα has any effect on cardiac gene transcription we studied the effect of TNFα on the expression of four genes considered to be important to the structure and function of myocytes. Luciferase reporter constructs containing promotor sequences from αMHC, βMHC, α–cardiac actin, and α-skeletal actin were transfected into myocytes and studied for expression. After transfection, cells were stimulated with 10 ng / mL of TNFα for 24 hours. We found that αMHC, α–cardiac actin, and α-skeletal actin gene expression was significantly reduced in response to TNFα compared to control cells ($p<.001$) (Figure 2). The extent of TNFα induced repression of βMHC expression was not as substantial as that seen with the other cardiac specific genes (βMHC, 82% $\pm$ 10, $p=.05$ versus αMHC 60$\pm$ 5, $p=.0006$). We also found that the inhibitory effect of TNFα was specific to cardiac genes as the Herpes Simplex thymidine kinase promotor was more resistant to the effects of TNFα than any of the cardiac genes tested (TK luc, 103% $\pm$ 13, $p = 0.4$).

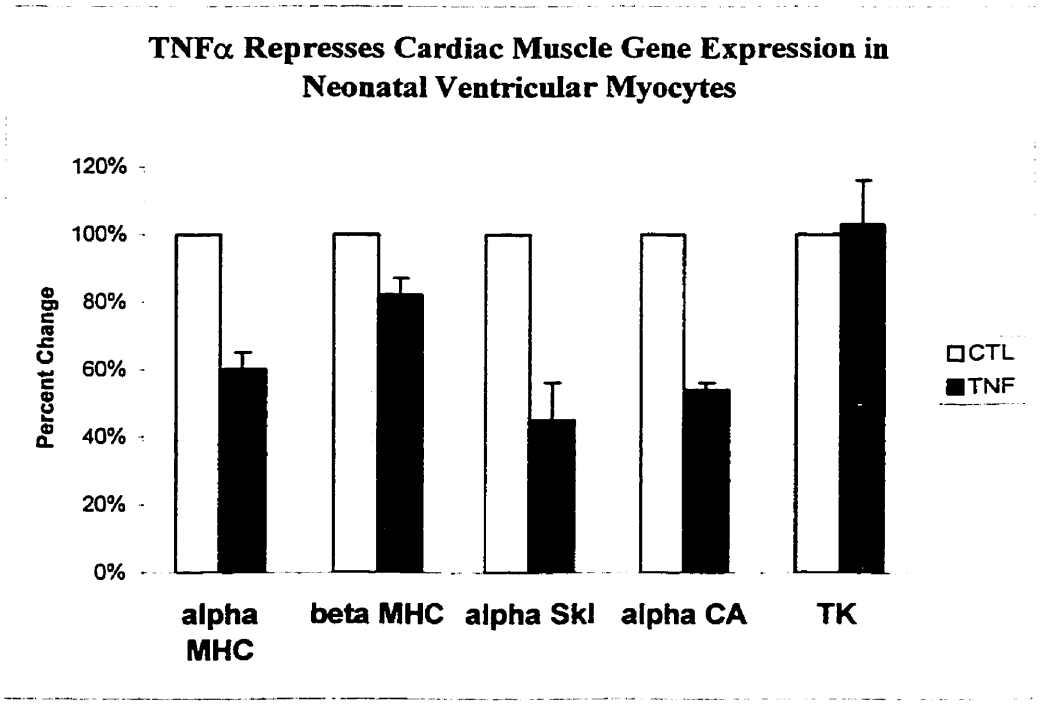


FIGURE 2. TNFα mediated repression of cardiac muscle genes in neonatal ventricular myocytes. Cells were transfected with luciferase reporter constructs (αMHC, βMHC, cardiac actin, α-skeletal actin, TK) and CMV beta gal to normalize for transfection efficiency. After 24 hours of incubation, cells were stimulated with 10 ng/mL of TNFα for 24 hours then analyzed for luciferase and beta galactosidase activity. TNFα repressed αMHC, βMHC, αskeletal actin, and cardiac actin by 60 ± 5, 82 ± 10, 45 ± 11, and 54 ± 2 respectively. Data represents the mean ± S.E. from at least 2 independent experiments with 3 replicates for each condition (n=6). Data are expressed as percent change with respect to the control value.

TNFα mediated repression of cardiac muscle gene expression is prevented by the iNOS inhibitor L-NAME

A well known downstream target of TNFα is the inducible nitric oxide synthase (iNOS) which results in the production of an important signaling molecule, nitric oxide (NO) (96). As a first step toward identifying the mechanism of TNFα induced cardiac gene modulation we used the nitric oxide synthase inhibitor L-NAME. L-NAME is an analog of L-arginine and acts by competing as substrate for the inducible nitric oxide synthase (iNOS). As NO production by iNOS has been shown to modulate cardiac gene expression (95), we feel that iNOS may be important for modulation of cardiac genes by TNFα. We found that 1mM L-NAME could prevent TNFα induced repression of αMHC reporter activity (Figure 3). This finding suggests that NO is activated and accounts for TNFα's inhibitory effect on cardiac muscle gene expression.

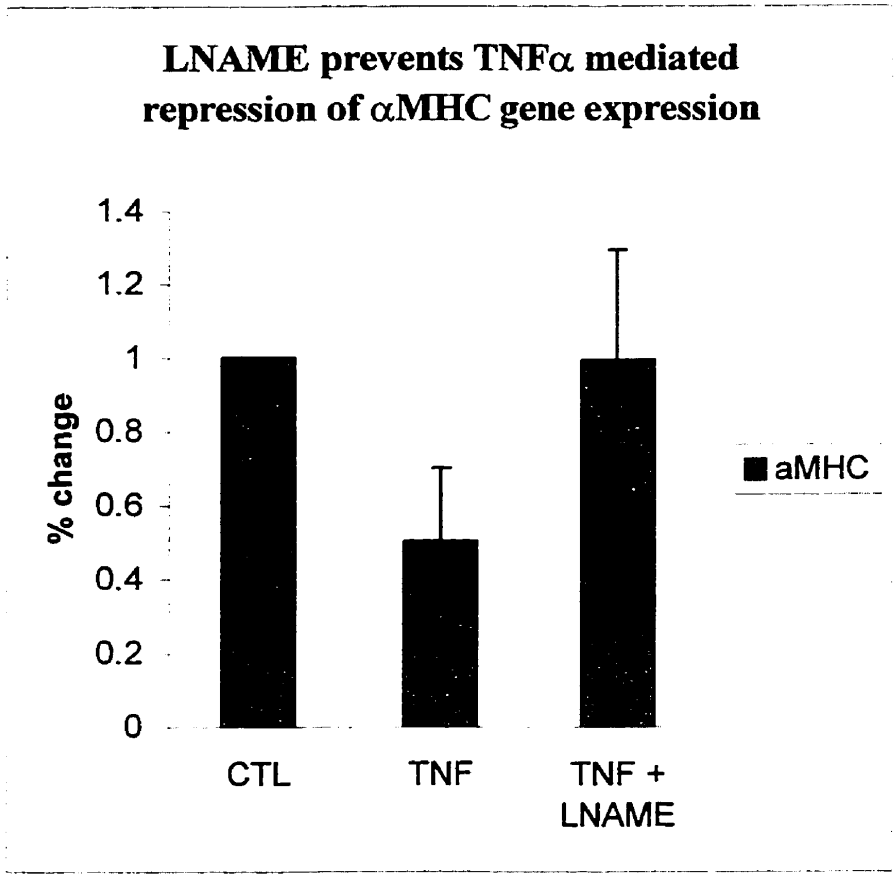


FIGURE 3. Inhibition of NO production using the iNOS inhibitor L-NAME rescues TNFα mediated repression of αMHC gene activity. Neonatal ventricular myocytes were transfected with the αMHC luciferase promotor. Twenty-four hours after transfection, cells were stimulated with 10 ng / mL of TNFα for 24 hours and analysed for luciferase activity. L-NAME prevented TNFα mediated repression of αMHC expression (TNFα 50 ± 22, TNFα + L-NAME 99 ± 34). Data represents the mean ± S.E. from 1 experiment with 3 replicates for each condition tested (n=3). Data are expressed as percent change with respect to the control value.

TNFα activates NFκB dependent gene transcription in neonatal ventricular myocytes

An important mediator of TNFα signaling is the ubiquitously expressed transcription factor NFκB (8, 32, 33, 37). NFκB may lie upstream of iNOS, as the iNOS gene contains multiple NFκB response elements in it's promotor. To test whether TNFα leads to NFκB activation, we transfected myocytes with a luciferase reporter construct containing cis acting NFκB response elements. Here, we demonstrate that TNFα produced an 11 fold increase in NFκB dependent transcription compared to control cells ($p<.05$) (Figure 4). This finding confirms that neonatal ventricular myocytes are functionally coupled to biological signals that lead to NFκB activation. Importantly, expression of the p65 subunit of NFκB resulted in a 22 fold induction of NFκB dependent gene transcription ($p<.05$). Thus, expression of active NFκB in neonatal ventricular myocytes is sufficient to transactivate NFκB dependent genes (Figure 5).

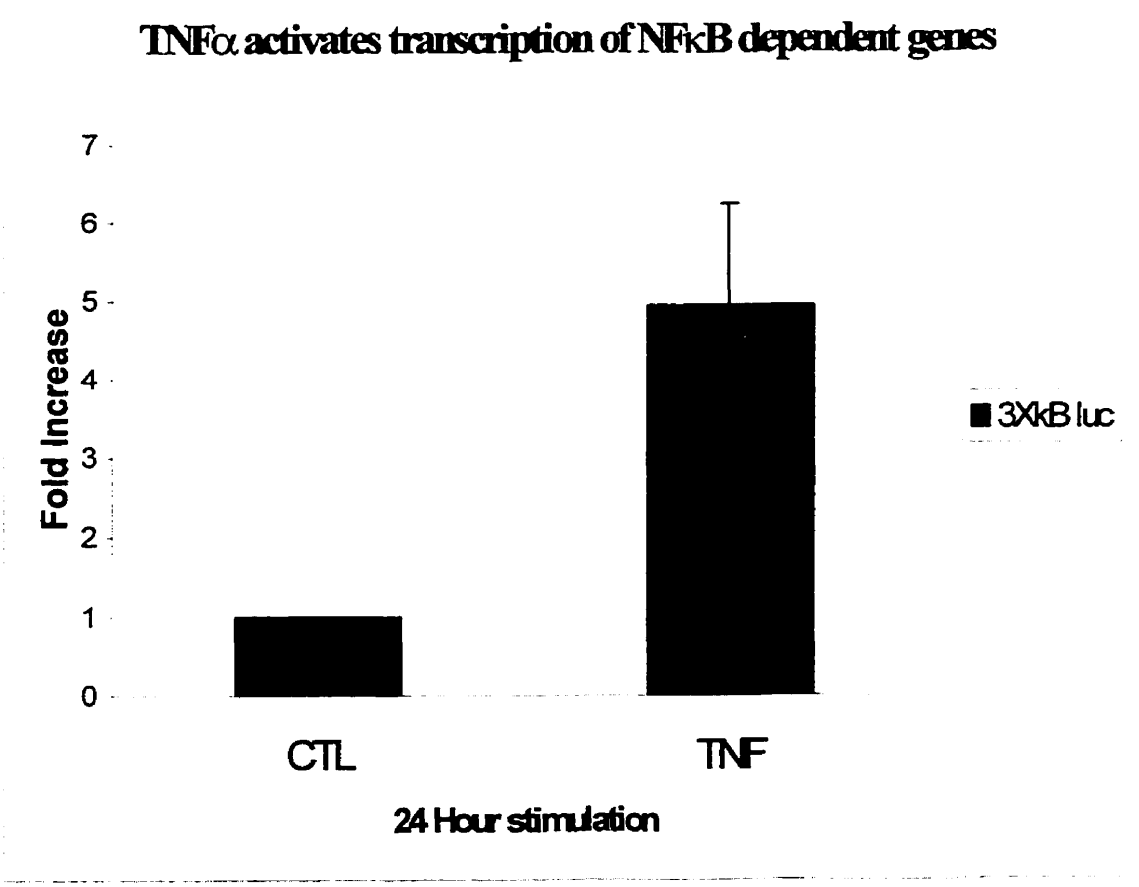


Figure 4. TNFα activates NFκB dependent gene transcription in neonatal ventricular myocytes. Cells were transfected with a luciferase reporter construct, containing 3 NFκB 'cis acting' elements (3XkB luc). Twenty-four hours after transfection the media was replaced with DF serum free. Cells were stimulated with 10 ng / mL of TNFα. TNFα strongly activated the 3XkB luciferase reporter construct (TNFα 4.9 $\pm$ 1.3, $p < 0.05$). Data represents the mean $\pm$ S.E. from 3 independent experiments with 3 replicates for each condition tested (n=9). Data are expressed as fold increase from control.

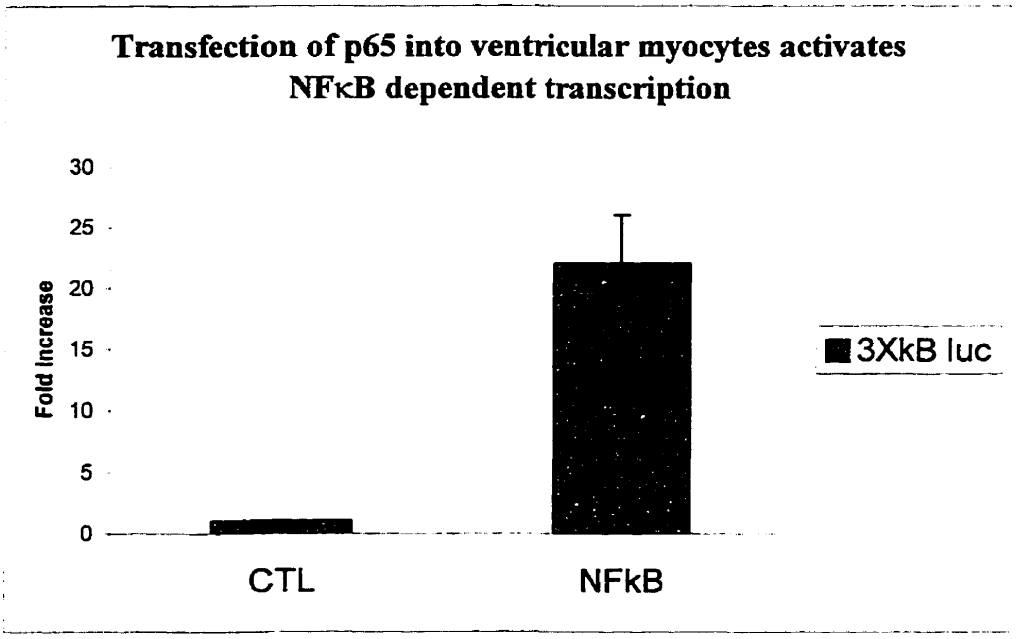


Figure 5. Expression of the p65 subunit of NFκB activates NFκB dependent gene transcription in neonatal ventricular myocytes. Cells were transfected 3XkB luciferase with and without 2 ug / mL of CMV p65. Fourty-eight hours after transfection, cell lystate was analyzed for luciferase. NFκB strongly activated the 3XkB luciferase reporter construct (NFκB 22 $\pm$ 4, $p < 0.05$). Data represents the mean $\pm$ S.E. from 1 experiment with 3 replicates for each condition tested (n=3). Data are expressed as fold increase with respect to the control value.

p65 kDa represses cardiac muscle gene expression

To further delineate the mechanism by which TNFα mediates repression of cardiac gene expression we tested whether NFκB expression could mimic TNFα's effects. Expression of p65 in myocytes resulted in the repression of cardiac muscle gene expression similar to that seen with TNFα. The αMHC, βMHC, α skeletal actin and cardiac actin showed significant reductions in response to p65 expression ($p<.05$) (Figure 6). This suggests that NFκB is responsible for the underlying effects mediated by TNFα in ventricular myocytes.

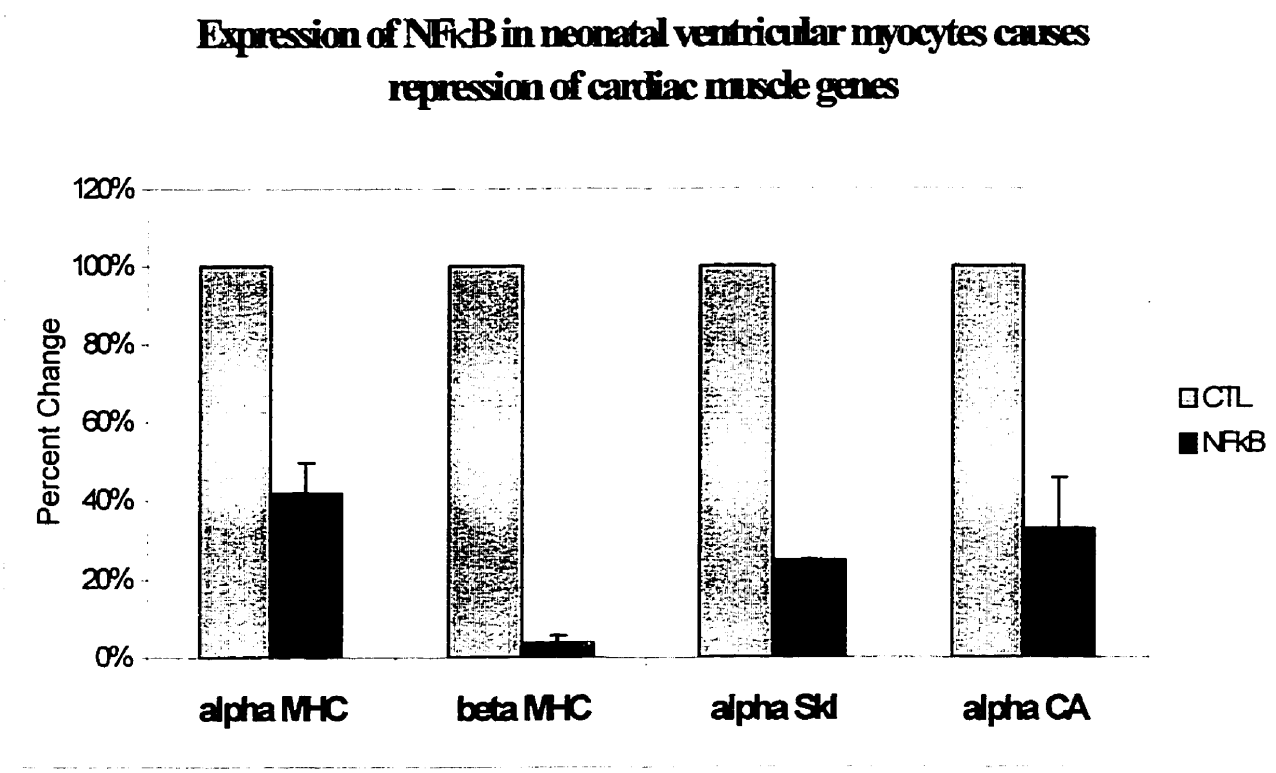


Figure 6. Expression of NFκB in neonatal ventricular myocytes causes repression of cardiac muscle gene expression. Cells were transfected with αMHC, cardiac α-actin, and α-skeletal actin, luciferase reporter constructs. Cells were also transfected with a plasmid coding for CMV p65. After 48 hours cells were analyzed for luciferase and normalized to beta galactosidase activity. NFκB repressed αMHC, βMHC, αskeletal actin, and cardiac actin gene expression by 42 ± 8, 4 ± 2, 25 ± 0.3, 33 ± 13 respectively. Data represents the mean $\pm$ S.E. from 1 experiment with 3 replicates for each condition (n=3). Data are expressed as percent change with respect to the control value.

IκBα mutant inhibits TNFα mediated activation of NFκB

Normally, NFκB remains inactive and sequestered in the cytoplasm of cells by the Inhibitory kappa B alpha protein (IκBα). TNFα signal transduction culminates in phosphorylation and subsequent degradation of IκBα, allowing NFκB translocation and transactivation of NFκB dependent genes. To test whether we could reverse the effect of TNFα, we expressed the IκBα protein in ventricular myocytes to prevent TNFα induced NFκB activation. We first examined whether expression of IκBα could prevent TNFα signaling of NFκB. Cardiac myocytes were co-transfected with the NFκB dependent luciferase reporter with and without IκBα. There was no statistical difference in TNFα induced NFκB activity in cells expressing the IkBα wild type versus control cells ($p = 0.2$). The IκBα wild type is an inhibitor of NFκB, however, it can be phosphorylated and subsequently degraded by stimuli such as TNFα thereby permitting NFκB activation (Figure 7).

Beaurle et al. (1996) determined that mutating IκBα's serine residues number 32 and 36 to alanine was sufficient to inhibit phosphorylation and subsequent degradation of IκBα (17). This IκBα mutant acts as a dominant negative inhibitor in many cell types by remaining constitutively bound to NFκB and sequestering it in the cytoplasm of the cell. We found that myocytes expressing the IκBα mutant were unable to activate NFκB in response to TNFα (Figure 7). The reason for the difference in inhibitory activity between the wild type and mutant IκBα molecules lies in the fact that the IκBα mutant is non-phosphorylatable and therefore constitutively active. Using the IκBα mutant we were successfully able to prevent TNFα signaling of NFκB in cardiac myocytes.

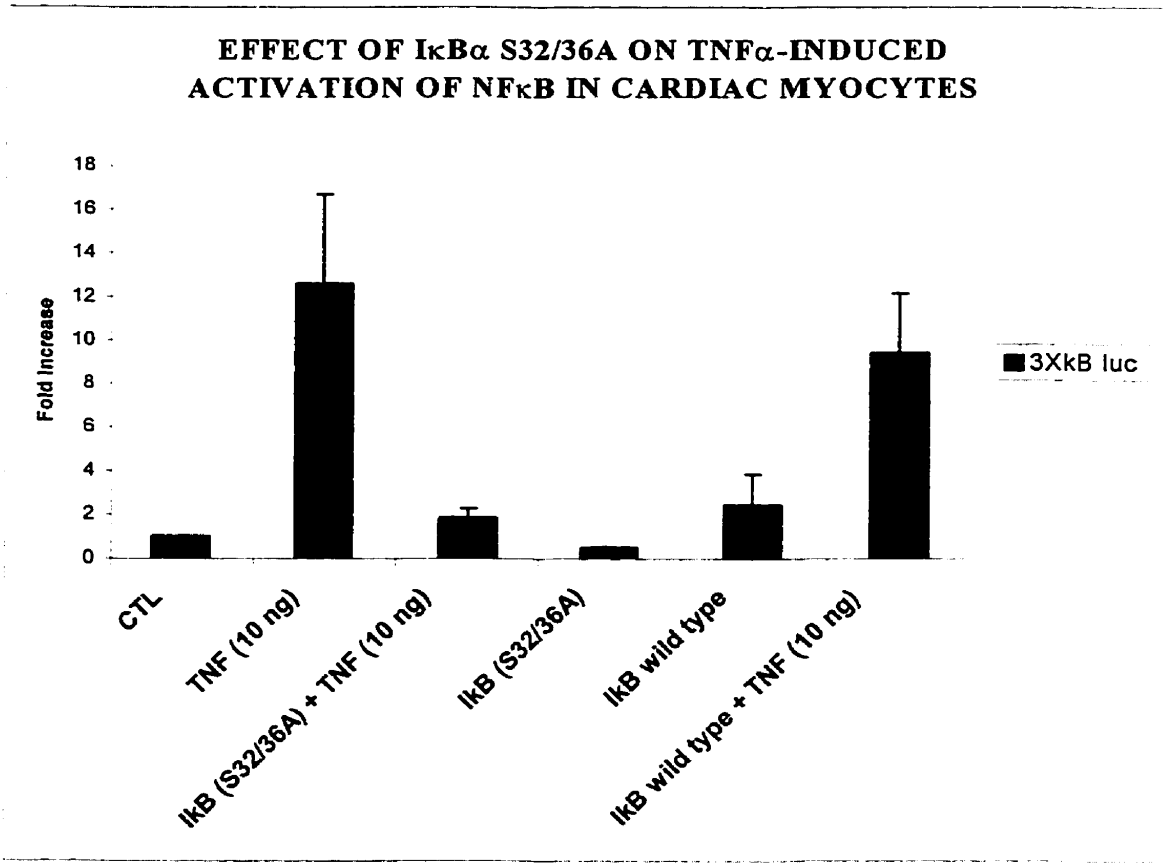


FIGURE 7. IκBα S32/36A blocks TNFα-induced activation of NFκB in ventricular cardiac myocytes. Cells were transfected with luciferase reporter plasmids containing NF-kB binding elements and a CMV driven beta-galactosidase plasmid to normalize for transfection efficiency. Stimulation with 10 ng per mL of TNFα resutled in a 12.6 fold induction of NFκB-dependent gene transcription. IκBα S32/36A blocked NFκB activation to near baseline levels while the IκBα wild type only moderately inhibited NFκB activation (TNFα 12.6 ± 2.9, TNFα + IκBα (S32/36A) 1.8 ± 0.3, IκBα (S32/36A) 0.5 ± 0.07, IκBα wt 2.0 ± 0.8, TNFα + IκBα wt 9.4 ± 2.0). Data represents the mean ± S.E. from 2 independent experiments with 3 replicates for each condition (n=6). Data are expressed as fold increase from control.

Adenovirus delivery of wild type and mutant IκBα in ventricular myocytes

To optimize transfection efficiency we utilized recombinant adenovirus technology. This technique allows almost 100 percent transfection of foreign DNA into mammalian cells. Western blot analysis confirmed expression of wild type and mutant IκBα proteins following adenoviral infection. Wild type IκBα, mutant IκBα and endogenous IκBα could be distinguished based on differences in molecular weight (Figure 8). The difference in size between the transfected wild type IκBα and mutant IκBα is attributed to the 3 kilodalton flag tag on the mutant IκBα. The endogenous rat IκBα is smaller than the human IκBα protein and hence is resolved slightly below the transfected wild type IκBα.

Having established that the transfected gene was expressed in ventricular myocytes we then tested the inhibitory effect of IκBα using the electromobility gel shift assay (EMSA). (Figure 9) This technique measures NFκB-DNA binding activity directly by using a radiolabelled ^{32}P oligonucleotide probe containing NFκB binding sites. The probe is incubated with nuclear extract from the myocyte cells and run on a non-denaturing polyacrylamide gel. Gel shift experiments revealed that TNFα significantly increased NFκB binding activity in ventricular myocytes compared to controls. The increase in DNA binding visualized by EMSA represents NFκB protein that has been translocated to the nucleus. TNFα was unable to activate NFκB in cells over expressing the IκBα mutant. This finding corroborates the transfection data that used a luciferase reporter construct with NFκB response elements (Figure 7). To confirm the identity of the complex containing NFκB, we used an antibody specific for the p65 subunit of

NFκB. The resulting band was super shifted, which confirmed that the migrating complex contained NFκB. A nonradioactive oligonucleotide probe specific for NFκB was used as a competitive inhibitor. The fact that we did not see any binding in the last lane confirms the specificity of the probe. Our initial goal was to block TNFα signaling in myocytes. Based on the transfection data and gel shift analysis we achieved complete blockage of TNFα signaling of NFκB using the 'Ad CMV IκBα (S32/36A)'.

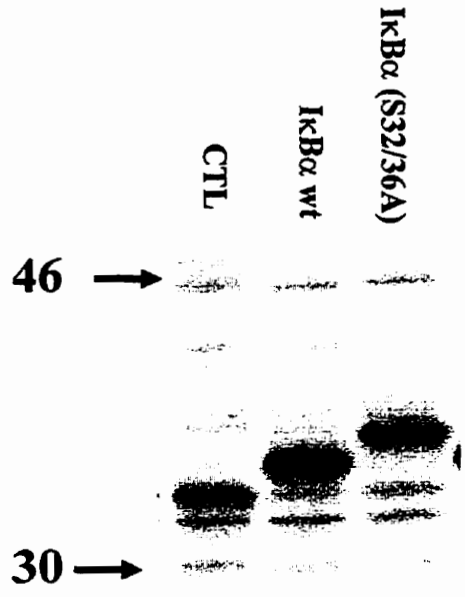


FIGURE 8. Adenoviral mediated overexpression of IκBα wild type and IκBα S32/36A mutant proteins in ventricular cardiac myocytes. Panel A Cardiac cell lysates expressing IκBα wild type and mutant proteins were analysed by SDS gel electrophoresis. The IκBα protein was visualized using a rabbit polyclonal antibody directed toward IκBα/MAD3 followed by a horseradish peroxidase-conjugated anti-rabbit IgG. Marker, Control (CTL), AdCMV IκBa wild type (2E8 pfu per mL), AdCMV IκBα S32/36A (2E8 pfu per mL), IκBα wt + TNFα (10 ng/ mL), IκBα S32/36A + TNFα (10 ng/mL), 293 cells.

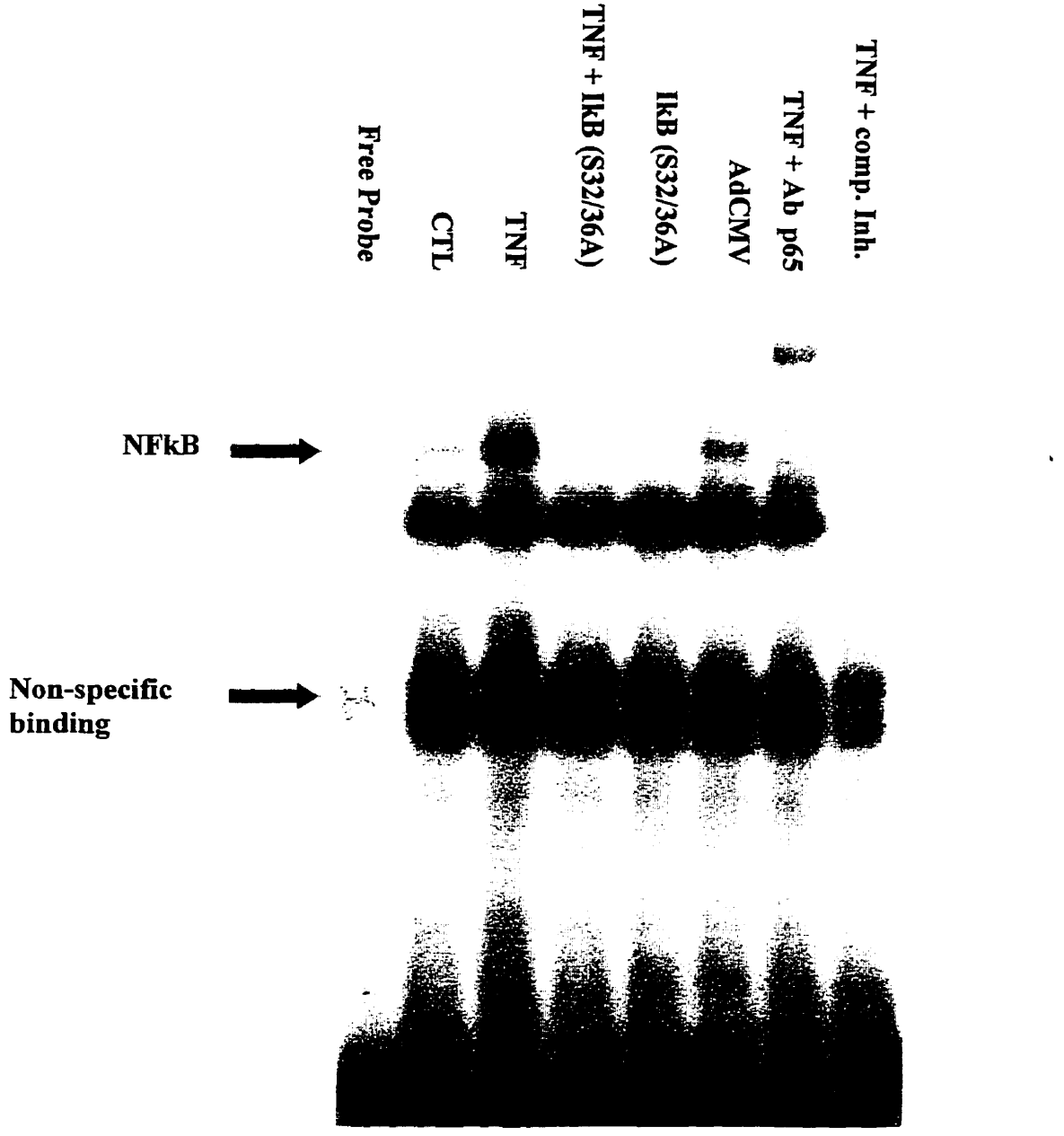


FIGURE 9. Electromobility gel shift assay of nuclear protein from ventricular cardiac myocytes. Equal amounts of nuclear protein extract from neonatal cardiac myocytes were prepared and analyzed for NF-kB binding activity using a p32 labelled oligonucleotide probe containing NF-kB binding sites. Lane 1 free probe, Lane 2 Control, Lane 3 TNFα (10 ng/mL), Lane 4 TNFα + IκBα S32/36A, Lane 5 AdCMV IκBα s32/36a, Lane 6 AdCMV, Lane 7 TNFα + p65 anti-body, Lane 8 TNFα + competitive inhibitor.

TNFα mediated repression of αMHC and βMHC gene expression is abrogated by expression of the IκBα mutant

To test the possibility that TNFα mediated repression of cardiac muscle gene activity was dependent on NFκB activation we used the dominant negative mutant, IκBα (S32/36A). We found that the IκBα mutant inhibited NFκB activation and TNFα mediated repression of αMHC and βMHC gene expression (αMHC, TNFα 68% ± 6, TNFα + IkB(S32/36A) 88% ± 2; βMHC, TNFα 67% ± 0.9, TNFα + IkB(S32/36A) 93% ± 10, $p<0.05$). Together, these data further implicate NFκB as a potential mediator of TNFα induced repression of cardiac muscle genes. Interestingly, the rescue seen be L-NAME is not as substantial as the rescue observed by overexpression of the IκBα mutant (Figure 10).

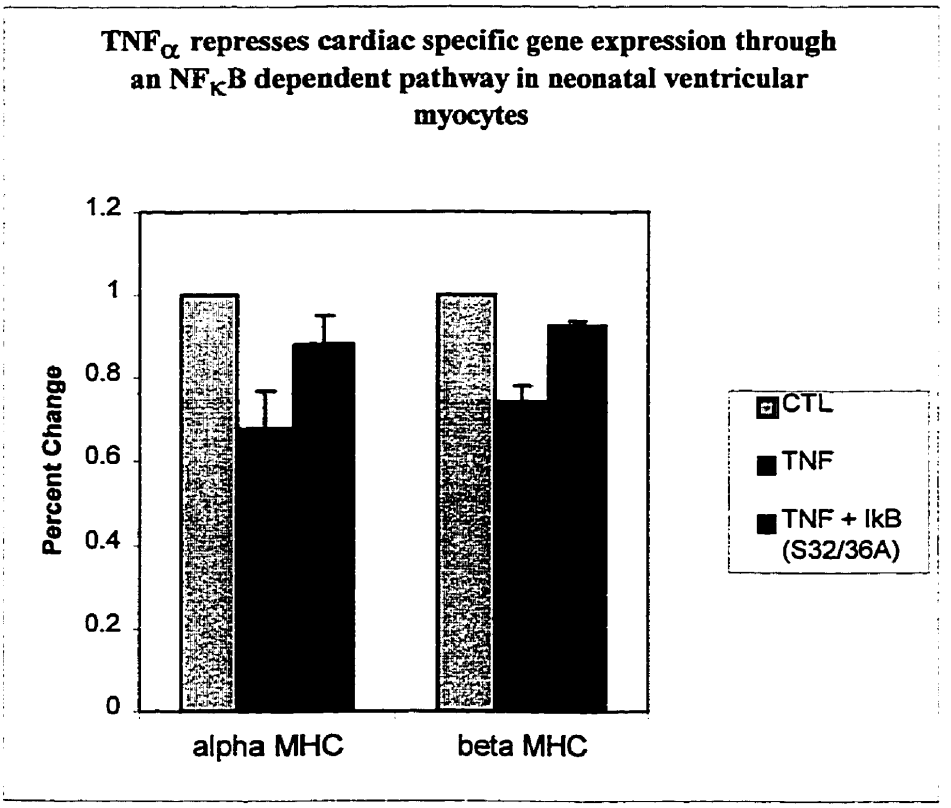


FIGURE 10. Inhibition of NFκB rescues TNFα mediated repression of αMHC and βMHC promotor activity. Neonatal ventricular myocytes were transfected αMHC and βMHC luciferase promotor constructs with and without CMV IκBα (S32/36A). Twenty-four hours after transfection, cells were stimulated with 10 ng / mL of TNFα for 24 hours and analysed for luciferase. The IκBα (S32/36) prevented TNFα mediated repression of αMHC expression (TNFα 68 ± 6, TNFα + IκBα (S32/36A) 88 ± 2). The IkBα (S32/36A) also rescued TNFα mediated repression of βMHC (TNFα 67 ± 0.9, TNFα + IkBα (S32/36A) 93 ± 10) Data represents the mean ± S.E. from 2 independent experiments with 3 replicates for each condition tested (n=6). Data are expressed as percent change with respect to the control values.

TRAF2 is required for TNFα induced NFκB activation in 293 cells

Previous studies in 293 cells and HeLa cells found that TRAF2 is important for TNFα signaling of NFκB (97 - 101). TRAF2 is composed of 4 structural domains (See Appendix). The N-terminal, composed of the ring finger and zinc finger domains, is of particular importance since it is thought to be involved in TNFα mediated activation of NFκB. A TRAF2 mutant with the first 79 amino acids at the N terminal deleted has been shown to act as a dominant negative inhibitor of TNFα mediated NFκB activation in some cell types (97,98). Our initial studies, demonstrate that TNFα activates NFκB dependent gene transcription in 293 cells and that the TRAF2 (80 – 501) can act in a dominant negative fashion to prevent TNFα signaling of NFκB in this cell type (Figure 11).

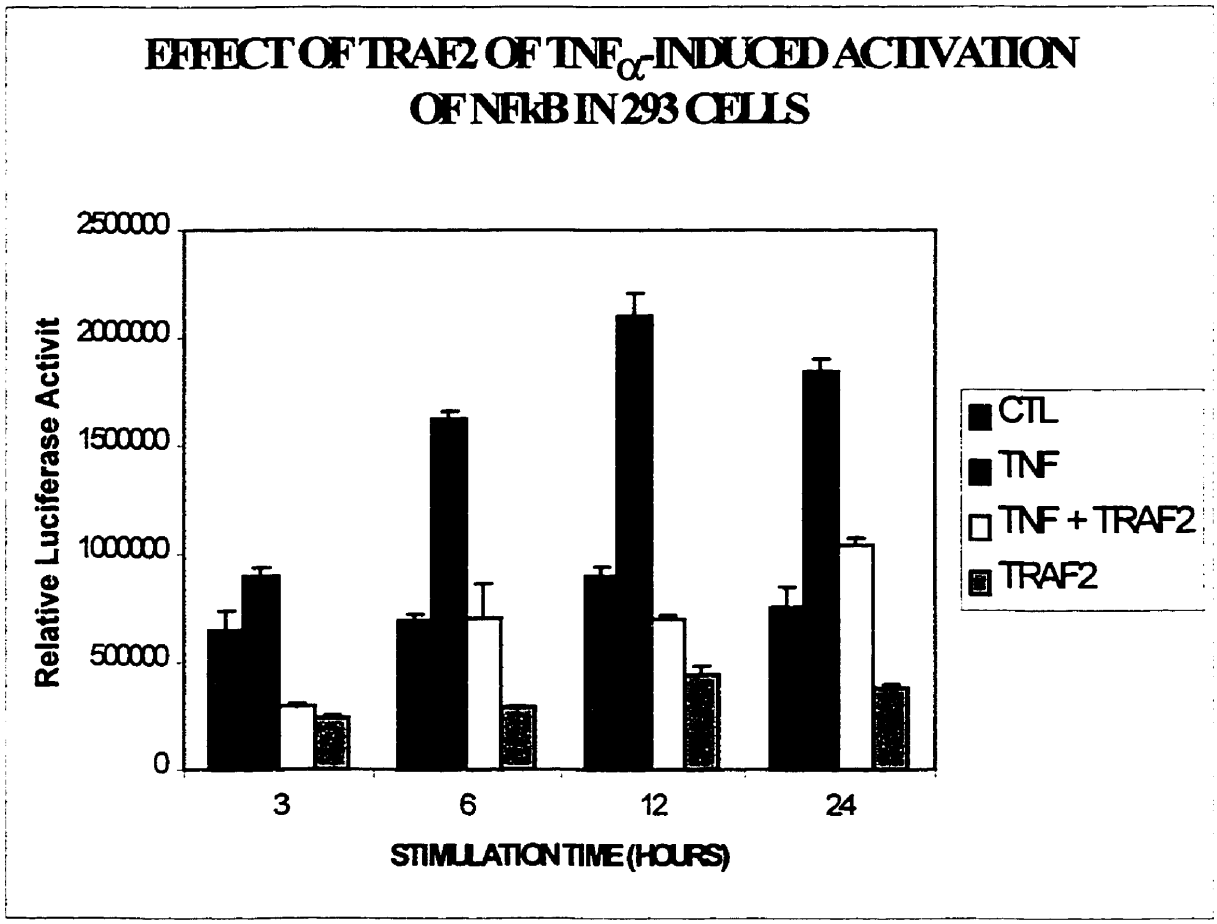


FIGURE 11. Overexpression of the TRAF2 (80 – 501) inhibits TNFα-induced activation of NFκB in 293 cells. Cells were co-transfected with 3XkB with and with out the TRAF2 (80 – 501). Twenty-four hours after transfection, cells were challenged with TNFα for 3, 6, 12 and 24 hours. Data represents luciferase activity normalized to beta-galactosidase activity and expressed as relative light units (n=3).

Is TRAF2 required for TNFα induced NFκB activation in neonatal ventricular myocytes?

To further delineate the mechanism by which TNFα activates NFκB in neonatal ventricular myocytes we examined the TRAF2. The TRAF2 (80 – 501) did not act as a dominant negative inhibitor of NFκB in myocytes as it had in the 293 cells (Figure 12). The differential effect of TRAF2 on TNFα's ability to activate NFκB in myocytes versus 293 cells may be attributed to a number of factors. To gain a better understanding of the role of TRAF2 and the function of its domains in ventricular myocytes we examined the TRAF2 wild type and other mutants with respect to NFκB activation.

As in previous experiments, TNFα could activate NFκB dependent gene expression. The TRAF2 wild type was capable of activating NFκB dependent gene expression to almost the same extent as TNFα, demonstrating that TRAF2 alone is capable of transducing intracellular signals in myocytes (Figure 13). The TRAF2 (80 – 501) also produced a significant increase in NFκB dependent transcription. To our surprise, its transactivating activity was equivalent to the wild type. This suggests that the ring finger domain of TRAF2 is not needed for activation of NFκB. To lend support to the possibility that the ring finger was dispensible for TRAF2 signaling of NFκB, we examined the effects of two other TRAF2 mutants. The TRAF2 (225 – 501) mutant, which lacks both the ring and zinc finger domains did not significantly activate NFκB. This suggests that the zinc finger domain in the absence of the ring may have NFκB activating ability in cardiac myocytes, lending support to the idea that the ring finger is not needed for signaling of NFκB. However, the TRAF2 (1 – 224), which only contains the ring and zinc finger domains had no effect on NFκB activity. The TRAF2 (1-224)

lacks the C-terminal domains (TRAF-N and TRAF-C) that are thought to be important for interaction with the TNFα receptor. Our findings suggest that the C-terminal domains may be needed for at least the zinc finger domain to be functional. In contrast, in 293 cells the TRAF2 mutants had no effect on NFκB activity while expression of the TRAF2 wild type had a moderate activating effect on NFκB (Figure 14).

Myocytes expressing the TRAF2 mutants showed no impairment in TNFα's ability to activate NFκB. (Figure 14) In fact, cells expressing the TRAF2 wild type and TRAF2 (80 – 501) showed a marked increase in TNFα mediated NFκB activation. The apparent potentiation of TNFα signaling of NFκB in cells overexpressing the TRAF2 wild type and TRAF2 (80 – 501) may be explained by additive effects; for example both TNFα and TRAF2 wild type can activate NFκB independently. In contrast, the 293 cells overexpressing the TRAF2 mutants (80 - 501) and (225 – 501) showed impaired TNFα signaling of NFκB. The TRAF2 (1 – 224) in fact had no effect on TNFα signaling of NFκB and did not act as a dominant negative inhibitor, consistent with findings in the literature. (Figure 14) Unexpectedly, 293 cells overexpressing the TRAF2 wild type demonstrated impaired TNFα signaling of NFκB.

The results suggest that TRAF2 may not be essential for TNFα signaling of NFκB. Furthermore, the observation that the TRAF2 (80 – 501) mutant was able to activate NFκB on its own to the same extent that the TRAF2 wild type suggests that the ring finger domain of TRAF2 is not required for NFκB activation in neonatal myocytes.

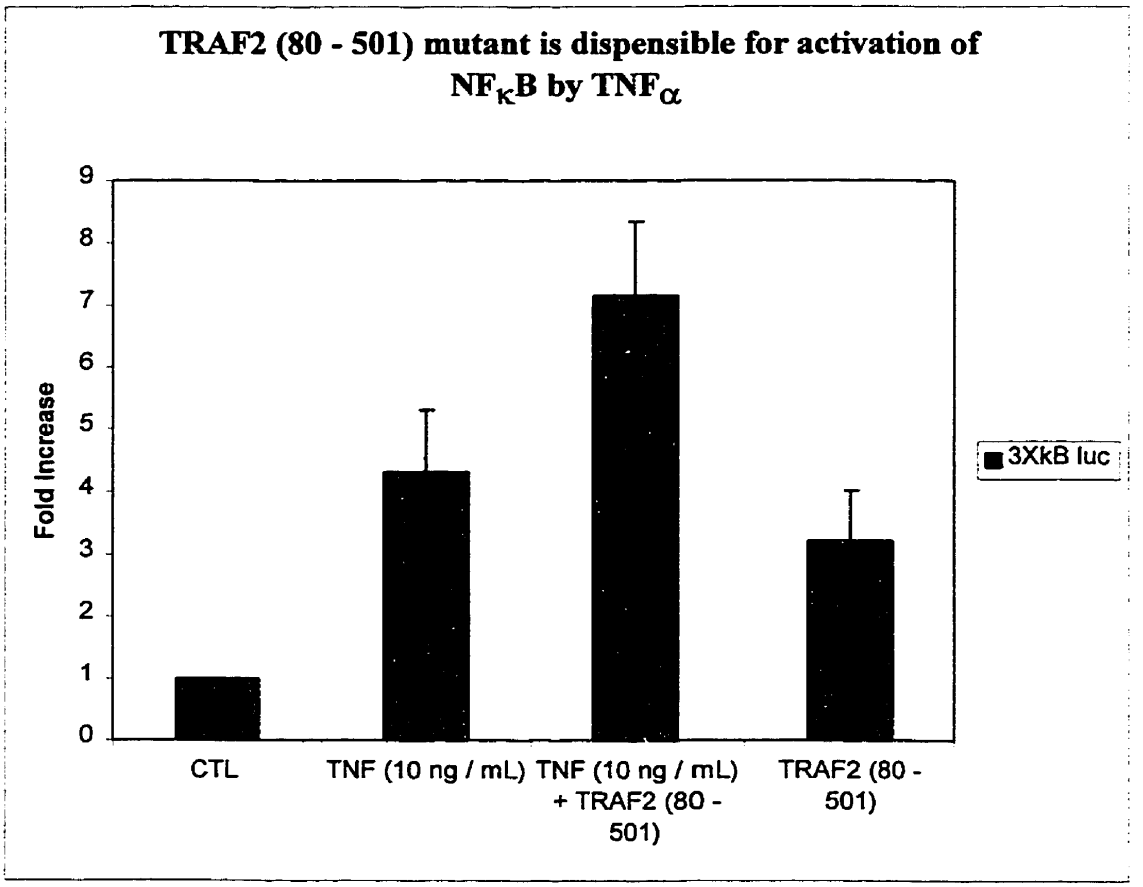


FIGURE 12. TNFα-Receptor Associated Factor-2 is unable to block NFκB activation by TNFα. Neonatal ventricular myocytes were co-transfected with 5 ug/mL of the 3XkB luciferase reporter with and with out 2 ug / mL of CMV TRAF2. Twenty-four hours after transfection, cells are stimulated with TNFα. Data represents the mean ± S.E. from 3 independent experiments with 3 replicates for each condition tested (n=9). Data are expressed as fold increase from the control value.

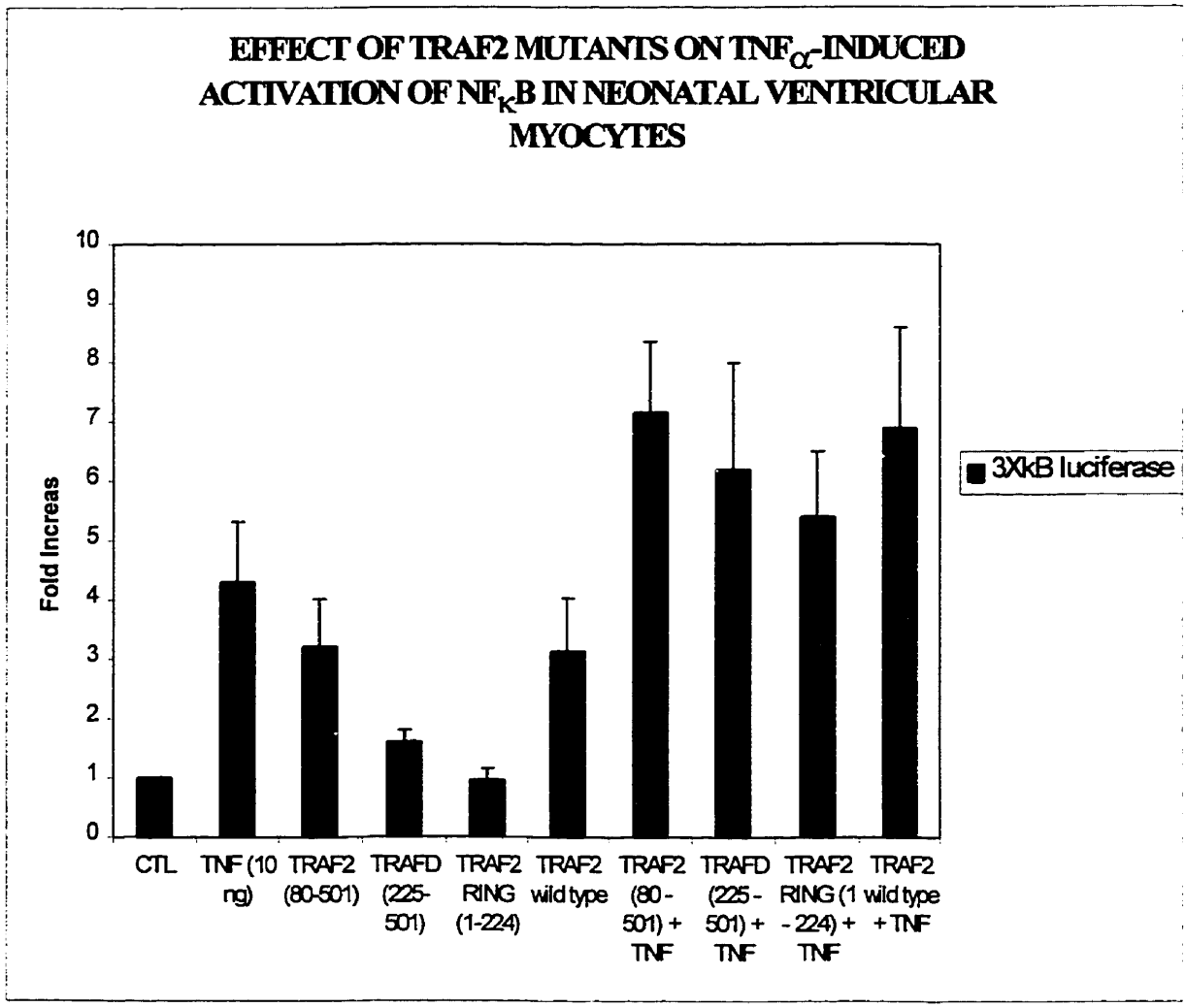


FIGURE 13. Effect of over expressing TRAF2 mutants on TNFα's ability to activate NFκB in neonatal ventricular myocytes. Cells were co-transfected with 3XkB luciferase with and with out 2 ug / mL of the TRAF2 structural mutants as described in methods. Twenty-four hours after transfection, cells were stimulated with TNFα for 24 hours. Data represents the mean ± S.E. from at least 3 independent experiments with 3 replicates for each condition tested (n=9). Data are expressed as fold increase from the control value.

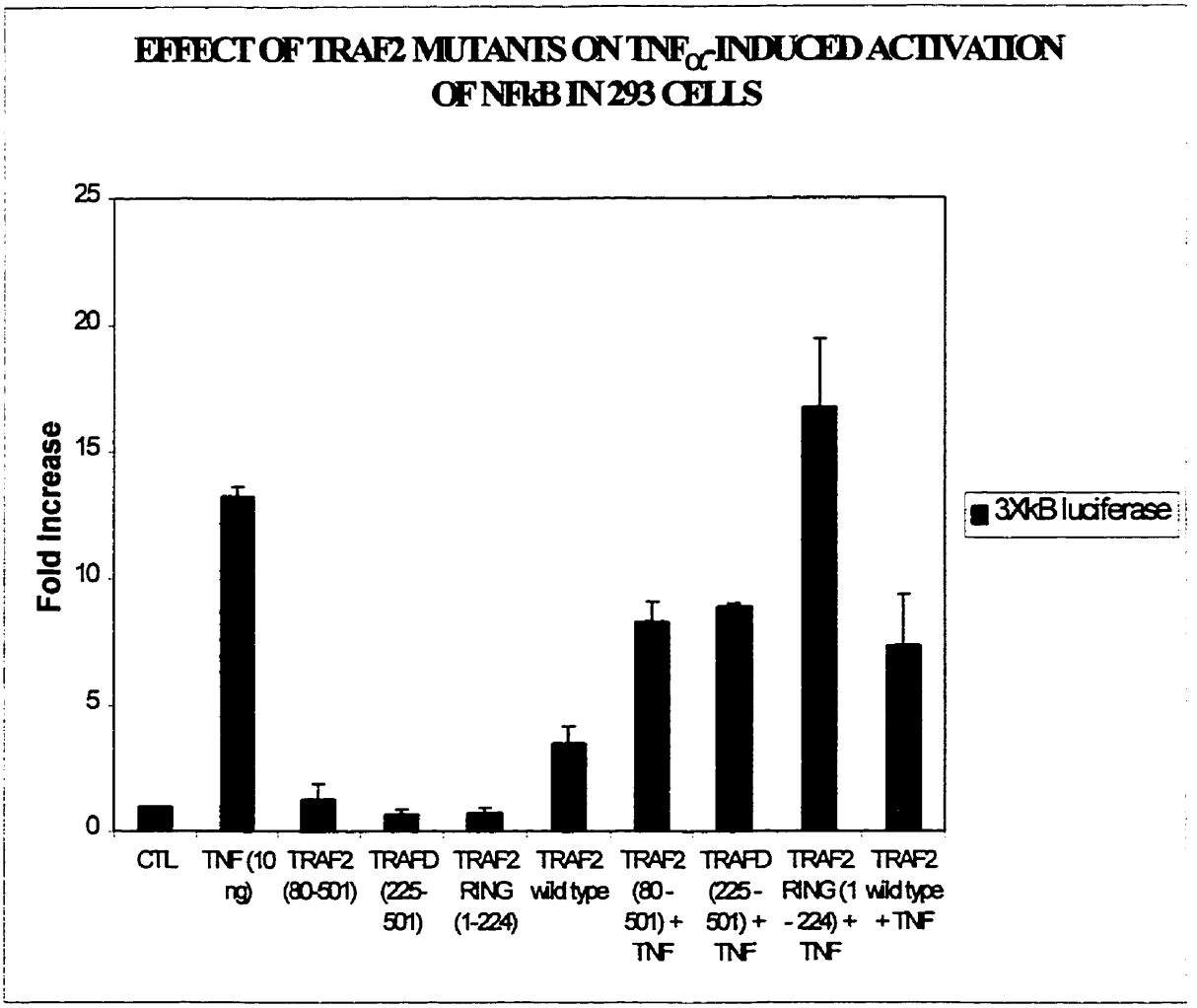


FIGURE 14. Effect of over expressing TRAF2 mutants on TNFα's ability to activate NFκB in 293 cells. Cells were co-transfected with 3XkB luciferase in the presence and absence of the TRAF2 mutants as described in the methods. Twenty-four hours after infection, cells were stimulated with TNFα and analyzed for luciferase activity. Data represents the mean ± S.E. from 2 independent experiments with 3 replicates for each condition tested (n=6). Data are expressed as fold increase from the control value.

V. DISCUSSION

For over two centuries cachexia has been recognized as a characteristic feature of patients with end-stage heart failure. Only recently, has TNFα been identified as a potential mediator of the symptoms of cachexia that is often associated with chronic diseases such as cancer, chronic infection and heart failure. Levine et al. (1990) first noted that elevated levels of serum TNFα were increased in patients with the most advanced symptoms of heart failure that include hypoperfusion and cachexia (173). Furthermore, interventions that improved patients heart function were accompanied by a decrease in serum TNFα (173). Others have documented a positive association between the severity of heart disease and the levels of circulating TNFα (89). It is apparent that TNFα can cause many of the clinical symptoms associated with cachexia however, TNFα may also contribute directly to the progressive ventricular function seen in heart failure (38). To date, the effects of elevated levels of circulating TNFα on the heart and its role in heart failure are not well defined.

TNFα has been shown to be cytotoxic to a number of cell types and it is conceivable that TNFα can trigger cell death of ventricular myocytes and in this way pose a serious threat to cardiac function (6,174). TNFα activates a number of signaling pathways after engaging its receptor. The TNFR1, in particular, has been shown to be essential for mediating TNFα induced programmed cell death in 293 and NIH 3T3 cells (174). The heart and ventricular myocytes in particular, express both the TNFR1 and TNFR2 and these receptors are dynamically regulated during the transition to heart failure suggesting that these receptors are biologically functional in the heart (48). A number of possible mechanisms for TNFα induced cell death have been postulated. In

some cell types, TNFα mediates the activation of sphingomyelinases leading to ceramide generation that can lead to apoptosis (175). Another potential mechanism involves TNFα mediated production of reactive oxygen intermediates (175). TNFα has also been reported to activate the programmed cell death machinery directly by engaging TNFR1 that can bind to specific death domain containing adapter proteins (TRADD and FADD) to initiate the cell death sequence (99). To elucidate the functional role of TNFα in the heart, we examined the effect of TNFα on neonatal ventricular myocytes. We first examined the effect of TNFα on cell viability and found that TNFα was not cytotoxic to neonatal ventricular myocytes at any of the time points tested. This suggests that the negative inotropic effects of TNFα in the heart may not be explained by its cytotoxicity to cardiac muscle cells.

Recent reports corroborate our findings that TNFα does not induce cardiomyocyte cell death. Ing et al. (1999) tested the effect of macrophage derived cytokines (IL-1β, TNFα and interferon-γ) on neonatal ventricular myocytes in regards to cell viability (111). In their study, TNFα alone could not induce cell death of isolated neonatal ventricular myocytes. However, cytokines applied in combination caused a time dependent induction of myocyte apoptosis beginning between 48 and 72 hours after treatment (111). There are a number of possible explanations for why TNFα does not induce cell death of neonatal ventricular myocytes. It is possible that neonatal myocytes lack one or more of the components required for TNFα signal transduction. In support of this hypothesis, Krown et al. (1998) determined that the mRNA transcript for TNFR1 is undetectable in cultured neonatal myocytes (110). By contrast, the TNFR1 mRNA and protein have been detected in adult myocytes. Two lines of evidence support the contrary

and suggest that components of the TNFα signaling pathway required for induction of apoptosis are present in neonatal ventricular myocytes. First, we previously demonstrated that TNFα provokes apoptosis of neonatal ventricular myocytes in the presence of the protein synthesis inhibitor, cycloheximide (31). This suggests that machinery needed to execute the apoptotic process exists within cardiomyocytes and can be activated by TNFα. Secondly, our current findings demonstrate TNFα stimulation results in nuclear translocation of NFκB and the transcription of NFκB dependent genes suggesting that the receptors required for a biological response are present in neonatal myocytes (Figures 7 and 10). However, our findings do not discriminate which receptor is being activated or whether both receptors are functional in our model system.

In vivo studies indicate that TNFα may not play a significant role in the induction of cardiomyocyte cell death. For example, transgenic mice that express myocardial TNFα demonstrate only rare myocyte apoptosis and necrosis (23). In a related study, Bozkurt et al. (1998) examined the effect of infusing pathophysiologically relevant concentrations of TNFα into rats for prolonged periods of time (15 days) (92). The presence of apoptosis in these animals was examined in situ using TUNEL and the double strand DNA ligase based method. The frequency of TUNEL positive nuclei in both control and TNFα treated hearts were below 0.002 percent. The authors did note a significant increase in TUNEL positive myocytes in the TNFα group compared to control. However, there was no evidence of double stranded DNA strand breaks which would be indicative of apoptosis. Despite the low levels of cell death detected in these in vivo studies, over expression of TNFα was sufficient to induce left ventricular dysfunction, cardiac myocyte shortening, and left ventricular dilatation thus mimicking

certain aspects of clinical models of heart failure. Our finding that TNFα does not induce cell death of neonatal ventricular myocytes may suggest that TNFα cytotoxicity does not explain its negative inotropic effects on the heart. What accounts for the cardiodepressive effects of TNFα?

The negative inotropic effects of TNFα in humans and experimental animals *in vivo* and *in vitro* are well documented (140,161,178). Clinical studies using TNFα to treat cancer patients provided some of the direct evidence of TNFα's ability to depress myocardial function (179). Yokoyama et al. (1993) studied the effect of TNFα on the contractility of isolated cardiac myocytes and found that TNFα decreased cardiomyocyte cell shortening (178). The precise mechanism of this effect, however, is unknown. By definition, cytokines have been shown to exert effects on cell growth, differentiation and gene expression. As cytokines are elevated in numerous cardiac related conditions, it is possible that cytokines contribute to the structural and functional alterations of the myocardium observed in heart disease by modulating cardiac gene expression. Interleukin-1 beta (IL-1β), a proinflammatory cytokine, has been shown to modulate the expression of genes associated with cardiac hypertrophy including the βMHC and atrial natriuretic protein (ANP) (96). Thaik et al. (1995) studied the effect of IL-1β on myocytes isolated from neonatal rat hearts (159). They found that IL-1β increased total cellular protein and specifically provoked the reexpression of the fetal genes ANP and βMHC in neonatal rat hearts. They also examined the effect of IL-1β on cardiac specific genes that are present in normal adult hearts including the sarcoplasmic reticulum calcium ATPase and calcium release channel and found that IL-1β repressed their expression. To test the possibility that TNFα can modulate cardiac specific gene

expression we examined the impact of TNFα on αMHC, βMHC, αSk, and CA gene expression.

Cardiac hypertrophy and to a very limited extent, cardiac repair are important phenomenon of the hearts' ability to adapt to increased stress. During cardiac hypertrophy there is a reversion or reactivation of the fetal genes βMHC, αSk and α-smooth muscle actin and a down regulation of the adult αMHC. Importantly, elevated levels of TNFα have been detected in cardiac hypertrophy and end-stage heart failure. We found that TNFα could repress αMHC, βMHC, αSk and CA gene expression. Is TNFα mediated modulation of cardiac genes compensatory or maladaptive? Although not proven, our data would suggest that elevated levels of TNFα may contribute to the progressive contractile dysfunction seen in cardiac hypertrophy and heart failure by repressing normal cardiac muscle gene expression. The αMHC, βMHC, αSk and CA are vital components of sarcomeric structure and contractile function. Alterations in the expression of actin and myosin in the heart may account for the impaired contractile function seen in the diseased myocardium. Indeed, mRNA encoding the αMHC isoform has been shown to be down regulated in the left ventricles of patients with heart failure while the mRNA for the βMHC isoform is induced (63). This apparent isoform switch is associated with decreased contractility, increased efficiency of contraction and a reduced velocity of contraction (63,86). Interestingly, our results indicated that TNFα did not aalter βMHC expression to the same extent as the other cardiac specific genes, suggesting that these cardiac muscle genes are differentially regulated.

Studies on the effect of TNFα on skeletal muscle have shown that TNFα causes a reduction in total protein content and, in specific, loss of adult myosin heavy chain

(MHC) content (176). Although the mechanism of this effect remains unknown, the authors contend that TNFα may be an important pathological contributor to the skeletal muscle atrophy and weakness that is seen in a variety of chronic diseases. One can speculate that elevated levels of TNFα in patients with end stage heart failure may promote decreased contractility by repressing the expression of cardiac muscle genes.

Previously, Finkel et al. (1992) found that the negative inotropic effects of TNFα on isolated papillary muscles could be completely abrogated by inhibiting the production of nitric oxide. As a first step toward identifying the signaling molecules important for TNFα signaling we tested nitric oxide, a known biological target of TNFα stimulation. We found that TNFα mediated repression of cardiac specific genes is dependent on nitric oxide production since LNAME, an inducible nitric oxide synthase (iNOS) inhibitor, could rescue TNFα-mediated repression.

The precise mechanism of TNFα mediated cardiac gene modulation is unknown. Our data suggests that nitric oxide may be an important mediator of TNFα signaling. The major source of nitric oxide production in cardiac myocytes is the inducible nitric oxide synthase (iNOS). The iNOS gene and the transcription factor NFκB are known to be activated by cytokine stimulation (105). Also, Taylor et al. (1998) discovered that there are multiple NFκB enhancer elements in the promotor of the human iNOS gene (96). To further delineate the mechanisms involved in TNFα induced NO production, we tested the role of a ubiquitously expressed transcription factor, NFκB. We hypothesized that NFκB is activated by TNFα and is involved in TNFα mediated repression of cardiac muscle genes. Three lines of evidence suggests that NFκB is involved in TNFα mediated repression of cardiac gene expression. First, TNFα stimulation resulted in the nuclear

translocation of NFκB and subsequent activation of NFκB dependent gene expression. Secondly, NFκB p65 mimicked the repressive effect of TNFα on cardiac specific gene expression. Third, by expressing the IkBα mutant we were able to prevent TNFα mediated repression of cardiac specific genes. Expression of the IkBα mutant in ventricular myocytes prevented TNFα mediated activation of NFκB. By inference, the discovery that the IkBα mutant prevents the inhibitory effects of TNFα suggests that NFκB is involved in this pathway. Therefore, TNFα likely exerts its effects through an NFκB and NO dependent pathway. As the inhibition of the TNFα signaling molecules NFκB and NO rescued TNFα mediated repression of cardiac muscle gene expression these mediators may serve as potential therapeutic targets to abrogate the negative effects of TNFα. The IkBα mutant did not completely prevent TNFα mediated repression of cardiac genes. One possible explanation is that NFκB indepentent pathways for TNFα mediated repression of cardiac muscle genes may be operational in neonatal ventricular myocytes.

It has been postulated that TNFα may function as a stress response factor (38). In support of this, TNFα stimulation of cardiac myocytes has been shown to induce the expression of heat shock proteins that play a protective role against different forms of enviromental stress (109). In further support, TNFα stimulation has been shown to confer resistance to hypoxic injury in the adult mammalian cardiac myocyte (21). In a recent paper by Wang et al. (1998), NFκB was found to activate anti-apoptotic pathways and suppress activation of Caspase 8 (25). These findings suggest that short exposures to TNFα at moderate concentrations may be cytoprotective to cardiomyocytes. Previously,

in our laboratory, we found that TNFα stimulation in the presence of a protein synthesis inhibitor, cycloheximide could induce apoptosis of neonatal ventricular myocytes (31). It seems cycloheximide is capable of unmasking the cytotoxic effects of TNFα. This finding implies that there are pre-formed cellular factors capable of initiating the apoptotic process. It also opens the possibility that TNFα in the absence of cycloheximide or other extenuating circumstances may activate the synthesis of anti-apoptotic proteins.

Krown et al. (1998) demonstrated that TNFα induces apoptosis of isolated adult ventricular myocytes in primary culture (110). The underlying cause of the apparent differential effect of TNFα on isolated adult myocytes versus neonatal myocytes is not known. It is interesting to speculate, however, that the difference may be dependent on the functionality of the downstream signaling pathways activated in neonatal and adult myocytes.

Given the importance of the TNFα – NFκB signaling axis we attempted to further delineate this signaling pathway by testing the requirement of the TNFα Receptor Associated factor 2 (TRAF2) for the activation of NFκB by TNFα. TRAF2 is a cytosolic protein that can interact with the intracellular membrane domain of the TNFR2 and indirectly with TNFR1 through TRADD. The TRAF2 is capable of transactivating NFκB and was initially thought to be indispensible for induction of NFκB by TNFα in some cell types. We demonstrated that a TRAF2 mutant lacking its N-terminal ring finger domain acts as a dominant negative inhibitor of TNFα-mediated NFκB activation in 293 cells. For 293 cells, the ring finger domain of TRAF2 is clearly important for NFκB signaling. In ventricular myocytes, however, we found that the TRAF2 (80 – 501)

was unable to inhibit TNFα-mediated NFκB activation. Furthermore, none of the other structural mutations of the TRAF2 molecule were able to prevent TNFα signaling of NFκB. Therefore, we concluded that TRAF2 was dispensible for NFκB activation in neonatal myocytes. This suggests that there are TRAF2 independent mechanisms for TNFα induced NFκB activation. Recent findings, in fact, are consistent with our findings. TRAF2 knockout mice and transgenic mice over-expressing the dominant negative TRAF2 mutants show only mild defects in NFκB activation (102). There are a number of possible explanations for these findings. Since there are 6 members of the TRAF family that may substitute for TRAF2 to mediate TNFα signaling of NFκB, our findings may point to the presence of redundancy in TRAF molecules that are capable of mediating TNFα signaling. Alternatively, it may be that TRAF2 is not important for TNFα signaling of NFκB in neonatal myocytes.

Importantly, the TRAF2 mutants, including the TRAF2 (80 – 501), were nonfunctional in 293 cells with respect to NFκB signaling while expression of the TRAF2 wild type significantly activated NFκB ($p<.05$). By contrast, expression of the TRAF2 (80 – 501) in neonatal myocytes activated NFκB to the same extent as the TRAF2 wild type. This suggests that the TRAF2 ring finger domain is dispensible for TRAF2 mediated NFκB activation in myocytes but not 293 cells.

VI. CONCLUSIONS

TNFα has a broad range of cell type specific physiological and patho-physiological functions. Importantly, elevated levels of serum TNFα have been reported in virtually all forms of cardiac injury. Using neonatal ventricular myocytes we studied the impact of TNFα with respect to cell death and changes in cardiac specific gene expression. We found that TNFα could repress cardiac specific genes however it was not cytotoxic to neonatal myocytes. A known downstream target of TNFα is the inducible nitric oxide synthase gene (iNOS). We found that L-NAME, an inducible nitric oxide synthase (iNOS) inhibitor, could abrogate TNFα-mediated repression. The transcription factor, NFκB is activated by TNFα in cardiac myocytes and may lie upstream of iNOS. We found that NFκB p65 mimicked the repressive effect of TNFα on cardiac specific gene expression. Furthermore, IkB (S32/36A), prevented TNFα mediated activation of NFκB and abrogated TNFα mediated repression of cardiac specific genes. It is conceivable that elevated levels of TNFα may contribute to the cardiac dysfunction through the inhibition of cardiac muscle gene expression. Furthermore, we suggest that TNFα represses cardiac specific gene expression through an NFκB and NO dependent pathway (see Appendix).

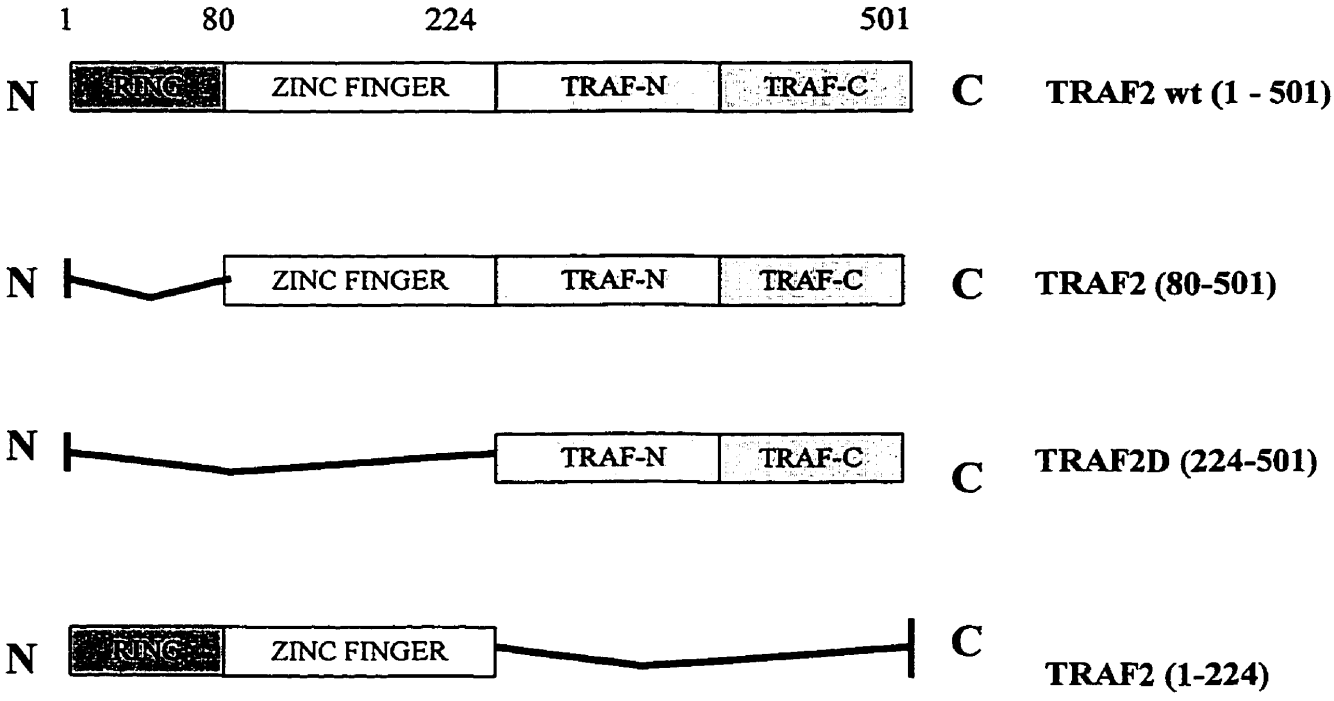
Next we tested the importance of the TNFα-Receptor Associated factor 2 (TRAF2) for the activation of NFκB. TRAF2 is capable of transactivating NFκB and was initially thought to be indispensible for induction of NFκB by TNFα in some cell types. We found that a TRAF2 mutant lacking its N-terminal RING finger domain acts as a dominant negative inhibitor of TNFα-mediated NFκB activation in 293 cells while

in myocytes, we found that TRAF2 (80 – 501) was unable to inhibit TNFα-mediated NFκB activation.

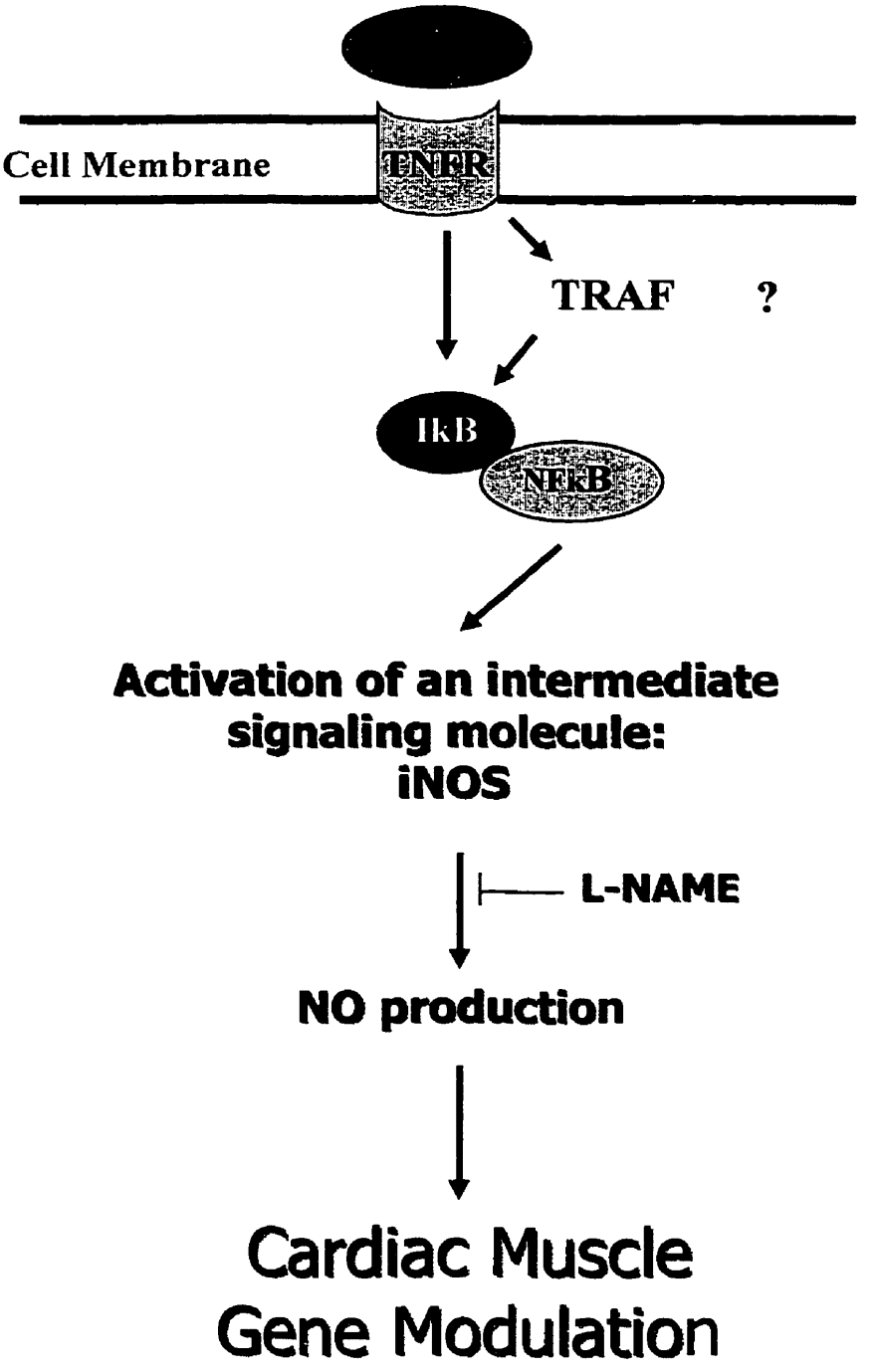
In summary we suggest that (1) elevated levels of TNFα may contribute to the progressive contractile dysfunction seen in cardiac hypertrophy and heart failure by repressing cardiac muscle gene expression; (2) TNFα likely represses cardiac gene expression through an NFκB and NO dependent pathway; (3) the ring finger domain of TRAF2 is dispensible for NFκB activation in myocytes but not 293 cells; and (4) there may be TRAF2 independent mechanisms present in myocytes for TNFα mediated activation of NFκB.

VII. APPENDIX

A. TRAF2 structural anatomy. Four distinct domains of the TRAF2 molecule are illustrated in the diagram below.



B. Proposed model for TNFa signaling in ventricular myocytes



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