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**CARDIAC SPECIFIC TRANSGENIC INHIBITION OF
NUCLEAR FACTOR – KappaB (NF- κ B) MITIGATES ADVERSE CARDIAC
REMODELING AND AMELIORATES CARDIAC DYSFUNCTION IN
DOXORUBICIN-INDUCED CARDIOMYOPATHY**

By

Santosh Kumar Sanganalmath, M.D., M.S.

Doctoral Dissertation

Presented to the Faculty of the Graduate School of the University of Louisville
in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

Department of Physiology and Biophysics
University of Louisville
Louisville, Kentucky

May 2010

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DEDICATION

This dissertation is dedicated to
my beloved wife, Shilpa Sanganalmath

and

my parents (Mrs. Jayashree Prabhu and Dr. Prabhulinga Swamy)
for the love, encouragement and support in my life.

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ABSTRACT

CARDIAC SPECIFIC TRANSGENIC INHIBITION OF NUCLEAR FACTOR – KappaB (NF- κ B) MITIGATES ADVERSE CARDIAC REMODELING AND AMELIORATES CARDIAC DYSFUNCTION IN DOXORUBICIN-INDUCED CARDIOMYOPATHY

Santosh Kumar Sanganalmath

April 29, 2010

Background

The anthracycline doxorubicin (DOX) is widely used as an effective antineoplastic drug. Cardiotoxicity leading to congestive heart failure is the primary factor limiting the clinical use of DOX. However, although a variety of approaches to protect the heart against DOX-induced cardiotoxicity have been attempted; treatment to prevent short and long term DOX-induced cardiac damage remains limited. Currently, there is no specific therapeutic strategy against this severe disease. Cardiac inflammation and myocardial apoptosis is known to contribute to DOX-induced cardiomyopathy. The nuclear factor-kappaB (NF- κ B) is a part of the innate immune system and is involved in cardiac stress reactions. Since NF- κ B might play a significant role in cardiac inflammatory

signaling and apoptosis, we investigated whether or not NF- κ B is involved in DOX-induced cardiotoxicity.

Methods and results

Age-matched wild-type (WT), nontransgenic littermates (NTg) and inhibitory kappaB transgenic mice (IkB Tg) mice were divided into six groups based on whether the mice receive either DOX or vehicle. DOX was injected at a dose of 7.5 mg/kg intraperitoneally (i.p.) 2 times over 2 wks. Group I, WT (vehicle, n=6); group II, NTg (vehicle, n=6); group III, Tg (vehicle, n=6); group IV, WT (DOX, n=14); group V, NTg (DOX, n=14); and group VI, Tg (DOX, n=11). Groups I, II and III served as control mice and received injections of saline as vehicle. 14 days after DOX therapy, cardiac function was assessed by echocardiography and hemodynamics using a micro conductance Millar catheter. Following invasive hemodynamics, the mice were euthanized and heart and lung weights were noted and hearts were perfusion fixed for pathology and morphometry. The left ventricular (LV) tissue was harvested for myocardial protein expression of proinflammatory cytokines and proapoptotic markers. DOX injection in WT and NTg mice resulted in significant impairment in LV function as evidenced by a decrease in LV ejection fraction (LVEF), LV systolic pressure (LVSP), LV rate of pressure development and pressure decay ($\pm$ dP/dt) and an increase in LV end-diastolic and end-systolic diameters, LV end-diastolic pressure and LV end-diastolic volume (LVEDV). Furthermore, WT and NTg mice after DOX therapy showed signs of congestive heart failure as evidenced by an increase in lung weight and development of pulmonary edema, which was also associated with

signs of adverse ventricular remodeling (increase in chamber diameters and decrease in wall thickness). In contrast to these findings, the heart weight, LVEDP, LV intrinsic diameters, LVEDV and lung weight were reduced, whereas the $\pm$ dP/dt, LVSP and LVEF were increased towards normal levels in I κ B Tg group.

In order to examine the effects of cardiac specific inhibition of NF- κ B on inflammatory response and myocardial apoptosis, LV samples were analyzed for the protein expression of key inflammatory cytokines [tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6) and inducible nitric oxide synthase (iNOS)] and proapoptotic proteins (Bax and p53) after DOX therapy. In WT and NTg hearts, therapy with DOX was associated with an increase in inflammation and apoptosis, as indicated by an up-regulation of TNF- α , IL-6, iNOS, Bax and p53. In contrast, I κ B Tg mice showed decreased expression of proinflammatory cytokines and proapoptotic proteins. Furthermore, survival rate was significantly higher in I κ B Tg mice than in WT and NTg mice 14 days after DOX injection (73% vs 43%, $P < 0.05$).

Conclusions

Cardiac specific inhibition of NF- κ B improves LV function and attenuates adverse cardiac remodeling in DOX-induced cardiomyopathy. These beneficial effects of inhibition of NF- κ B may be due to the decrease in the development of cardiac inflammation and myocardial apoptosis in DOX-induced cardiotoxicity. These findings may have important therapeutic implications of NF- κ B in DOX-induced congestive heart failure.

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CHAPTER I

INTRODUCTION

Doxorubicin (DOX) was first isolated in early 1950s and is considered one of the most effective antineoplastic agents used in the treatment of solid and hematopoietic tumors. However, this drug has proven to be a double-edged sword since it can also cause severe cardiotoxicity leading to a dilated cardiomyopathy, which results in congestive heart failure that is usually refractory to common medications (1-3). Therefore, several investigators are currently focusing their research to better understand the mechanisms underlying DOX's cardiotoxicity. This may enable development of therapies, which may potentially prevent and/or treat the heart failure it causes. Although the precise cellular mechanisms responsible for the cardiotoxic effects of DOX remain enigmatic, it is believed that the antitumor activity of DOX is likely to be distinct from the mechanism of its cardiotoxicity (4, 5). It has been suggested that the DOX-induced cardiomyopathy may be due to the formation of reactive oxygen species, expression of proinflammatory genes, apoptosis, and altered molecular signaling (2, 3, 6). Until to date, taking these various mechanisms into consideration, a variety

of approaches, which were aimed at preventing or mitigating the cardiotoxicity of DOX have been tried, but so far, the ability of these treatments to protect the heart from damage has not been successful. Therefore, to understand the pathogenesis of DOX-induced cardiac dysfunction, inflammation and apoptosis, we investigated the activation of NF- κ B in myocardium.

NF- κ B, a ubiquitous transcription factor, was first identified as a regulator of the expression of the kappa light-chain gene in murine B lymphocytes (7) but has subsequently been found in many different cells. NF- κ B is found to be activated by a wide range of inducers, such as cytokines, ultraviolet irradiation, and chemotherapeutic agents including DOX. In quiescent cells, NF- κ B is maintained as an inactive form in the cytoplasm where it is bound to I κ B inhibitor proteins, which prevent NF- κ B from entering the nuclei. Upon cellular activation by extracellular stimuli, I κ B is phosphorylated and proteolytically degraded by proteases. This proteolytic process causes the release and translocation of NF- κ B complex into the nucleus, where it can mediate gene transcription by binding to specific sequences in the promoter regions of its target genes. Products of these target genes initiate or regulate inflammatory responses, apoptosis and carcinogenesis. NF- κ B promotes or inhibits programmed cell death (8, 9). The opposing effects of NF- κ B are dependent on cell type and/or the nature of stimuli. It remains unclear whether NF- κ B regulates inflammation and myocardial apoptosis in DOX-induced cardiomyopathy. Therefore, we speculated that suppression of inflammation and apoptosis may largely rescue DOX-triggered

cardiotoxicity. In the present study, we induced DOX cardiomyopathy in a novel mouse model, which can specifically inhibit NF- κ B signaling pathway in the heart and examined the effect of NF- κ B inhibition on the following: (1) preservation of the systolic and diastolic heart dysfunction; (2) mitigation of inflammatory response; (3) attenuation of cardiomyocyte apoptosis; and (4) prevention of mortality.

CHAPTER II

BACKGROUND AND LITERATURE REVIEW

DOX as an Anticancer Agent

DOX belongs to a group of anthracycline drugs, which was originally isolated from the pigment-producing bacterium *Streptomyces peucetius* in the early 1950s. DOX is one of the most effective anticancer agents (10) used in the treatment of various neoplasms including acute leukemia, Hodgkin's, and non-Hodgkin's lymphoma, and breast cancer. Although DOX is an effective anticancer agent, it can cause tumor cell resistance and cardiotoxicity leading to dilated cardiomyopathy and congestive heart failure, which is refractory to common medications (11-14). In recent years, the use of higher doses of DOX has dramatically increased the incidence of dilated cardiomyopathy. Figure 1 depicts the chemical structure of DOX in which the side chain terminates with a primary alcohol. Although the precise mechanisms of action of DOX on tumor cells have not been fully understood, various mechanisms have been suggested by investigators such as: generation of reactive oxygen species leading to DNA damage and peroxidation of lipids; inhibition of protein synthesis by interacting

with DNA; inhibition of topoisomerase II, resulting in apoptosis and DNA breakdown; cross-linking and alkylation of DNA, and blocking DNA strand separation and helicase activity (15, 16). The critical component of the antitumor activity of DOX is by generation of reactive oxygen species and by DNA replication. DOX interacts with mitochondrial enzymes via their quinone ring and produces huge amount of reactive oxygen species (17, 18). Furthermore, DOX can directly act on DNA base pairs, inhibit DNA topoisomerase II, and lead to DNA replication (19-21).

Figure 1: Chemical structure of DOX: DOX consists of a tetracyclic ring, with the sugar daunosamine attached by a glycosidic linkage. Structurally, doxorubicin is related to daunorubicin and differs only in hydroxyl group substitution (instead of hydrogen) at the alkyl side chain, at position '9' of the 'A' ring.

Epidemiology and Clinical Features of DOX-induced Cardiomyopathy

DOX-induced cardiomyopathy is similar to other forms of dilated cardiomyopathy characterized by enlarged ventricles, dilation of all chambers, and presence of mural thrombi in both ventricles. DOX causes both acute and chronic cardiotoxicity. The acute effects occur in approximately 11% of patients during or soon after initiation of DOX treatment and are usually reversible. The symptoms include arrhythmias (supraventricular tachycardia and ventricular premature beats), hypotension, and nonspecific electrocardiographic changes (11, 22, 23). The chronic cardiotoxic effects of DOX occur in approximately 2% of patients, and the prognosis is dismal with a mortality rate >50%. In chronic DOX-induced dilated cardiomyopathy, the condition is typically irreversible leading to congestive heart failure (11, 14).

Mechanisms of DOX-induced Cardiomyopathy: *Antitumor Activity versus Cardiotoxicity*

The antitumor activity of DOX is mainly due to initiation of DNA damage via the inhibition of topoisomerase II. DOX-induced cardiotoxicity involves multiple other mechanisms including oxidative stress, mitochondrial functional impairment, cellular necrosis, and induction of apoptosis (2, 16). Therefore, despite the fact that the exact mechanism of DOX-induced cardiomyopathy still remains unknown, most investigators believe that an increase in oxidative stress and generation of reactive oxygen species (24-26), peroxidation of lipids (24, 27, 28), and reductions in the levels of antioxidants and sulfhydryl groups (29-31), play a

crucial role in the pathogenesis of DOX induced cardiomyopathy (24-26, 32). In addition, various other mechanisms suggest that DNA breakdown and protein synthesis (33-35); release of vasoactive amines (histamine, serotonin and catecholamines) (36); alteration in adrenergic activity (37); mitochondrial dysfunction (38); changes in lysosomes (28); alterations in sarcolemmal Ca^{2+} handling by Na^+/K^+ ATPase and Ca^{2+} -ATPase activities (39) (40); impairment of mitochondrial enzymatic activity (41); and production of highly reactive peroxynitrate and nitric oxide and inactivation of myofibrillar creatine kinase (42, 43). Furthermore, DOX also induces apoptosis in vascular smooth muscle cells and cardiomyocytes by activating caspases and DNA degradation (44-46), which eventually results in cardiomyocyte loss and thus can exacerbate heart failure (47-51). Figure 2 shows the proposed mechanisms of interaction between DOX and Nuclear Factor - kappaB (NF- κ B) in pathogenesis of dilated cardiomyopathy and congestive heart failure.

Figure 2: Proposed mechanisms of NF-κB and DOX-induced cardiomyopathy leading to heart failure. DOX is known to increase oxidative stress, which in turn leads to increase in inflammation, subcellular remodeling and apoptosis via NF-κB dependent mechanism that eventually results in progressive dilated cardiomyopathy and congestive heart failure.

DOX-induced Toxicity - Why does DOX Target Heart?

As stated earlier, the exact mechanism of DOX-induced cardiotoxicity still remains unclear. DOX is a highly lipophilic drug and has a relatively long half-life in the body (16, 52). The presence of quinone ring in the DOX makes it prone to the generation of free radicals by redox cycling. Under aerobic conditions, a single electron reduction in the DOX molecule causes formation of a semiquinone free radical, which donates its unpaired electron to oxygen leading to the formation of superoxide radical (26, 53). Mizutani et al. show that a relatively small amount of DOX is sufficient to catalyze the generation of numerous superoxide radicals (54). As cardiomyocytes constantly require high energy phosphates and thus rely on oxidative phosphorylation, these cells are more prone to apoptosis than any other cell in the body. Also, as cardiac myocytes contains low levels of enzyme catalase, and DOX can readily inactivate glutathione peroxidase-1 present in these cells, cardiomyocytes are further more susceptible to DOX injury (55, 56). Li et al. show that DOX decreases protein levels of cytosolic glutathione peroxidase in cardiomyocytes, which is responsible for scavenging of free radicals (57). It should also be noted that as cardiomyocytes are considered as terminally differentiated cells, any loss of these cells due to necrosis or apoptosis results in paucity of contractile elements, eventually leading to cardiac dysfunction. Additionally, Singal et al. (58) suggests remodeling of subcellular organelles such as sarcolemma, mitochondria, sarcoplasmic reticulum and myofibrils causes alteration in

intracellular Ca^{2+} dynamics and leads to contractile dysfunction in the cardiomyocytes (58).

Nuclear Factor - kappaB (NF- κ B)

NF- κ B was discovered in 1986 via its interaction with an 11-base pair sequence in the immunoglobulin light-chain enhancer in murine B lymphocytes (59). It is a widespread dimeric transcription factor that exists in the cytosol complexed to an inhibitory kappaB (I κ B) monomer. The complex of NF- κ B consists of either a p50 (NF- κ B1) or a p52 (NF- κ B2) subunit complexed to another protein from the Rel group [which includes p65 (RelA), c-Rel and RelB] (60, 61). NF- κ B is activated by various stimuli including ischemia, cytokines, hypoxia, free radicals and oxidants, lipopolysaccharide (LPS), activators of protein kinase C, ultraviolet radiation, and viruses (60). The activation of NF- κ B due to these stimuli translocates NF- κ B to the nucleus, which in turn binds to specific DNA binding sites (called κ B), present within the promoter regions of various genes including those encoding p65, I κ B α , tumor necrosis factor- α (TNF- α), interleukin (IL)-2, IL-6, IL-8, interferon (IFN)- β , inducible nitric oxide synthase (iNOS), and several adhesion molecules (60, 61). This activation of NF- κ B is controlled by site-specific phosphorylation and ubiquitination of I κ B proteins that target I κ B for proteasome-mediated degradation (62). Although, there are currently seven known I κ B isoforms (I κ B α , I κ B β , I κ B γ , Bcl-3, I κ B ϵ , I κ B-R, and I κ B-L) (61), only I κ B α and I κ B β have been studied most extensively, and are thought to be the key regulators of NF- κ B nuclear translocation. Traenckner et al. show that phosphorylation of serine

residues at positions 32 and 36 is critical for ubiquitination and degradation of I κ B α (63) with consequent release and nuclear migration of NF- κ B.

Figure 3: The NF- κ B pathway is activated by various pathologic stimuli, which leads to phosphorylation of I κ B and proteolytic degradation by proteases. This proteolytic process causes the release and translocation of NF- κ B complex into the nucleus, where it can mediate gene transcription by binding to specific sequences in the promoter regions of its target genes. Products of these target genes initiate or regulate inflammatory responses, apoptosis and carcinogenesis. The figure also depicts the overexpression of I κ B α , which inhibits the process of phosphorylation of I κ B by I κ B kinase thereby inhibiting the NF- κ B pathway.

Generation of I κ B α ^{S32A, S36A} transgenic mice with Cardiac-Specific Inhibition of NF- κ B

To specifically determine the role of NF- κ B in pathophysiologic processes requires specific inhibition of the transcription factor in the heart *in vivo*, which thus far has not been possible. As a result, the contribution of NF- κ B to cardiovascular pathophysiology in the intact animal has been inferred either from correlative evidence (e.g., association of NF- κ B activation with a certain outcome) or by inhibition with pharmacologic agents of limited specificity (64, 65). Although there are numerous pharmacologic agents to inhibit NF- κ B, which are available for *in vivo* use, none of these agents have sufficient specificity to provide conclusive evidence of NF- κ B function (64, 65). Therefore, in order to determine whether expression of a transdominant mutant I κ B α abrogates NF- κ B activation specifically in the heart, we have created a transgenic mouse with cardiac-specific expression of I κ B α ^{S32A, S36A} (Figure 3) (66). In this mouse model, the α -MyHC promoter was chosen to drive the expression of I κ B α ^{S32A, S36A} since this promoter directs high level, cardiac-specific gene expression in a position-independent, copy-number-dependent manner (67, 68). As demonstrated by Dawn et al., this I κ B α ^{S32A, S36A} transgenic mouse exhibits normal cardiac morphology and histology and the expression of the mutant I κ B α completely blocks NF- κ B activation by both TNF- α and LPS (two important agents, which elicits NF- κ B activation and orchestrates NF- κ B signaling) (68-70). Therefore, these mice provide an *in vivo* system to study and conclusively determine the role of NF- κ B in specific cardiac pathophysiological states. In this mouse model,

a transgene was constructed for the expression in the murine heart of a transdominant human $\text{I}\kappa\text{B}\alpha$ cDNA (with serine residues at positions 32 and 36 replaced by alanine, $\text{I}\kappa\text{B}\alpha^{\text{S32A, S36A}}$). The human $\text{I}\kappa\text{B}\alpha^{\text{S32A, S36A}}$ cDNA was released from the original plasmid (pSVK3) (71) by restriction digestion with *Sma* I and subcloned between the 5.5 kb α -MHC promoter and the 736 bp human growth hormone polyadenylation sequences (72) (Figure 4). Transgenic mice were made by pronuclear microinjection of the isolated purified linear transgene into the male pronuclei of fertilized mouse oocytes (FVB/N strain) (73). The founder transgenic mice were genotyped by PCR, using α -MHC-specific primer (5'-AAGCCT- AGCCCACACCAGAAATGACAGACA-3') and an $\text{I}\kappa\text{B}\alpha$ -specific primer (5'-AGTAGCCGCTCCTTC-TTCAGCCCGTC-3').

Figure 4: Diagrammatic representation of the transgenic promoter construct. The 1.1 kb human $\text{I}\kappa\text{B}\alpha$ cDNA ($\text{I}\kappa\text{B}\alpha^{\text{S32A, S36A}}$) was subcloned between the 5.5 kb α -MyHC promoter and the 736 bp human growth hormone (Hgh) polyadenylation signal. The cDNA was released from plasmid pSVK3 by excision with *Sma* I and cloned into the *Sal* I restriction site of the α -MyHC vector. The transgene was excised from the plasmid by restriction with *Not* I and used for pronuclear microinjection.

Uses of I κ B α ^{S32A, S36A} transgenic mice

Although several pharmacologic inhibitors of NF- κ B activation are currently available, they lack specificity, and thus, do not yield valid results. For example, dithiocarbamates are antioxidants and iron chelators (64), and therefore would be expected to produce a multitude of effects, including inhibition of other transcription factors like AP-1 (74) in addition to inhibition of NF- κ B. Peptide inhibitors of NF- κ B that have been used in isolated cells (75) are not effective *in vivo*. Furthermore, using pharmacologic inhibitors *in vivo*, it is impossible to localize the effect of inhibition of NF- κ B activity to a specific organ. The I κ B α ^{S32A, S36A} transgenic mouse provides a novel tool to investigate specifically and conclusively the role of NF- κ B in cardiac pathophysiology. As a result of the cardiac-specific expression of the transgene, these mice enable assessment of the role of NF- κ B specifically in the heart. Accordingly, the I κ B α ^{S32A, S36A} transgenic mice should be useful not only for signal transduction research, but also to verify the utility and efficacy of new therapeutic approaches in modifying NF- κ B activity and in modulating the effects of TNF- α and LPS in the setting of myocardial ischemia-reperfusion injury, heart failure, septic shock, cardiac hypertrophy, atherogenesis, and acute coronary syndromes.

CHAPTER III

FUNDAMENTAL QUESTION

Phenomenon

The prognosis of patients with DOX-induced cardiomyopathy and congestive heart failure is dismal and mechanisms leading to this cardiotoxicity remain elusive. Also, various approaches to protect the heart against this severe cardiac damage have been unsuccessful.

Proposed Concepts

Although end-stage ischemic cardiomyopathies could be manageable by using procedures such as revascularization and therapeutic angiogenesis, nonischemic cardiomyopathies can only be treated radically by heart transplantation (76, 77). Cardiac transplantation has several limitations such as availability of donors, graft rejection and adverse effects of immunosuppressive medications (76, 77). DOX is widely used as a broad-spectrum antineoplastic drug in the treatment of both solid tumors and leukemia. Unfortunately, despite its broad spectrum effectiveness, long-term therapy with DOX is associated with a high incidence of a cumulative and irreversible dilated cardiomyopathy followed by congestive

heart failure, which is often fatal to the patients (2, 11, 78). The morphology of the heart of DOX-induced cardiomyopathy is similar to dilated cardiomyopathy, in which progressive heart failure and sudden cardiac death from ventricular arrhythmia occur (79).

Experimental Design

To address these issues, a series of experiments were designed to test the following hypothesis. The experimental protocol is shown in Figure 5.

Figure 5: Experimental Protocol. WT, NTg and I κ B Tg mice were divided into six groups based on whether the mice received either DOX or vehicle. DOX was injected at 7.5 mg/kg dose intraperitoneally (i.p.) 2 times over 2 wks. Group I, WT (vehicle); group II, NTg (vehicle); group III, Tg (vehicle); group IV, WT (DOX); group V, NTg (DOX); and group VI, Tg (DOX). Echocardiography was performed at baseline and at 2 wks after DOX/saline therapy. At the end of study period (2wks after first DOX dose), after an

invasive hemodynamics, heart and lungs were pathologically assessed and LV samples were frozen for the expression of proinflammatory cytokines and apoptotic proteins.

Hypothesis

Sustained over-expression of proinflammatory cytokines provokes the induction of cardiomyocyte apoptosis in DOX-induced cardiomyopathy, which contributes to adverse ventricular remodeling and cardiac dysfunction eventually progressing to congestive heart failure.

AIM #1: To test the hypothesis that cardiac-specific transgenic inhibition of NF- κ B mitigates doxorubicin-induced adverse ventricular remodeling and cardiac dysfunction.

AIM #2: To test the hypothesis that cardiac-specific transgenic inhibition of NF- κ B reduces the expression of proinflammatory cytokines in doxorubicin-induced cardiomyopathy.

AIM #3: To test the hypothesis that cardiac-specific transgenic inhibition of NF- κ B reduces myocardial apoptosis in doxorubicin-induced cardiomyopathy.

CHAPTER IV

AIM #1

Cardiac-Specific Transgenic Inhibition of NF- κ B Mitigates Doxorubicin-induced Adverse Ventricular Remodeling and Cardiac Dysfunction

AIM #1 ABSTRACT

Background

The use of the DOX is associated with adverse cardiac remodeling with impaired ventricular function that can progress to congestive heart failure and has important implications for survival. In spite of several pharmacologic and surgical interventions for cardiomyopathy, many patients continue to experience a progressive decline in cardiac function. Therefore, improved methods to attenuate ventricular remodeling and function in dilated cardiomyopathy are needed. Although NF- κ B has been implicated in heart failure due to ischemia and cardiac hypertrophy, its role on detrimental cardiac remodeling and cardiac dysfunction in DOX-induced cardiomyopathy are unknown. We sought to examine if cardiac specific transgenic inhibition of myocardial NF- κ B activation

could prevent structural cardiac remodeling and mitigate cardiac dysfunction in dilated cardiomyopathy due to DOX treatment.

Methods and Results

Age-matched wild-type (WT, n=14), nontransgenic littermates (NTg, n=14) and I κ B Tg (n=11) mice received intraperitoneal injections of DOX twice over 2 wks (total dose of 15 mg/kg BW). Cardiac function and remodeling were assessed using echocardiographic techniques and *in vivo* hemodynamic measurements. Saline injected WT, NTg and I κ B Tg mice served as controls. At 14 days after DOX therapy, WT and NTg mice showed marked increase in heart weight, left ventricular (LV) end-diastolic pressure (LVEDP), LV intrinsic diameters, LV end-diastolic volume (LVEDV) and reduction in rates of pressure development and decay ($\pm$ dP/dt), LV systolic pressure (LVSP) and LV ejection fraction (LVEF). WT and NTg mice also showed signs of heart failure as evidenced by an increase in lung weight and development of pulmonary edema. The heart weight, LVEDP, LV intrinsic diameters, LVEDV and lung weight were reduced, whereas the $\pm$ dP/dt, LVSP and LVEF were increased towards control levels in I κ B Tg group compared with WT and NTg groups. Total mortality during 2 wk follow-up period averaged 57% in WT and NTg mice and 27% in I κ B Tg mice.

Conclusions

The results suggest that cardiac specific abrogation of NF- κ B in DOX-induced cardiomyopathy decreases mortality and has beneficial effects on LV function and cardiac remodeling in congestive heart failure. These findings may have important therapeutic implications of NF- κ B in DOX-induced cardiac dysfunction.

INTRODUCTION

Heart failure induced by DOX has been characterized by bilateral ventricular enlargement followed by thinning of the ventricular wall, and reduction in the LVEF (80). After DOX treatment, the LVEF measured by echocardiography is shown to be decreased from 54% to 35% while the cardiac output decreased from 5.6 to 3.9 L/min in 2 wks. Dilation of the ventricles as evidenced by increase in LV end-diastolic and end-systolic diameters (LVEDD and LVESD) has been shown to increase by 10% and 30%, respectively (80). In recent years, a growing body of evidence indicates the crucial role of NF- κ B as a central mediator of various cardiac pathologies. Therefore, complete inhibition of this pathway will be beneficial in the setting of stress and heart disease. In these lines, several investigators have used the classic model of NF- κ B activation to develop therapeutic strategies that inhibit various steps in the canonical signaling cascade. The appreciation of the importance of the NF- κ B family of transcription factors in cardiac disease has stimulated the development of multiple therapeutic strategies based on modifying this signaling pathway. The aim of this study was to examine the effects of cardiac specific inhibition of NF- κ B activation on the improvement of cardiac function and attenuation of adverse cardiac remodeling in DOX-induced cardiomyopathy and congestive heart failure.

MATERIALS AND METHODS

Animals

All animal studies were performed in accordance with the guidelines of the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and University of Louisville. Mice were housed in a facility with a 12 hr/12 hr light/dark cycle and were fed rodent chow and water *ad libitum*. The room was kept specific pathogen-free. Age-matched wild-type (WT), nontransgenic littermates (NTg) and I κ B Tg mice were divided into six groups based on whether the mice receive either DOX or vehicle. We injected a 7.5 mg/kg dose of DOX intraperitoneally (i.p.) 2 times over 2 wks. Group I, WT (vehicle, n=6); group II, NTg (vehicle, n=6); group III, Tg (vehicle, n=6); group IV, WT (DOX, n=14); group V, NTg (DOX, n=14); and group VI, Tg (DOX, n=11). Groups I, II and III served as control mice and received injections of saline as vehicle.

Echocardiography

Echocardiography was performed prior to DOX injection (baseline) and at the time of sacrifice (2 wks after the first DOX dose). Mice were weighed and anesthetized with isoflurane (ISF) inhalation. Induction was performed over 2 minutes with 3% ISF in 100% O₂. Anesthesia was maintained with 1% ISF in 100% O₂ at a flow rate of 1 L/min via a small nose cone. The mice were kept on a heating pad in a supine position with a slight tilt to the left decubitus position. The heating pad temperature was adjusted to maintain mice rectal temperature

between 37 and 38⁰ C. The anterior chest was shaved and the mice were placed in the left lateral decubitus position. Echocardiographic images were obtained using a Philips HDI 5000 SonoCT ultrasound system equipped with a 12-5 MHz phased-array probe fitted with a 0.3 cm standoff and a 15-7 MHz broadband linear probe. LV structure and function were assessed by previously validated two-dimensional, M-mode, and Doppler echocardiographic techniques.

Echocardiography - Analysis

Digital images were analyzed off-line by a single blinded observer using the ProSolv image analysis software (ProSolv, Inc., Indianapolis, IN). At least three consecutive beats were analyzed and averaged for the reported measurement.

M-mode LV diameter and wall thickness:

LVEDD and LVESD were measured from M-mode tracings obtained at the mid-papillary level and analyzed according to the modified American Society of Echocardiography standards (posterior wall leading-edge to leading-edge and anterior wall trailing-edge to trailing-edge).

2-D Cross-sectional Anatomy:

LV areas in diastole and systole were measured using the American Society of Echocardiography standards.

- Teichholz EDV: EDV was calculated using the Teichholz formula:

$$\text{EDV } (\mu\text{L}) = [7 / (2.4 + \text{LVEDD})] \times (\text{LVEDD}^3)$$

- LVEF was calculated as:

$$\text{EF } (\%) = [(\text{LVEDD}^2 - \text{LVESD}^2) / \text{LVEDD}^2] \times 100$$

Hemodynamics

The hemodynamic studies were performed just before euthanasia (2 wks after the first DOX dose). Animals were anaesthetized (thiopental 50 mg/kg; i.p.), intubated and artificially ventilated. A 1.4 F microconductance pressure catheter (Millar-Instruments, Inc., Texas, USA) was inserted into the right carotid artery and advanced into the LV under pressure control as described (81). After stabilization for 15 mins, the signals were recorded continuously with a Powerlab/4SP A/D converter (AD Instruments), stored, and displayed on a personal computer. The maximal LVSP and LVEDP, maximal slope of systolic pressure increment ($+dP/dt$) and diastolic pressure decrement ($-dP/dt$) were calculated and corrected according to *in vitro* and *in vivo* volume calibrations with a cardiac pressure-volume analysis program (PVAN 2.9; Millar instruments).

Pathology and Morphometry

Animals were euthanized at 2 wks after the first dose of DOX therapy. Following euthanasia, the heart was removed, washed in cold PBS, and weighed with an analytical balance. Pathologic heart weight was noted. The heart was then cannulated via the aorta and retrogradely perfused at a constant perfusion pressure equivalent to 100 cm H₂O. The heart was perfusion-fixed in 10% neutral-buffered formalin and was sectioned serially perpendicular to the longitudinal axis, processed, and embedded in paraffin. The morphometric variables were calculated by computerized planimetry (Image-Pro Plus) of digital images of three Masson's trichrome-stained serial sections taken at 0.5 to 1.0

mm intervals along the longitudinal axis. The midsection was used to measure LV chamber diameter. LV wall thickness was also measured. All anatomical variables were corrected according to a uniform sarcomere length.

Statistical Analysis

Data are reported as mean $\pm$ SEM. Data were measured with a two-way (time and group) ANOVA followed by Student's *t* tests with the Bonferroni correction (82). A '*P*' value less than 0.05 was considered statistically significant. All statistical analyses were performed using the SPSS software (version 8, SPSS, Inc., Chicago, IL). Mortality was analyzed using Kaplan Meier survival analysis. Comparison of Kaplan Meier survival curves was performed by using a log-rank test.

RESULTS

Survival Rates

Survival rates were compared between WT, NTg and I κ B Tg mice up to 14 days after DOX injection. As a consequence of preserved LV function in I κ B Tg mice after DOX, survival was significantly higher in I κ B Tg mice than in WT and NTg mice. Out of 28 mice that received DOX therapy in WT (n=14) and NTg (n=14) groups, 8 mice died in WT and 8 mice in NTg groups at the end of 2 wk follow-up period, which corresponds to 57% mortality. We lost 3/11 mice during the 2 wk period in I κ B Tg group. There was no animal loss in the vehicle treated groups of

18 mice. The data on mortality, as well as Kaplan Meier Survival plot for all groups is shown in Figure 6.

Figure 6: Survival curves after DOX injection in WT, NTg and I κ B Tg mice at 2 wks after DOX therapy. Survival curves were created by Kaplan-Meier method and compared by log-rank test. Percentages of surviving WT, NTg and I κ B Tg mice were plotted.

Clinical Manifestations and Pathologic Changes in Organs after DOX Treatment

WT and NTg mice had clinical signs of congestive heart failure, i.e. lung congestion as reflected by the increased lung weight, ventricular chamber enlargement, pulmonary edema and ascites. After echocardiography and hemodynamic studies, mice were euthanized with a lethal injection of sodium pentobarbital (50 mg/kg) intraperitoneally, and heart and lung weights were measured in all the groups. Although body weight was the similar in all groups, heart and lung weights were less in I κ B Tg mice than in WT and NTg mice after DOX administration (Figures 7A and 7B). Gross anatomical examinations at the end of the study period showed the hearts of DOX-treated WT and NTg mice were dilated and both the atria and ventricles were enlarged and hypertrophic (Figure 8). To confirm the improvement of the morphology of the hearts in I κ B Tg mice, we examined Masson's trichrome-stained serial sections taken at 0.5 to 1.0 mm intervals along the longitudinal axis at 2 wks after the DOX therapy. As shown in Figure 9, hearts in WT and NTg groups showed thin LV walls compared with the control hearts, whereas hearts in I κ B Tg groups showed thicker LV walls than in WT and NTg mice, being a similar morphology to that of a normal heart. Thus, we confirmed that DOX induces cardiomyopathy and that inhibition of NF- κ B improved morphology and function of the DOX injured heart.

Figure 7: Pathologic heart weight (A) and lung weight (B) in vehicle-treated (A), WT DOX-treated (B), NTg DOX-treated (C) and *IkB* Tg DOX-treated (D) mouse heart at 2 wks after DOX/saline therapy. The *IkB* Tg mice showed decrease in heart and lung weights compared to WT and NTg mice after DOX treatment.

Figure 8: Gross anatomical changes in vehicle-treated (A), WT DOX-treated (B), NTg DOX-treated (C) and IκB Tg DOX-treated (D) mouse heart at 2 wks after DOX/saline therapy. The IκB Tg heart exhibits less chamber dilation and thicker ventricular walls compared to WT and NTg mice.

Figure 9: Assessment of LV remodeling. Representative Masson's trichrome-stained myocardial sections from vehicle-treated (A), WT DOX-treated (B), NTg DOX-treated (C) and IκB Tg DOX-treated (D) mice at 2 wks after DOX/saline therapy. The LV cavity is smaller and the ventricular wall is thicker in the IκB Tg mice compared with the WT and NTg mice.

I κ B Tg Mice Are Resistant to DOX-induced Cardiac Dysfunction

Echocardiography

Before DOX injection (baseline), all variables of LV function, measured by echocardiography, were similar in WT, NTg and I κ B Tg groups (Figure 10); indicating that the extent of injury sustained during DOX therapy was comparable. WT and NTg mice exhibited a progressive deterioration in LVEF from baseline to 2 wks; this worsening in LVEF was attenuated in I κ B Tg mice, resulting in a markedly greater LVEF at 2 wks compared with WT and NTg mice. During follow-up, LVEDD and LVEDV increased progressively in all groups, consistent with postinfarct LV remodeling (Figure 10). However, in I κ B Tg mice LVEDV at 2 wks was significantly smaller compared with WT and NTg mice, indicating sustained improvement in LV remodeling with NF- κ B inhibition (Figure 10). Shown in Figure 11 are representative two-dimensional and M-mode echocardiographic tracings from a WT, NTg and I κ B Tg animal at 2 wks after DOX treatment. In contrast to the LV wall motion in the control group (Figure 11A), the waveforms from the WT (Figure 11B) and NTg (Figure 11C) animals clearly demonstrated blunted anterior and posterior wall motion, consistent with decreased EF ($P < 0.05$), indicating a decrease in myocardial performance in WT and NTg mice after DOX treatment. The decrease in the LV wall motion was improved in I κ B Tg mice (Figure 11D).

Figure 10: Quantitative assessment of LV systolic function. Echocardiographic measurements of LVEF (A), LVEDV (B), LVEDD (C) and LVESV (D) in WT, NTg and *IkB* Tg mice at baseline and at 2 wks after DOX/saline therapy. The *IkB* Tg mice showed an improved LVEF and a decreased LVEDV at 2 wks after DOX therapy. Also, compared with the WT and NTg hearts, the *IkB* Tg mouse exhibited smaller LV diameters. Data are mean $\pm$ SEM.

Figure 11: Echocardiographic data. (A–D) Representative 2-dimensional and M-mode echocardiographic images from a normal healthy mouse with vehicle injection (A), WT DOX-treated (B), NTg DOX-treated (C), and IκB Tg DOX-treated (D) mice at 2 wks after DOX/saline therapy. Compared with the WT and NTg hearts, the IκB Tg heart shows a smaller LV cavity and thicker anterior wall. Contractile activity in the anterior wall is present in the IκB Tg heart and virtually absent in the WT and NTg heart.

Hemodynamics

Hemodynamic parameters obtained from the different experimental groups 2 wks after DOX injection are shown in Figure 12. WT and NTg mice showed LV dysfunction with decreased LVSP, decreased rate of LV pressure development (+dP/dt), pressure decay (-dP/dt) and an elevated LVEDP compared to control animals. The LVEDP was reduced, whereas the ±dP/dt and LVSP was increased towards sham levels in IκB Tg group compared with WT and NTg mice.

Figure 12: Hemodynamic measurements of LVSP (A), LVEDP (B), +dP/dt (C) and -dP/dt (D) in WT, NTg and lkb Tg mice at 2 wks after DOX/saline therapy. A decrease in LVSP, performance (+dP/dt) and relaxation (-dP/dt) were observed in WT and NTg mice. This was improved in lkb Tg mice. Data are mean $\pm$ SEM.

Figure 13: Morphometric assessment of LV anatomy. Pathologic measurements of chamber diameter (A) and wall thickness (B) in WT, NTg and lkb Tg mice at baseline and at 2 wks after DOX/saline therapy. The lkb Tg mice showed a significant decrease in LV chamber diameter and an increase in LV wall thickness at 2 wks after DOX therapy.

Cardiac Specific Transgenic Inhibition of NF- κ B Halts LV Remodeling in DOX-induced Dilated Cardiomyopathy:

By echocardiography, the LVEDD and LVESD were markedly increased in WT and NTg mice after DOX injection, as shown in Figures 10 and 11. However, in I κ B Tg mice, LV dilatation after DOX therapy was prevented, and LVEDD and LVESD were significantly smaller than in WT and NTg mice. Similar to these findings, morphometric assessment of heart sections showed smaller LV chamber diameter in I κ B Tg mice treated with DOX when compared with WT and NTg groups. Also, wall thickness was significantly improved in I κ B Tg mice treated with DOX when compared to WT and NTg mice (Figure 13).

CONCLUSIONS:

This study was undertaken to test the beneficial effects of cardiac specific transgenic inhibition of NF- κ B on LV function and ventricular remodeling in DOX-induced cardiomyopathy. From the results obtained in this study, the following conclusions can be drawn -

- 1) Cardiomyopathy in mice due to DOX therapy was evidenced by decrease in LVSP, increase in LVEDP and LV diameters followed by LV dilation. These changes in LV lead to impairment in systolic and diastolic function.
- 2) In view of observed alterations in cardiac morphology in WT, NTg and I κ B Tg hearts, it is suggested that ventricular remodeling, systolic and

diastolic dysfunction in CHF due to DOX therapy, may be partly due to the NF- κ B activation in the failing heart.

- 3) Cardiac specific transgenic inhibition of NF- κ B significantly decreased mortality and improved the LV function and reversed the adverse ventricular remodeling due to DOX and such changes may be indirectly due to decrease in preload and afterload.

CHAPTER V

AIM #2

Cardiac-Specific Transgenic Inhibition of NF- κ B reduces the Expression of Proinflammatory Cytokines in Doxorubicin-induced Cardiomyopathy

AIM #2 ABSTRACT

Background

The NF- κ B proteins are known to play a critical role in mounting the immune response and in controlling genes of proinflammatory cytokines. The expression of innate immune response proteins such as tumor necrosis factor- α (TNF- α), interleukin (IL-6) and inducible nitric oxide synthase (iNOS) occurs in the cardiac tissue of humans and experimental animals with heart failure. Therefore, due to the profound cardiodepressant effects of proinflammatory cytokines, there is a well-justified interest to identify the signaling pathways that drive the expression of these proteins in cardiomyocytes.

Methods and Results

Age-matched wild-type (WT, n=14), nontransgenic littermates (NTg, n=14) and I κ B Tg (n=11) mice received intraperitoneal injections of DOX twice over 2 wks

(total dose of 15 mg/kg BW). Myocardial protein expression of proinflammatory cytokines, TNF- α , IL-6, and iNOS were assessed by Western immunoblotting. At 14 days after DOX therapy, we found a significant increase in the expression of TNF- α , IL-6, and iNOS in NTg mice treated with DOX. This increase in expression of TNF- α , IL-6, and iNOS were significantly attenuated by DOX-treated I κ B Tg mice.

Conclusions

Cardiac specific inhibition of NF- κ B has preventive effects on cardiac inflammation induced by DOX. These findings suggest that NF- κ B is detrimental in DOX-induced inflammatory processes. These insights might have useful implications for future studies utilizing NF- κ B antagonists for treatment of human DOX-induced cardiomyopathy.

INTRODUCTION

The activation and translocation of NF- κ B to the nucleus is followed by transcription of various pro-inflammatory genes, including tumor necrosis factor- α (TNF- α), interleukin (IL-6) and inducible nitric oxide synthase (iNOS) (83, 84). NF- κ B acts on genes for proinflammatory cytokines, chemokines (chemotactic cytokines that attract inflammatory cells to sites of inflammation), enzymes that generates mediators of inflammation, immune receptors, and adhesion molecules that play a crucial role in the initial recruitment of leukocytes to sites of inflammation. The activation of NF- κ B leads to a coordinated increase in the expression of many proinflammatory genes whose products mediate

inflammatory and immune responses. Therefore, NF- κ B is an obvious target for new types of anti-inflammatory treatment. Although various proinflammatory cytokines are not constitutively expressed in the normal heart (85, 86), the failing heart produces robust quantities of these cytokines that can be found both in the heart and in the peripheral circulation of patients with heart failure. Upregulation and production of these cytokines represent an intrinsic or an innate stress response against myocardial injury (87). When selectively overexpressed in hearts of mice, these cytokines effects the development of a dilated cardiomyopathy that closely mimics the pathophysiology seen in human failing hearts (88, 89). One pathway that appears to play a role in proinflammation in dilated cardiomyopathy is the ubiquitous transcription factor NF- κ B. Therefore, the objective of this study was to investigate the role of cardiac specific inhibition of NF- κ B in DOX-induced myocardial inflammation. Using a well established model of DOX-induced cardiomyopathy and heart failure in mice, we examined whether the inflammatory processes associated with DOX-induced cardiomyopathy is a result of an increase in the expression of proinflammatory cytokines.

MATERIALS AND METHODS

Animals

All animal studies were performed in accordance with the guidelines of the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and University of Louisville. Mice were housed in a facility with a 12

hr/12 hr light/dark cycle and were fed rodent chow and water *ad libitum*. The room was kept specific pathogen-free. Age-matched wild-type (WT), nontransgenic littermates (NTg) and I κ B Tg mice were divided into six groups based on whether the mice receive either DOX or vehicle. We injected a 7.5 mg/kg dose of DOX intraperitoneally (i.p.) 2 times over 2 wks. Group I, WT (vehicle, n=6); group II, NTg (vehicle, n=6); group III, Tg (vehicle, n=6); group IV, WT (DOX, n=14); group V, NTg (DOX, n=14); and group VI, Tg (DOX, n=11). Groups I, II and III served as control mice and received injections of saline as vehicle. At the end of study period (14 days after first DOX dose), heart was removed, the atria and the large vessels were carefully trimmed, and the ventricles were separated. The LV (including septum) was dissected and the tissue was frozen at -70°C for protein expression of TNF- α , IL-6 and iNOS.

Preparation of Homogenates and Cytosolic and Membranous Fractions

Homogenates, cytosolic and membranous proteins of heart tissue was prepared as previously described (90-92). Briefly, myocardial samples were homogenized for 2 x 10 seconds in *buffer A* [25 mmol/l Tris · HCl (pH 7.5), 0.5 mmol/l EDTA, 0.5 mmol/l EGTA, 1 mmol/l phenylmethylsulfonyl fluoride, 2 μ mol/l leupeptin, 1 μ mol/l pepstatin, 1 μ mol/l aprotinin, 10 mmol/l NaF, and 100 μ mol/l dephostatin] as total cellular homogenates. After that, homogenates were centrifuged at 14,000 *g* for 15 mins, and the resulting supernatants were collected as cytosolic fractions. Pellets were incubated in a lysis buffer (*buffer A* + 1% Nonidet P-40) for 4 hrs, centrifuged, and the resulting supernatants used as membranous

fractions. The protein content in the homogenates, cytosolic, and membranous fractions was determined using the Bradford technique (Bio-Rad).

Western Immunoblotting

The expression of proteins was assessed using standard SDS-PAGE Western immunoblotting techniques as previously published methods (90-92). Briefly, 70 µg of cytosolic proteins or membranous proteins were electrophoresed on a denaturing SDS gel for 2 to 3 hrs. After electrophoresis, the proteins on the gel were electrophoretically transferred to nitrocellulose membranes (Bio-Rad) overnight at 4°C. Gel transfer efficiency was carefully recorded by making photocopies of membranes dyed with reversible Ponceau staining; gel retention was determined by Coomassie blue staining as previously described. The membranes were incubated in 5% nonfat dry milk in TBST buffer (10 mmol/L Tris-HCl [pH 7.2], 0.15 mol/L NaCl, and 0.05% Tween-20) followed by incubation with specific primary antibodies (Santa Cruz Biotechnology, Inc). After extensive rinsing with TBST buffer, blots were incubated with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies and developed with the use of an enhanced chemiluminescence system (ECL kit, Amersham). After washing with TBST buffer, blots were air-dried and exposed to Kodak films in x-ray cassettes with intensifying screens. The signals detected by immunoblotting and the corresponding records of Ponceau stains of nitrocellulose membranes were quantitated using an image scanning densitometer (Personal PI, Molecular Dynamics). Anti TNF- α , anti IL-6 antibodies were purchased from Santa Cruz

Biotechnology, Inc., and anti iNOS antibodies were purchased from BD Transduction Laboratories.

Statistical Analysis

Data are reported as means $\pm$ SEM as previously described methods (90-92). Measurements were analyzed by ANOVA followed by unpaired Student's *t* tests with the Bonferroni correction. In all Western blot analyses, the content of the specific protein of interest was expressed as a percentage of the corresponding protein in the anterior LV wall of NTg vehicle-treated mice.

Figure 14: Western blots of membranous (Panel A) and cytosolic (Panel B) fraction of TNF- α protein at 2 wks after DOX/saline treatment in NTg mice compared with I κ B Tg mice. Bar graph shows densitometric quantification from 5 individual hearts per group. Data are mean $\pm$ SEM.

Figure 15: Western blots of IL-6 (Panel A) and iNOS (Panel B) proteins at 2 wks after DOX/saline treatment in NTg mice compared with I κ B Tg mice. Bar graph shows densitometric quantification from 5 individual hearts per group. Data are mean $\pm$ SEM.

RESULTS

TNF- α , IL-6 and iNOS protein expression

Protein expressions of both membranous and cytosolic fractions showed a markedly elevated expression of TNF- α after DOX treatment in NTg mice. However, these increases in TNF- α were suppressed in I κ B Tg mice compared with NTg mice after DOX therapy ($P < 0.05$) (Figure 14). Furthermore, DOX injection led to an increased expression of IL-6 and iNOS in NTg mice compared to vehicle treated NTg and I κ B Tg mice hearts. Although I κ B Tg mice also decreased expression of both IL-6 and iNOS compared to NTg mice, the protein expression of iNOS did not display a significant difference in comparison to NTg mice after DOX therapy (Figure 15 A and 15 B).

CONCLUSIONS

NF- κ B regulates gene transcription of many pro-inflammatory cytokines (61) and myocardial overexpression of NF- κ B initiates an inflammatory cascade and elevates the expression of TNF- α , IL-6, and iNOS. This study shows an increase in the expression of TNF- α , IL-6, and iNOS in the LV of mouse hearts after DOX therapy. However, when I κ B transgenic mice were treated with DOX, the induction of TNF- α , IL-6 and iNOS was significantly reduced. Thus inhibition of NF- κ B activity may serve as a potential mechanism for regulating inflammatory responses.

CHAPTER VI

AIM # 3

Cardiac-Specific Transgenic Inhibition of NF- κ B reduces Myocardial Apoptosis in Doxorubicin-induced Cardiomyopathy

AIM #3 ABSTRACT

Background

Clinical use of DOX is limited by its cardiotoxic effects, which have been shown to be due to increased oxidative stress and apoptosis. It is unclear if inhibition of NF- κ B has the protective effects on myocardial apoptosis induced by DOX. To elucidate the possible role of cardiac specific inhibition of NF- κ B during the development of DOX cardiomyopathy, we studied I κ B Tg mice by investigating cardiac apoptosis after induction of DOX-induced cardiomyopathy.

Methods and Results

Age-matched wild-type (WT, n=14), nontransgenic littermates (NTg, n=14) and I κ B Tg (n=11) mice received intraperitoneal injections of DOX twice over 2 wks (total dose of 15 mg/kg BW). Myocardial expression of proapoptotic

proteins Bax and p53 were assessed by Western blot analysis. At 14 days after DOX therapy, we found a significant increase in the expression of both Bax and p53 in NTg mice treated with DOX. This increase in expression of Bax and p53 were attenuated by DOX-treated IκB Tg mice.

Conclusions

Cardiac specific inhibition of NF-κB has preventive effects on myocardial apoptosis induced by DOX, which is probably associated with inhibiting binding activity of NF-κB and further regulating apoptosis-related protein expression and translation, and inhibiting myocardial apoptosis. These findings suggest that NF-κB is detrimental in DOX-induced cardiomyopathy in that it mediates apoptosis. These insights might have useful implications for future studies utilizing NF-κB antagonists for treatment of DOX cardiomyopathy.

INTRODUCTION

Apoptosis (also known as programmed cell death), is a highly conserved evolutionary event that is crucial for normal development and cell homeostasis. It is well accepted paradigm that cardiomyocyte apoptosis could play a key role in the aggravation of heart failure (49, 93). Recent evidence from both *in vitro* and *in vivo* studies demonstrate that exposure to DOX can trigger cardiomyocyte apoptosis and that this mode of cell death represents the predominant form of myocyte injury seen in this toxicity (44, 94). Furthermore, morphological features of hearts from patients with DOX-induced cardiomyopathy suggest that myocyte cell death may be mediated at least in part through an apoptotic process (58).

Therefore, the objective of this study was to investigate the antiapoptotic role of cardiac specific inhibition of NF- κ B in DOX-induced myocardial apoptosis. Using an established model of DOX-induced cardiomyopathy and heart failure in mice, we examined whether the myocyte loss associated with DOX-induced cardiomyopathy is a result of an increase in the incidence of apoptosis. Protein expression of apoptosis regulatory products such as Bax and p53 were examined.

MATERIALS AND METHODS

Animals

All animal studies were performed in accordance with the guidelines of the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and University of Louisville. Mice were housed in a facility with a 12 hr/12 hr light/dark cycle and were fed rodent chow and water *ad libitum*. The room was kept specific pathogen-free. Age-matched wild-type (WT), nontransgenic littermates (NTg) and I κ B Tg mice were divided into six groups based on whether the mice receive either DOX or vehicle. We injected a 7.5 mg/kg dose of DOX intraperitoneally (i.p.) 2 times over 2 wks. Group I, WT (vehicle, n=6); group II, NTg (vehicle, n=6); group III, Tg (vehicle, n=6); group IV, WT (DOX, n=14); group V, NTg (DOX, n=14); and group VI, Tg (DOX, n=11). Groups I, II and III served as control mice and received injections of saline as vehicle. At the end of study period (14 days after first DOX dose), heart was removed, the atria and the large vessels were carefully trimmed, and the

ventricles were separated. The LV (including septum) was dissected and the tissue was frozen at -70°C for protein expression of Bax and p53.

Preparation of Homogenates and Cytosolic and Membranous Fractions

Homogenates, cytosolic and membranous proteins of heart tissue was prepared as previously described (90-92). Briefly, myocardial samples were homogenized for 2 x 10 seconds in *buffer A* [25 mmol/l Tris · HCl (pH 7.5), 0.5 mmol/l EDTA, 0.5 mmol/l EGTA, 1 mmol/l phenylmethylsulfonyl fluoride, 2 µmol/l leupeptin, 1 µmol/l pepstatin, 1 µmol/l aprotinin, 10 mmol/l NaF, and 100 µmol/l dephostatin] as total cellular homogenates. After that, homogenates were centrifuged at 14,000 *g* for 15 mins, and the resulting supernatants were collected as cytosolic fractions. Pellets were incubated in a lysis buffer (*buffer A* + 1% Nonidet P-40) for 4 hrs, centrifuged, and the resulting supernatants used as membranous fractions. The protein content in the homogenates, cytosolic, and membranous fractions was determined using the Bradford technique (Bio-Rad).

Western Immunoblotting

The expression of proteins was assessed using standard SDS-PAGE Western immunoblotting techniques as previously published methods (90-92). Briefly, 70 µg of cytosolic proteins or membranous proteins were electrophoresed on a denaturing SDS gel for 2 to 3 hrs. After electrophoresis, the proteins on the gel were electrophoretically transferred to nitrocellulose membranes (Bio-Rad) overnight at 4°C. Gel transfer efficiency was carefully recorded by making

photocopies of membranes dyed with reversible Ponceau staining; gel retention was determined by Coomassie blue staining as previously described. The membranes were incubated in 5% nonfat dry milk in TBST buffer (10 mmol/L Tris-HCl [pH 7.2], 0.15 mol/L NaCl, and 0.05% Tween-20) followed by incubation with specific primary antibodies (Santa Cruz Biotechnology, Inc). After extensive rinsing with TBST buffer, blots were incubated with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies and developed with the use of an enhanced chemiluminescence system (ECL kit, Amersham). After washing with TBST buffer, blots were air-dried and exposed to Kodak films in x-ray cassettes with intensifying screens. The signals detected by immunoblotting and the corresponding records of Ponceau stains of nitrocellulose membranes were quantitated using an image scanning densitometer (Personal PI, Molecular Dynamics). Anti-Bax, and anti-p53 antibodies were purchased from Santa Cruz Biotechnology, Inc.

Statistical Analysis

Data are reported as means $\pm$ SEM as previously described methods (90-92). Measurements were analyzed by ANOVA followed by unpaired Student's *t* tests with the Bonferroni correction. In all Western blot analyses, the content of the specific protein of interest was expressed as a percentage of the corresponding protein in the anterior LV wall of NTg vehicle-treated mice.

Figure 16: Western blots of Bax (Panel A) and p53 (Panel B) protein at 2 wks after DOX/saline treatment in NTg mice compared with I κ B Tg mice. Bar graph shows densitometric quantification from 5 individual hearts per group. Data are mean $\pm$ SEM.

RESULTS

Attenuation of Bax and p53-mediated apoptosis by cardiac specific inhibition of NF- κ B

As shown in Figure 16A, the cardiac protein expression of the proapoptotic protein Bax was significantly increased in DOX-treated NTg mice when compared with healthy sham controls. Cardiac specific inhibition of NF- κ B led to a suppression of DOX-induced Bax over expression. Furthermore, we examined the changes in protein expression of another proapoptotic factor p53 in NTg mice and I κ B Tg mice with or without DOX treatment. Protein expression of p53 was significantly increased after DOX administration in NTg mice. Although the upregulation of p53 was partially prevented by I κ B Tg mice, the value did not reach statistical significance (Figure 16B).

CONCLUSIONS

In the present study, we determined a significant induction of myocardial apoptosis following DOX administration in mice, which correlated with abnormal expression and translation of proapoptotic proteins Bax and p53. Our results showed that cardiac specific inhibition of NF- κ B protects myocardium from apoptosis by inhibiting NF- κ B activation in DOX-induced cardiomyopathy, and further regulating expression, and translation of apoptosis-related genes Bax and p53. As it is well documented that apoptosis may contribute to cardiomyocyte loss and structural changes accompanied by up-regulation of the proapoptotic Bcl-2 family member Bax (95), we examined this protein in mice after DOX

therapy and found a DOX-mediated increase in the cardiac protein expression of Bax which was reduced by cardiac specific inhibition of NF- κ B. Furthermore, DOX-induced apoptosis in cardiomyocytes and endothelial cells is more likely attributable to H₂O₂ production (44, 45), and there is increasing evidence connecting p53 to intracellular oxidant formation (96, 97). Also, activation of p53 may directly induce activation of Bax gene, which contains a p53 binding site. Since both superoxide and H₂O₂ induce p53, we conducted further studies to detect the protein expression of p53 in DOX-treated hearts. Although the precise mechanisms of DOX-induced cardiotoxicity remain elusive, there is increasing evidence that DOX exposure can trigger cardiomyocyte apoptosis, and that this type of cell death represents the predominant form of myocyte damage seen in this setting (98). These results suggest that DOX induces myocardial apoptosis through activation of NF- κ B in mice and NF- κ B activation requires I κ B α degradation. Therefore, regulation and control of NF- κ B activation may be a powerful therapeutic strategy for reducing DOX-induced myocardial apoptosis.

CHAPTER VII

SUMMARY AND FUTURE DIRECTIONS

Summary of Findings

In this study, we have shown for the first time that NF- κ B contributes to systolic and diastolic cardiac dysfunction, adverse ventricular remodeling, cardiac inflammation and apoptosis in experimental DOX-induced cardiomyopathy. In agreement with other investigators, WT and NTg mice treated with DOX were found to display severe systolic and diastolic LV dysfunction, resulting in impaired cardiac function as assessed by echocardiography and invasive hemodynamics (99-101). In I κ B Tg mice treated with DOX, global LV function as indexed by EF, LVSP, LVEDP, and $\pm$ dP/dt, was improved as a result of enhanced systolic and diastolic performance. In the present study, I κ B Tg mice also showed lower production of proinflammatory cytokines and less activation of proapoptotic proteins after DOX than did WT and NTg mice. Consequently, survival rate was higher in I κ B Tg mice compared with WT and NTg mice. This study provides direct evidence of the involvement of NF- κ B mediated signaling pathways in DOX-induced cardiomyopathy. One possible mechanism for

such a protective effect of NF- κ B inhibition is that I κ B α expression would directly inhibit NF- κ B activation, resulting in attenuation of the inflammatory response and subsequent myocardial damage, because the expression of many cytokines, including TNF- α , IL-6, and iNOS, is regulated by NF- κ B activation. These cytokines are not constitutively expressed in the normal heart. Upregulation and production of these cytokines represent an intrinsic or innate stress response against myocardial injury (102, 103). In this investigation, we found that TNF- α , IL-6, and iNOS levels decreased noticeably in I κ B Tg mice compared with WT and NTg mice, further indicating that I κ B α overexpression attenuates inflammatory response mediated by DOX-induced cardiomyopathy.

As inflammation might also be associated with induction of apoptosis and as TNF- α induces increase in reactive oxygen species production and oxidative stress in cardiomyocytes and lead to apoptosis (49, 104), we further investigated the expression of proapoptotic proteins Bax and p53 in mice hearts after DOX therapy. Since cardiomyocytes are terminally differentiated cells, any loss of these cells due to cell death can have critical functional consequences (105). In agreement with *in vitro* and *in vivo* studies using myocytes, we also found a marked up-regulation of the pro-apoptotic protein p53 14 days after DOX administration in our model (106, 107). This effect was blunted in I κ B Tg mice, suggesting that NF- κ B contributes to pro-apoptotic activation in DOX-induced cardiomyopathy. Furthermore, to investigate possible mechanisms, which may lead to attenuated apoptosis in DOX-treated I κ B Tg mice, we measured the expression of protein Bax, which is a pivotal regulator of mitochondrial apoptosis.

The Bax content of I κ B Tg mice were significantly decreased when compared to WT and NTg mice suggesting protection from apoptosis due to NF- κ B deficiency. In summary, the effects of DOX-induced cardiomyopathy included cardiac dysfunction, detrimental cardiac remodeling, inflammatory response, and apoptosis leading to LV dysfunction. NF- κ B deficiency attenuated these mechanisms known to be critical for the development of DOX-induced heart failure. Our data demonstrate a relevant role of NF- κ B in the development of this disease under experimental conditions. We have shown the preservation of cardiac function, increase in survival rate, and attenuation of myocardial apoptosis after DOX in I κ B Tg mice. These findings strongly implicate that DOX induces myocardial apoptosis through activation of NF- κ B in mice and NF- κ B activation require I κ B α degradation.

Future Directions

In clinical settings, the typical time course of DOX-induced cardiac toxicity is over years after multiple low dose treatments. As such, these temporal differences as well as other differences between rodents and humans do not allow our results to be directly extrapolated to the clinical arena (52, 108, 109). However, further clinical trials and basic research will have to verify this concept and furthermore, it needs to be elucidated whether or not NF- κ B inhibition counteracts with the oncological effect of DOX. In these lines, we need better understanding of the molecular mechanisms underlying DOX-induced cardiac toxicity. Aside from mechanistic aspects, the present investigations suggest the cardioprotective role

of NF- κ B inhibition in DOX-induced cardiomyopathy, which may be of therapeutic benefit for patients receiving DOX. Thus, cardiac specific NF- κ B inhibition might constitute a potentially new therapeutic option to prevent DOX-induced cardiotoxicity.

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Invited Presentations in International Meetings:

- 1) **American College of Cardiology 59th Annual Scientific Sessions (Cardiac Function and Heart Failure).** Therapy with Adult Bone Marrow-derived Mesenchymal Stem Cells Ameliorates Doxorubicin-induced Cardiomyopathy, March 15 2010, Atlanta, Georgia, USA.

- 2) **American College of Cardiology 58th Annual Scientific Sessions (Cardiac Function and Heart Failure).** Genetic Deletion of IL-6 Attenuates Pressure Overload-Induced Left Ventricular Hypertrophy and Dysfunction, March 29 2009, Orlando, Florida, USA.
- 3) **International Society for Heart Research (ISHR) annual meeting.** Atorvastatin Preserves Left Ventricular Function and Ameliorates Adverse Remodeling after Myocardial Infarction. XXXth Annual Meeting of the ISHR, American Section, June 17-20, 2008, Cincinnati, OH, USA.
- 4) **International Society for Heart Research (ISHR) annual meeting.** Cardiac-Specific Transgenic Inhibition of NF- κ B Protects Against Pressure Overload-Induced Left Ventricular Hypertrophy and Systolic Dysfunction. XXXth Annual Meeting of the ISHR, American Section, June 17-20, 2008, Cincinnati, OH, USA.
- 5) **International Academy of Cardiology, 13th World Congress on Heart Disease Annual Scientific Sessions.** Beneficial Effects of Sarpogrelate (an antiplatelet agent) in Congestive Heart Failure, Invited Lecture, July 29, 2007, Vancouver, Canada.
- 6) **American College of Cardiology 56th Annual Scientific Sessions (Myocardial Function and Heart Failure: Basic Novel Therapies).** Antiplatelet Therapy Attenuates Subcellular Cardiac Remodeling by Modifying Ca^{2+} Dynamics in Ischemic Heart Failure, March 24-27, 2007, New Orleans, LA, USA.
- 7) **American Heart Association (AHA) Scientific Sessions.** The Beneficial Effects of Cytokine Combinations are sustained during Long-term Follow-up. International Meeting of American Heart Association, November 2006, Chicago, USA.
- 8) **International Academy of Cardiovascular Sciences, Progress in Molecular and Cellular Cardiology and Cardiac Surgery.** Antiplatelet Therapy Attenuates Ventricular Remodeling. Global

Conference on Heart Health and Disease, Global Conference on Heart Health and Disease, October 15, 2006, Winnipeg, Canada.

- 9) **Canadian Cardiovascular Congress.** Effects of Phosphodiesterase-III Inhibition on Left Ventricular Remodeling and Mortality in Chronic Heart Failure. International Meeting of Canadian Cardiovascular Society, October 2005, Montreal, Canada.
- 10) **Young Investigator's Forum.** Serotonin Antagonism Improves Cardiac Performance and Reduces QTc Prolongation in Heart Failure. YiForum, April 2005. Winnipeg, Canada.
- 11) **Young Investigator's Forum.** Mechanisms of Isoproterenol Induced Increase in Intracellular Calcium in Isolated Cardiomyocytes. YiForum, May 2004, Winnipeg, Canada.

Research Expertise:

➤ *Experimental assessment of cardiac function:*

- 1) Echocardiographic imaging in rats, mice and Pigs: 2D-mode, M-mode and Color Doppler.
- 2) In vivo hemodynamic measurements by inserting Millar's catheter through carotid arteries in rats.

➤ *Animal models of heart disease:*

- 1) Aortic banding in mice to induce pressure-overload induced heart failure.
- 2) Doxorubicin model of dilated cardiomyopathy.
- 3) Induction of non-ischemic heart failure in rats by inducing autoimmune myocarditis.
- 4) Rat model of myocardial infarction: by permanently occluding the left anterior descending coronary artery.

➤ *Molecular Techniques:*

- 1) Isolation of myofibrils from the heart tissue.

- 2) Estimation of myofibrillar ATPase activities and myofibrillar protein content.
- 3) m-RNA isolation and Northern blotting.
- 4) Myosin heavy chain isoforms, Myosin light chain phosphorylation and Troponin-I phosphorylation.
- 5) Isolation of sarcoplasmic reticulum and estimation of sarcoplasmic reticular calcium uptake and release activities.

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- 1) **Sanganalmath SK**, Babick AP, Barta J, Kumamoto H, Takeda N, Dhalla NS. Antiplatelet therapy attenuates subcellular remodelling in congestive heart failure. *J Cell Mol Med.* 2008;12:1728-1738.
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