GILZ: A Novel Glucocorticoid Induced Cytoprotective Protein in Cardiomyocytes

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A Dissertation Submitted to the Faculty of the

Department of Medical Pharmacology
In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2012

APPROVAL PAGE

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ACKNOWLEDGEMENTS

First and foremost I would like to acknowledge my advisor Dr. Qin Chen. Without her continued guidance and patience the completion of this dissertation would not be possible. I would also like to acknowledge my committee members: Dr. Doug Larson, Dr. Todd Vanderah, Dr. John Bloom and Dr. Georg Wondrak. Their insight and expertise have provided me training in critical thinking, experimental design and analysis that I am looking forward to apply in my future scientific endeavors. I would also like to acknowledge my lab members Dr. Thai Dinh, Dr. Jack Zhang, Dr. Beibei Xu, Dr. Lena Sheveleva, Dr. Sang (General) Lee, Dr. Kyle Kappeler and Josh Strom. I have been extremely lucky to have had the opportunity to work with skilled scientists throughout my graduate training. Moreover, my lab mates provided me a family that has supported me throughout my graduate career. I would like to give special thanks to Dr. Kyle Kappeler, Josh Strom, and Dr. Mark Stevens (NIH). They have been extremely helpful during the editing and revision process of my dissertation.

DEDICATION

I dedicate this dissertation to my mother Christina Anna Wells, who supported me from day 1 and has been my rock to lean on throughout the entire process

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ABSTRACT

Glucocorticoids (GCs) are frequently prescribed pharmacological agents most notably for their immunosuppressant effects. Endogenous GCs mediate biological processes such as energy metabolism and tissue development. At the cellular level, GCs bind to the Glucocorticoid Receptor (GR), a cytosolic receptor that translocates to the nuclei upon ligand binding and alters gene transcription. Among a long list of genes activated by GCs is the Glucocorticoid Induced Leucine Zipper (GILZ). Although GC induced GILZ expression has been well established in lymphocytes, little is known whether cardiomyocytes respond to GCs by inducing GILZ. Unlike lymphocytes, in which GCs induce apoptosis and GILZ mediates GC induced apoptosis, cardiomyocytes respond to GCs by gaining resistance against apoptosis. We determined GILZ expression pattern in cardiomyocytes *in vivo* and *in vitro*. Our data demonstrate GILZ induction in cardiomyocytes both *in vivo* and *in vitro* by GCs and point to H9C2 cell line as a valid model for studying the biological function of GILZ in cardiomyocytes. I have also determined GILZ functions as GC induced cytoprotective protein against the known cardiac toxicant Doxorubicin. Finally I have determined GILZ stabilizes Bcl-xL pro-survival protein, providing a possible mechanism of cytoprotection in cardiomyocytes.

CHAPTER 1: INTRODUCTION

1.1 Heart Disease and Doxorubicin Cardiotoxicity.

Heart disease is the leading cause of death in the United States and it has been estimated that 935,000 heart attacks occur annually. In fact, every 25 seconds, an American will experience a coronary event [1, 2]. According to the Center for Disease Control and Prevention, the total cost of cardiovascular disease in 2010 was estimated to be $444 billion dollars in the United States.

Although many studies have been conducted concerning cardiovascular disease, questions remain regarding the mechanisms of myocardial injury. Experimental evidence suggests that oxidative stress and apoptotic cell death are associated with cardiac dysfunction. Increased levels of hydroxyl radicals generated from superoxide has been reported in the failing hearts [3]. Therefore, it is important to understand how the heart regulates the different mechanisms to combat oxidative stress and the subsequent loss of cardiomyocytes. In addition, it is essential for the identification of pharmacologic agents targeting such pathways that will prevent myocardial injury consequential to cardiac stress. Drug-induced cardiomyopathy is a major problem for patients who rely on medications that cause cardiotoxicity as a side effect. For example, certain chemotherapeutic agents can improve and extend the quality of life of cancer

patients, however they can act as a double edged sword and kill healthy cells. As a result, disease states such as cardiomyopathy and ultimately heart failure can occur. The anthracyclin chemotherapeutic agent, Doxorubicin, is among these agents inducing cardiomyopathy.

1.2 The Anthracycline Antibiotic Doxorubicin, and Cardiotoxicity

Anthracyclines are a class of chemotherapeutic agents known to elicit cardiotoxicity [3]. One of these anthracyclines, Doxorubicin (Dox) is commonly used for treatment of hematological malignancies, carcinomas and soft tissue sarcomas [4]. Cancer patients treated with Dox have an increased survival rate; however, long-term survival may face the side effect of Dox-induced cardiotoxicity shown as dilated cardiomyopathy [4]. Anthracycline cardiotoxicity was observed in up to 20% of patients receiving the treatment [4]. Cardiac toxicity is dependent on cumulative dose of Dox, showing as fatal arrhythmia for acute toxicity or cardiomyopathy developed chronically [5, 6].

Acute cardiotoxicity by Dox can be identified through the various symptoms associated with cardiovascular diseases. These symptoms include hypotension, tachycardia, arrhythmia, and transient depression of left ventricular function [7]. At the molecular level cardiotoxicity, is associated with DNA damage, protein synthesis inhibition, myofiber degeneration and apoptosis. Free

radical induced mitochondrial damage is suggested to be a primary contributor to cell death [8]. Modifications of proteins, lipids and DNA as a result of oxidative stress have also been reported to contribute to early injury of cardiomyocytes [9] [10, 11].

Chronic cardiotoxicity is represented by the development of cardiomyopathy and ultimately congestive heart failure. Symptoms often mimic those of dilated cardiomyopathy and heart failure [12, 13]. Life threatening symptoms can occur weeks or months after treatment. About 30% of patients treated for carcinoma using Dox exhibited extreme hypotension, decreased QRS activity, cardiac dilatation and ventricular failure [14]. Unfortunately symptoms of chronic Dox toxicity can manifest in the absence of acute toxicity [15, 16]. Additionally, chronic toxicity is irreversible, whereas acute toxicity is "manageable" [17]. There is therefore a necessity to develop strategies for alleviating chronic Dox-cardiotoxicity.

Dox dosing regimens are strictly monitored because its cardiotoxicity is dependent on accumulative doses. Weekly regimens have been shown to decrease toxicity as compared to treatment of every three weeks [18, 19]. Doxorubicin is primarily administered via intravenous administration to patients. Dox is formulated as a sterile lyophilized orange powder for intravenous use and is dissolved in sterile water before injection. According to Daunorubicin HCL package insert distributed by Gensia Sicor Pharmaceuticals listed on pateintconsultants.com/druginserts/daunorubicin.pdf, the desired dose is then

drawn into a 0.9% NaCl solution and then injected into tubing with a rapidly flowing IV infusion of 0.9% NaCl and 5% dextrose or 0.9% NaCl alone. According to meds.com, the most commonly administered dose is 60-75mg/m^2 injected at 21-day intervals. When used in combination with other chemotherapeutic drugs, such as herceptin, the most common dose of Dox is 40 to 60 mg/m^2. Even with strict dosing regimens to lessen cardiac effects, Dox-induced cardiotoxicity remains a problem to some cancer patients.

Pharmacokinetics of Dox has been well studied and after intravenous dosing, Dox blood levels fall dramatically as the drug distributes into tissues. A slow elimination phase due to renal and biliary clearance follows tissue distribution [20]. Aldo-ketoreductases in the liver and other tissues reduce side chain carbonyl groups of Dox, leading to the formation of doxorubicinol [21]. Approximately 40% of the dose can be detected as doxorubicinol in the serum after only 30 minutes according to Daunorubicin HCl package insert as listed in patientconsultants.com. Additionally Daunorubicin HCl package insert states 25% of original dose is excreted in the urine and 40% via biliary excretion. Further metabolism of Dox can occur by reductive cleavage of the sugar moiety to form 7-hydroxy aglycone [22].

1.3 ROS Mediate Toxicity of Dox, a Closer Look

The generation of free radicals leading to increases in oxidative stress by Dox exposure has been suggested to contribute to cardiotoxicity [23]. Free radicals lead to disturbance of the energy-producing mitochondria, damage genomic DNA, lipid peroxidation and ultimately cause cell death [24]. Oxidative stress is caused by an imbalance between the production and detoxification of reactive oxygen species. Certain elements and compounds can lead to ROS production. Heavy metals such as iron and copper can produce hydroxyl radicals in the presence of H_2O_2 via Fenton reaction [25]. Modification of these trace metals contributes to coronary heart disease [26, 27]. Lipid peroxides are produced when hydroxyl radicals interact with cellular lipids. The result of lipid peroxidation is the disruption of membrane integrity and DNA damage [28]. In relation to the heart, high levels of lipid peroxide have been reported in diseased myocardium samples linking this form of oxidative damage to the damaged heart [29-32].

The cellular antioxidant system is also affected in damaged myocardium leading to further imbalances of ROS in heart muscle. Patients with chronic heart failure have reduced levels of the antioxidant, glutathione, in atrial samples suggesting that proper reduction-oxidation (redox) balance is necessary for maintaining cardiac homeostasis [33]. Glutaredoxin 2, an enzyme that repairs S-glutathionylation of proteins in the mitochondria, is also shown to protect against the myocardium from Dox toxicity in mice [34]. These and other studies have

undoubtedly linked Dox toxicity to the generation of free radicals, the resulting cellular damage and alterations in antioxidant enzyme activity.

We and others have shown doxorubicin induces apoptotic cell death in cardiomyocytes [35-37]. Dox-induced apoptosis may involve direct damage to DNA or destabilization of mitochondrial membrane integrity. Evidence for each of these mechanisms has been presented previously. For example, Dox has been reported to induce the formation of 8-oxo-7, 8-dihydro 2'-deoxyguanosine, an indicator of DNA damage caused by hydroxyl radical attacking to guanine base in DNA [38]. Patients in end stage heart failure presenting with idiopathic dilated cardiomyopathy were further reported to have increased DNA fragmentation, a common biomarker for apoptosis [39]. In mitochondria, Dox can alter membrane lipids, decreasing oxidative phosphorylation and allowing the release of cytochrome c, which triggers caspase activation and the cascade of apoptosis. In addition to these, Dox toxicity causes cytoskeleton remodeling characterized by a decrease in myofibrillar bundles, alteration of z-disc structure and depolymerization of actin filaments [40-42]. Damage caused by Dox may lead to ischemic states via decreased contractility.

Studies have shown that the anti-apoptotic Bcl family member, Bcl-xL, prevents Dox induced apoptosis by maintaining the integrity of the mitochondrial membrane [107]. Bcl-xL normally resides on the mitochondrial membrane and helps to maintain mitochondrial integrity [43]. Bcl family proteins' role in apoptosis is discussed in further detail in section 1.8.

1.4 Glucocorticoids as a Physiological and Pharmacological Agent

Glucocorticoids (GCs) are stress response hormones secreted by the adrenal glands and help to maintain biochemical homeostasis during stress. Cortisol, also known as hydrocortisone, is the major GC in humans, whereas corticosterone is the primary GC in rodents. GCs are synthesized from the precursor cholesterol in the Zona Fasciculate of the adrenal cortex. The Cytochrome P450 isoform 11A (CYP11A) converts cholesterol to pregnenolone as the initial step of GC synthesis. This rate-limiting step is controlled by adrenocorticotropic hormone (ACTH) released from the anterior pituitary. Cortisol is produced from 17-αhydroxyprogesterone, whereas11-doxycorticosterone is the precursor for corticosterone. Furthermore enzymatic activation of 11β-dehydrogenases (11β-HSD) converts inactive cortisone to the biologically active cortisol. GC levels vary throughout the day and follow a diurnal rhythm.

Serum GC levels vary with changes in ACTH levels. ACTH is released from the anterior pituitary when stressful events cause the hypothalamus to release corticotrophin releasing hormone (CRH). CRH then stimulates the anterior pituitary to release ACTH. When ACTH is released, it circulates to the adrenal cortex where it binds to its receptor in the plasma membrane of cells in the Zona Fasciculate and Reticularis. As a result adenyl cyclase is activated, which causes the level of cAMP to increase. Increases in cAMP in these cells

result in the activation of enzymes leading to the biosynthesis of cortisol from cholesterol.

A variety of factors such as clinical depression, physiologic stress, illness, fever, trauma, surgery, fear, pain, physical exertion or extreme temperatures increase ACTH levels. Transient stress can increase serum GC levels significantly as early as in 30 minutes [44]. Secretion of cortisol can be suppressed by negative feedback regulation. High levels of cortisol in the serum inhibit the release of CRH from the hypothalamus. The highest and most frequent release of cortisol is in the morning, and lowest in the night [45]. This timing correlates to levels of stress one typically experiences throughout a normal work day.

GCs have a wide range of functions including the regulation of energy metabolism, inflammation, development and immune function. Early studies described GC's role in maintaining normal concentrations of blood glucose. Cortisol stimulates gluconeogenesis, mobilizes amino acids from extra hepatic tissues, and stimulates fat metabolism in adipose tissue, which are important for the maintenance of homeostasis in all living systems. GCs can also inhibit glucose uptake in muscle and adipose tissue and increase overall protein and fat metabolism. Therefore, it is critical for living systems to tightly regulate the balance of GCs.

Excessive GC levels can be reached by consumption as a therapeutic agent or by individuals whom suffer from hyperadrenocorticism. GCs have been

implicated in several diseases in humans, including Cushing's and Addison's diseases. Cushing's syndrome is a rare disorder characterized by over-production or over-exposure to glucocorticoids. Symptoms of Cushing's syndrome include depression, abnormal fat deposit, muscle wasting and hypertension [46]. Addison's disease is characterized by an insufficient amount of glucocorticoid production. Symptoms of Addison's disease include lethargy, decreased cardiac function, stomach irritability and darkening of the skin [47]. Glucocorticoid-related syndromes exhibit systemic effects on the body. The central nervous system, body fat, skin and heart are all affected in these diseases. Thus, the GC system has become an important pharmacological target in the treatment of such disorders.

Glucocorticoids (GCs) are a mainstay in the treatment of inflammation and immune suppression. Dr. Phillip Hench pioneered the work describing what eventually became to be known as compound "E" isolated from adrenal glands of animals. In 1948, the first human patient was administered compound "E". The patient, a 29 year old female, was immobile due to erosive arthritis. After four days of administration she was able to walk out of the hospital. Dr. Hench won the Nobel Prize in Medicine in 1950 for his work. In current medicine, a large number of synthetic GCs are available for treatment with designated short, intermediate or long half life. A short acting GC is cortisone. Intermediate acting includes prednisone, prednisolone, methylprednisolone and triamcinolone. Long acting GCs includes paramethasone, betamethasone and dexamethasone. GCs

that are used as therapies can be given orally, i.v., i.m., s.c., or topically. Pharmacokinetics of GCs determines their effectiveness. The majority of circulating GC is bound to plasma proteins with only about 10% free to interact with target tissues. Free steroids are lipid-soluble and can diffuse through the cell membranes where they bind to its receptor to exert effects.

1.5 Glucocorticoid Receptor

Glucocorticoids interact with an intracellular nuclear receptor protein termed the Glucocorticoid Receptor (GR). The activation of GR has a direct or indirect transcriptional effect on 5-20% of the human genome [48]. The glucocorticoid receptor is ubiquitously expressed in almost all human tissues.

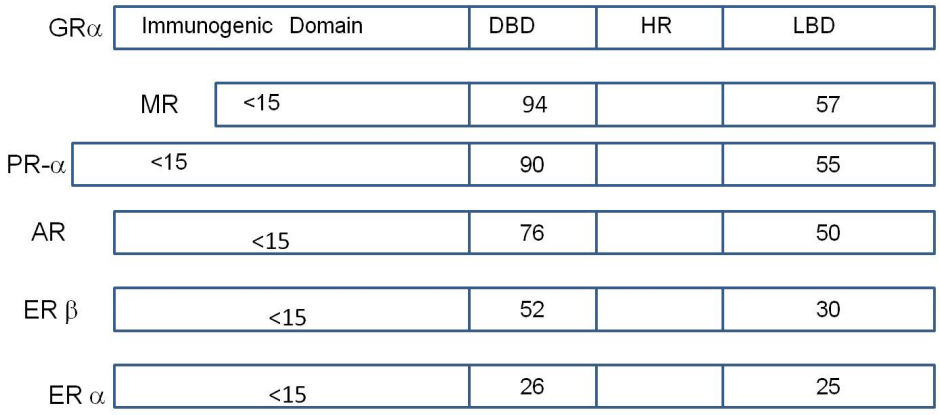
GR is a member of the steroid hormone receptor subfamily of nuclear receptors. Six evolutionary related receptors exist in the family: alpha and beta estrogen receptors (ERα and ERβ), androgen receptors (ARα and ARβ), progestin receptors (PRα and PRβ), glucocorticoid receptors (GRα and GRβ), and mineralocorticoid receptors (MRα and MRβ). All these receptors share sequence homology. The N-terminal domain of GR encodes an immunogenic domain, which has about 15% sequence homology between the nuclear receptor families. In addition, the DNA binding domain, a hinge region and a ligand binding domain are also conserved within this receptor family (Figure 1). Human GR gene can undergo alternative splicing, resulting in two isoforms, alpha (97 kDa) and beta

(94 kDa). The transcriptionally inactive GRβ heterodimerizes with the alpha isoform to prevent GC binding. Therefore, GRβ acts as an inhibitor of GRα activity [49]. However, GRα possess two zinc finger domains that have been shown to interact directly with promoter sequences of the Glucocorticoid Responsive Elements (GRE) [50, 51]. GRE is composed of an inverted hexameric palindromic sequence separated by three base pairs. The sequence reads PuGNACANNNTGTNCPy, with each GR binding to one of the palindromes [52].

In the cytosol, GR interacts with cytoplasmic chaperone Heat Shock Protein 90 (Hsp 90). The dissociation of GR from Hsp90 is critical for GR ligand binding and translocating to the nucleus [50, 53, 54]. After ligand is bound, GR associates with importin and translocates to the nucleus because of the presence of nuclear translocation signals on GRα, NL1 and NL2. Upon DNA binding, activated GRα recruits initiation complexes of transcription including RNA polymerase II [55]. Once transcription of target genes is complete, GR dissociates from the ligand and forms a heterocomplex with heat shock proteins to be shuttled out of the nucleus [56-58]. While in the nucleus, however, GR can serve an inhibitory role via its interaction with transcription factors other than GR.

Figure 1

Sequence Homology of Nuclear Receptors



Comparison of steroid nuclear receptor family. Numbers indicate percentages correlating to homology as compared to GRα. The domains represented are the immunogenic domain, DNA binding domain (DBD), hinge region (HR), and ligand binding domain (LBD).

Adapted from http://www.endotext.org/adrenal/adrenal6/adrenalframe6.htm

The significance of GR's promiscuity in binding other transcription factors was illustrated in transgenic mice harboring a mutant GRα incapable of binding to DNA. The mutant retained the ability to homodimerize and heterodimerize with other transcription factors such as NFκB, AP-1 and STATs [59-61]. Mice were able to survive and reproduce as opposed to null mice containing a deletion for the entire GR gene that died immediately after birth [62, 63]. The experiment suggests that GR's ability to binding its partners is necessary for survival, independent of its ability to induce transcription of target genes in the nucleus. The transrepression caused by GR heterodimerization is thought to be important for suppression of inflammatory and immune response genes.

The biology of GC-GR signaling in humans has also demonstrated its importance as a pharmacological target. People with a haplotype of GR consisting of the polymorphism 9βA/G (haplotype 3) are prone to heart failure due to low grade inflammation [64]. In these respects, GCs and GR have been described to contribute to a number of cellular processes, including cell survival and apoptosis, in a tissue specific manner.

1.6 Role of Glucocorticoids in Apoptosis

Glucocorticoids have been described to be both pro-survival and pro-apoptotic depending on cell type [65]. For example, in lymphocytes, GCs

increase T cell apoptosis by inhibiting the expression of T cell growth factor and IL-2 genes [66]. GCs also promote apoptosis of eosinophils by suppressing the expression of the growth factors, cytokines and their receptors essential for maturation and survival. In lymphocytes, GC-induced apoptosis involves increased expression of pro-apoptotic Bcl-2 family members such as Bad [67], as well as a decrease in pro-survival family members such as Bcl-xL and Bcl-2 [68]. Interestingly, GCs protect against apoptosis in endometrium, ovarian follicle, hepatocytes and fibroblasts [69]. In lung cancer cells, dexamethasone (Dex) inhibits the toxicity of the chemotherapeutic drugs cisplatin and gemcitabine. In cultured rat cardiomyocytes, corticosterone is capable of preventing Dox from inducing apoptosis [107].

The effects of GCs on the cardiovascular system have not been widely studied. Our group has reported that GC pre-conditioning is protective in a myocardial infarction mouse model [70]. Moreover a synthetic GC, methylprednisolone, protected feline hearts against myocardial ischemia [71]. Although a potential for cytoprotection has been reported in the heart, long term administration of GCs has proven to be detrimental [72]. Long term elevated GCs have been linked to atherosclerosis, hyperglycemia, hypertension, and dyslipidemia. However it remains important to elucidate the mechanism of acute cytoprotective effects elicited by GCs in cardiomyocytes. This allows us to gain a better understanding of specific pathways that can be targeted during the design

of novel therapeutics, and potentially circumvent the more broad effects elicited by long term GC treatment.

1.7 Glucocorticoid Induced Leucine Zipper (GILZ) and its Role in Apoptosis

Several studies have been designed to address the mechanism of GC induced apoptosis [35, 58, 68]. One such study identified that Glucocorticoid Induced Leucine Zipper (GILZ) mRNA was significantly up regulated in cells preconditioned with GC [73]. This study identified GILZ as a novel dexamethasone induced leucine zipper protein mediating TCR/CD3 induced cell death in T lymphocytes [73]. The biological importance of GILZ includes the modulation of T-lymphocyte activation, regulation of T-helper (Th) cell differentiation and dendritic cell (DC) function, IL-2 production, apoptosis and cell proliferation [74-76]. In addition, GILZ has been shown to increase Na^+ transport via the epithelial Na^+ channel (ENAC) in kidneys [77, 78]. GILZ has also been shown to mediate carcinogenesis by inhibiting Ras-driven tumorigenisis [76].

GILZ is a member of the TGFβ- stimulated clone 22 (TSC-22) family of leucine zipper proteins, therefore also named as TSC22D. Other members of the family include TSC22, THG-1, KIAA0669, DIP, shc and *Drosophila* bunched gene [79]. Several isoforms and splice variants have been characterized and named to GILZ 1-4, according to their order of discovery [80].

GILZ, a 135 amino acid protein, has many domains that have been previously characterized. GILZ is composed of three distinct domains: the N-terminal domain, leucine zipper (LZ), and a C-terminal domain containing a proline and glutamic acid rich region (PER). Because of the internal LZ domain, it was thought originally that GILZ acts as a transcription factor. However, the lack of a canonical DNA binding domain quickly ruled out a role as a transcription factor [65]. Although the LZ domain did facilitate transcriptional regulation, it served as a site for homodimerizing and heterodimerizing with other LZ proteins (29). In addition, GILZ can interact with proteins that lack a LZ domain. The N-terminal domain contains a tuberous sclerosis complex box (TSC-box), allowing protein-protein interaction.

Molecular targets of GILZ include c-Fos, NF-κB, Raf-1 and Ras[81]. The first 29 amino acids of the proline rich region in GILZ interacts with p65 and p52 subunits of NF-κB in T-Cells, preventing nuclear translocation and transactivation of NF-κB (30). The TSC box of GILZ is critical for the interaction with Ras and Raf-1, resulting in blockage of MAP-kinase/ERK-kinase phosphorylation and AP-1 dependent transcription (68). Since GILZ transcription can be turned by AP-1 transcription factor, there is a feedback mechanism regulating GILZ expression.

The regulation of GILZ has been extensively described. The promoter region of GILZ gene contains six GREs and putative binding sites for STAT6, nucleofactor of activated T cell (NFAT), Oct-1, c-myc, Forkhead transcription factor, cyclic AMP response element binding protein (CREB), and estrogen [82,

83]. GILZ is constitutively expressed in various murine and human tissues [73, 84]. GCs induce expression of GILZ rapidly in several cell types including T cells [85], human airway epithelial cells [11, 85], human lens epithelial cells [10] and mesenchymal stem cells [14, 86, 87]. In addition, GILZ has been reported to be expressed in the absence of GCs. IL-2 deprivation in T lymphocytes [82] as well as vasopressin and aldosterone administration in kidney epithelial cells have been reported to induce the expression of GILZ [78]. IL-10 stimulation induces GILZ in murine macrophage and endothelial cells [88, 89]. Down regulation of GILZ has been reported in human airway epithelial cells by inflammatory cytokines, e.g. IL-1, TNF-α, and IFN-γ [90]. Down regulation of GILZ has also been reported by anti-CD3 activation in T lymphocytes. In MCF-7 breast cancer cells the down regulation of GILZ has been reported by estrogen stimulation [83].

GILZ, much like GC treatment, can elicit tissue specific effects and play different roles in cell survival or cell death depending on cell type and the context [81]. GCs potential to attenuate or induce apoptosis in a tissue specific manner combined with the tissue specific regulation of Bcl-2 family members by GC begs the question as to which additional proteins may contribute cell survival or apoptosis. Our lab has focused on understanding how GC regulates cell survival in the heart. As mentioned previously GILZ appears to mediate apoptosis in lymphocytes. Interestingly transgenic mice overexpressing GILZ in T cell lineage cells were reported to inhibit the expression of Bcl-xL and augment apoptosis [75]. It has been reported that NF$-\kappa$B directly activates transcription of Bcl-

xL[91]. Therefore a potential explanation for the down-regulation of Bcl-xL in transgenic mice overexpressing GILZ in a T-cell lineage could be GILZ ability to bind and inhibit NF-κB. Tissue specific effects of GC in mediating changes in apoptosis have been reported in the thymus and ovaries. GCs are pro-apoptotic in the thymus and anti-apoptotic in the ovaries [92]. It is reasonable to predict molecules under the control of GC, such as GILZ, may exhibit distinct roles depending on the context of tissues.

1.8. Bcl-2 Family

Unraveling genetic code from *Caenorhabditiselegans* (C. Elegans) revealed a unique set of proteins able to regulate programmed cell death. Ced-9 and the mammalian Bcl-2 were found to be structural homologs [93]. Ced-3 belongs to a family of proteases now known as caspases, which play a role in the sequential activation and cleavage of proteins that ultimately leads to dismantling the cell [94]. Ced-4 is the homolog of Apaf1 (Apoptotic Protease Factor 1), which upon binding to cytochrome C and ATP forms an oligomeric apoptosome. The apoptosome binds and cleaves caspase 9 and initiates the caspase cascade ultimately leading to cell death. Ced-3 and Ced-4 were essential for programmed cell death during development and Ced-9 was able to inhibit their function [95].

About 20 years ago, McDonnel *et al*. made the observation that overexpression of Bcl-2 caused polyclonal expansion of lymphocytes, which

resulted in carcinogenesis [96]. Bcl-2 family members can be pro-apoptotic or pro-survival. Membrane permeabilizing family members such as Bax and Bak are potent inducers of programmed cell death. Anti-apoptotic members such as Bcl-2, Bcl-xL, and Mcl-1 help maintain mitochondrial membrane integrity and prevent the release of Cytochrome C. Protein levels of Bcl family members have been reported to be regulated in response to stress via transcription, protein turnover, post translational modifications, changes in binding partners and subcellular localization [97]. A balance of pro-survival to other family members is necessary for homeostasis involving cell survival.

Bcl-2 family members are involved in maintaining the integrity of the mitochondrial outer membrane. BAX and BAK, pro-apoptotic proteins, disrupt mitochondrial membrane in a process called mitochondrial outer membrane permeabilization (MOMP). When this occurs, proteins are released from the intermembrane space of mitochondria and gain access to the cytosol. This permeabilization can generate holes in the order of 25-100 nm in diameter [98]. The mechanism as to how BAX and BAK cause such permeabilization is unclear. Inactive structures of BAX and BAK have been determined; however, the active structure remains unsolved. Activation and oligomerization are assumed to be responsible for MOMP [99-101]. This subsequently leads to apoptosis via activation of caspases and cysteine proteases [102]. There have been reports of cells recovering from MOMP if sufficient glycolysis is maintained [103]. The anti-apoptotic BCL-2 family members Bcl-xL, BCL-W, A1/Bfl 1 and MCL-1 can block

MOMP and inhibit permeabilization of BAX and BAK. BH3 family members provide further checkpoints in survival by binding to and inhibiting anti-apoptotic proteins with differing efficiency [104, 105]. Interestingly, some BH3 members: BID, BIM and PUMA have also been shown to activate pro-apoptotic effectors [106, 107].

Identifying underlying mechanisms of Bcl family member protein stability and degradation will provide an insight as to how they are maintained in the cell. For example, BAX can be inhibited by the function of peptidyl-prolineisomerase PIN1 [108]. BAX is targeted by the E3 ligase IBRDC2 for ubiquitination and degradation [109]. BAX b, a splice variant of BAX, is constitutively expressed in the brain; however, it is rapidly degraded by the proteasome [110]. MCL 1 is the shortest lived anti-apoptotic proteins. Protein levels of MCL-1 are mediated by ubiquitin dependent and independent mechanisms [97].

Bcl-xL protein stability is directly regulated by proteasome degradation and enzymatic cleavage. Skin cells damaged by UVB irradiation were shown to have a significant drop in Bcl-xL protein levels [103]. The addition of MG132 proteasome inhibitor was able to partially restore Bcl-xL protein levels in skin cells after UVB treatment. This implied that Bcl-xL was regulated by proteasome degradation [111]. Bcl-xL was reported to be cleaved by caspase 3-like proteases [112, 113]. Additionally, the inhibition of the cysteine protease calpain was shown to increase protein levels of Bcl-xL[114].

The binding of Bcl family members to each other offers a powerful level of regulation, as is in the case of the BH3 only family member PUMA. PUMA is a BH3 only protein that binds to and inhibits all of the anti-apoptotic proteins [104, 115]. Puma, a transcriptional target of p53 and FOXO3a, has been shown to activate BAX and BAK directly [97]. PUMA deletion is a common genetic abnormality that correlates with a wide range of tumor formation. However, genetic ablation of PUMA did not cause spontaneous oncogenesis in mice and actually prolonged the time course for formation of tumors [116], this finding was contrary to what was expected based on the observation from human genetics. Furthermore, mice lacking PUMA exposed to radiation were resistant to radiation induced transformation [97]. PUMA reinforces how much work has yet to be done on understanding the many facets of the Bcl-2 family members and their role in cell survival.

We have previously reported GCs induce cytoprotection in cardiomyocytes. This protection is, in part, attributed to increases of the pro-survival protein Bcl-xL [35]. Elucidating the exact mechanism(s) by which GCs alter protein levels of Bcl-xL will allow us to understand how GCs elicit their cytoprotective effects and provide insight as to design potential therapeutic regimens to protect patients at risk of cardiac failure.

Bcl-2 family members have been previously reported to play a significant role in GC mediated changes in apoptosis [35]. GCs have been shown to directly regulate the transcription of Bcl-2 family proteins [92]. In thymocytes,

anti-apoptotic Bcl-2 family members are down regulated while pro-apoptotic family members are up-regulated in the presence of GCs [117]. In cells where GCs have been described to contribute to cytoprotection, increases of pro-survival Bcl-2 and Bcl-xL were reported [69]. For example, survival in serum starved endometrial cells was restored with the administration of dexamethasone and increases in Bcl-xL mRNA were observed [118]. Additionally, in mouse mammary epithelial cells (HC11), hormone treatment lead to increases in the transcripts of Bcl-xL as well as the pro-apoptotic isoform Bcl-x_s. However, Bcl-xL mRNA was increased at a higher ratio relative to Bcl-x_s thus promoting cell survival [92, 119]. GCs have been shown to induce increases in Bcl-xL in rat hepatoma cells, podocytes, glomerular endothelial cells, glioma cells and human gastric cell line [120]. It is predicted that up-regulation or down regulation of Bcl-xL protein as a result of GC stimulation is tissue specific and is highly regulated in target tissues [121].

Bcl-xL originates from a spliced product from the Bcl-X gene [116]. Differential Bcl-X promoter activity predicts which splice variant will be produced. Human and mice Bcl-X genes contain two promoter regions deemed P1 and P2. TATA box is absent in both promoter regions. However, further analysis of the 5'-flanking revealed three consensus TATA box at -1889 (P3), -2721(P4) and -3412 (P5) upstream of the translation initiation site [122]. In addition to alternative transcription start sites, several isoforms of mRNA from Bcl-X exist due to differential splicing [123-125]. Five promoters have been characterized

(P1-P5) in the mouse Bcl-X gene giving rise to at least 5 different mRNAs with different 5'- untranslated regions. Translation initiation site is the same for all products. With the exception of the most proximal P1 product, all other products are generated via alternative splicing [122]. P1 predominately gives rise to Bcl-xL; whereas, P2 generates Bcl-xL, Bcl-X_S, and Bcl-$X_{\gamma,}$ and P3 yields Bcl-X_γ [122]. Additionally, dexamethasone treatment activates P4 and P4 is also known to induce Bcl-xL [92]. These promoters display tissue specific activity as well, reinforcing tissue specific activity of the Bcl-X genes. As mentioned earlier, Bcl-xL isoform protects against apoptosis, while Bcl-x_S is pro-apoptotic. Bcl delta TM is a third isoform and was found to be pro survival [124]. Bcl-X_β was identified in cerebellum, heart, and thymus and was found to be pro apoptotic [125]. The anti-apoptotic Bcl-X gamma is induced in lymphocytes [126]. Bcl-X_{ES} (extra short) is found in numerous human cancer cell lines and has been shown to be pro survival [127].

1.9. Statement of Scientific Questions Addressed in this Dissertation

Doxorubicin is frequently used in the clinic for cancer therapy. Advances in chemotherapeutics have improved the patient's chance of surviving cancer, however, the side effects may not be evident for months to years after treatment. Cardiotoxicity is a major concern for patients receiving Dox treatment. Molecules protecting the heart from cardiotoxicity induced by Dox will be very important to circumvent this issue. GCs are frequently prescribed pharmacological agents and are already used as an anti-emetic in cancer therapy. Our group has recently found GCs are protective against Dox-induced apoptosis in cardiomyocytes. Identifying molecules involved in GC- induced cardioprotection will be important in developing new therapies, not only just for drug-induced but also other forms of cardiomyopathy that plagues the population.

GILZ is a novel glucocorticoid-induced protein that mediates the biological function of GCs in the hematopoietic system. There is no literature regarding the role of GILZ in cardiomyocytes. Given the emerging cytoprotective role of GCs and the lack of knowledge about GILZ in cardiomyocytes, it is necessary to address whether GC induces GILZ and the function of GILZ in cardiomyocytes. In this dissertation, I have addressed the hypothesis that GILZ mediates glucocorticoid-induced cytoprotection via following specific aims:

Specific Aim 1: Characterize GILZ expression in cardiomyocytes.

Specific Aim 2: Determine the significance of GILZ expression in cardiomyocytes by measuring its potential for cytoprotection.

Specific Aim 3: Describe mechanisms by which GILZ elicits cytoprotective effects.

CHAPTER 2:MATERIALS AND METHODS

Chemicals. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Pharmacological inhibitors were obtained from Enzo Life Sciences (Plymouth Meeting, PA).

***In Vivo* Study**. Male C57BL6 mice at 8 weeks old were administered 20 mg/kg Dex or vegetable oil vehicle control. At 24 hrs after, the hearts were removed and immediately frozen in liquid nitrogen. Proteins were extracted by grinding tissues with a mortar and pestle in a lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Sodium Pyrophosphate, 1 mM beta-Glycerophosphate, 1 mM Na_3VO_4, 1 mg/mL Leupeptin, from Cell Signaling, Boston, MA). After lysis in a 4ºC orbital shaker for 1 hr, protein concentration was measured by the Bradford method (Bio-Rad, Hercules CA) and Western blot analysis was performed to determine GILZ protein levels.

Cell Culture and Treatment of Drugs. Primary cultured cardiomyocytes were prepared from 1 to 2 days-old neonatal Sprague-Dawley rats as described [128]. These myocytes were seeded at a density of $2x10^6$ cells per 100 mm dish or $0.3x10^5$ cells per well in 6-well plates in low glucose Dulbecco's Modified Eagle

Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin. Three days after plating, cells were treated with Dex.

H9C2 cells (obtained from ATCC) were seeded in 6-well plates at $3x10^5$ cells per well and grown to 80% confluency. The cells were starved in serum free media 16 hr before treatment with corticosterone (CT), estrogen, cholesterol, testosterone or L-Thyroxine at a final concentration of 1 mM for 9 hrs. Inhibitors were administered 1 hr before 1 μM CT treatment. Final concentrations of inhibitors were as follows: PD-98059 20 μM, Wortmannin 100 nM, LY 294002 10μM, H-89 10μM, mifepristone 1μM, SB-202190 10 μM, FR 180204 (Santa Cruz Biotechnology Inc, CA) 10 μM and Rapamycin 5 ng/ml. Cells were harvested lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Sodium Pyrophosphate, 1 mM beta-Glycerophosphate, 1 mM Na_3VO_4, 1 mg/mL Leupeptin, from Cell Signaling, Boston, MA) for measurements of GILZ protein by Western blot analyses.

Real-Time PCR (qPCR).

Total RNA extracted using TRIzol was used as templates for RT-PCR. cDNA was synthesized using cDNA synthesis kit (Fermentas) with random hexamer as primers. For PCR, specific primers against rat GILZ and GAPDH were purchased from the Integrated DNA Technologies (IDT, San Diego CA) with the sequence of 5'-AGC TGA ACA ACA TAA TGC GCC AGG-3' or 5'- ATC TTG TTG TCT AGG GCC ACC ACA-3' as forward or reverse primer for GILZ, and 5'-

CCT CTC TCT TGC TCT CAG TAT-3' or 5'-GTA TCC GTT GTG GAT CTG ACA-3' as forward or reverse primer for GAPDH. Real time PCR was performed on the CFX 96 Thermal Cycler (Bio-Rad) using Cyber Green (Fermentas) for product detection after 10 mins of denaturation at 95ºC followed by 35 cycles of 95ºC for 15 sec, 60ºC for 30 sec and 72ºC for 30 sec. At the end of PCR, melting curve analysis was carried out to verify product specificity.

Caspase Activity Assay.

Detached cells were collected by centrifugation (3000xg for 5 minutes) and subsequently combined with adherent cells from the same well lysed in 200 μl of lysis buffer (0.5% Nonidet P-40, 0.5 mM EDTA, 150 mM NaCl, 50 mM Tris pH 7.5) for measurements of caspase activity using 40 μM N-acetyl-Asp-Glu-Val-Asp-7- amino-4-methyl coumarin (N-acetyl-DEVD-AMC; Alexis Biochemical, San Diego, CA) as a substrate. The released AMC was measured as the relative fluorescence unit using a 96-well fluorescence plate reader (Biotek Synergy 2) with an excitation wavelength of 365 nm and an emission wavelength of 450 nm.

Transient and Stable Transfection.

Fugene HD transfection reagent (Roche) was used for transient transfection of H9C2 cells with pcDNA3 plasmid containing full length GILZ. The stable transfectant was generated by nucleofection (Lonza, Basel, Switzerland) with 2 μg pcDNA3 plasmid containing full length mouse GILZ linearized with Bam HI

digestion (Fermentas). Transfected cells were seeded in 6-well plates and grown to 70% confluency before subsequent subculture and selection using 500 μg/mL G418

MTT Assay for Cell Viability.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich) was added to cells in 6-well plates at a final concentration of 0.5 mg/ml. After 20-min incubation in a 37°C tissue culture incubator, culture medium was removed, and the resulting formazan crystals were dissolved in isopropanol for quantification by a spectrophotometer for absorbance at a wavelength of 570 nm.

Western Blot.

Cells in 100-mm dishes or 6-well plates were lysed by scraping in cell lysis buffer (20 mM Tris-HCL pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM N_3VO_4, 1 μg/mL leupeptin, Cell Signaling, Boston MA). Protein concentration was measured by the Bradford method (Bio-Rad, Hercules, CA). Proteins were separated by SDS-polyacrylamide gel electrophoresis (12% gel) and transferred to a PVDF membrane at 60 Volts for 3 hr. Membranes were blocked in 10 % milk/TBS-tween solution for 2 hours, and then incubated with rabbit polyclonal or mouse monoclonal antibodies against p21, Bax, Bcl-2 and GILZ (Santa Cruz Biotechnology) at 1:200 dilutions. Monoclonal antibody against Bcl-xL (Cell

Signaling) was diluted at 1:1000. Vinculin, used as an internal loading control, was recognized by a monoclonal antibody (Sigma-Aldrich). The bound antibodies were detected using an enhanced chemiluminescence reaction after blotting with secondary antibodies conjugated with horseradish peroxidase.

siRNA Transfection.

6-well plates containing H9C2 cardiomyocytes at 90% confluency were transfected with 1 μg of siRNA against GILZ or Bcl-xL. GILZ siRNA was generated via *in vitro* transcription with the sequence of 5'-GAGGGUAUUCUCACGCUCCAGCUGCGA-3'. siRNA against Bcl-xL was obtained from Ambion (Austin, TX) and has a sequence of 5'-GGCUGGGCGAUGAGUUUGAATT-3'. The siRNA oligo was transfected into H9C2 cells using Fugene HD at 4 μl per 1μg RNA. Following overnight incubation, cells were treated with 1 μM corticosterone for 8 hr followed by 0.75 μM Dox treatment for 24 hr.

Statistical Analysis. ANOVA one-way analysis of variance ($P<0.05$) followed by Bonferroni correction was used to verify the significant difference using Prism Graph Pad 4.0 Software (La Jolla, CA).

Chapter 3: Glucocorticoids Regulated Expression of Glucocorticoid Induced Leucine Zipper (GILZ) in Cardiomyocytes

Abstract

Glucocorticoids (GCs) are frequently prescribed pharmacological agents most notably for their immunosuppressant effects. Endogenous GCs mediate biological processes such as energy metabolism and tissue development. At the cellular level, GCs bind to the Glucocorticoid Receptor (GR), a cytosolic receptor that translocates to the nuclei upon ligand binding and alters gene transcription. Among a long list of genes activated by GCs is the Glucocorticoid Induced Leucine Zipper (GILZ). Although GC induced GILZ expression has been well established in lymphocytes, little is known whether cardiomyocytes respond to GCs by inducing GILZ. Unlike lymphocytes, in which GCs induce apoptosis and GILZ mediates GC induced apoptosis, cardiomyocytes respond to GCs by gaining resistance against apoptosis. We determined GILZ expression pattern in cardiomyocytes *in vivo* and *in vitro*. Expression of GILZ in mouse hearts as a result of GC administration was confirmed by Western blot analyses. GCs induced dose and time dependent elevation of GILZ expression in primary cultured rat neonatal cardiomyocytes. Dexamethasone as low as 0.1 μM was sufficient to induce GILZ expression. Time course analysis indicated that GILZ protein levels increased at 8 hr and peaked at 48 hr after exposure to 1 μM dexamethasone. H9C2 rat cardiomyocyte cell line showed similar response of GILZ induction by dexamethasone, providing a convenient model for studying the

biological significance of GILZ expression. With corticosterone (CT), an endogenous form of corticosteroid in rodent, 0.1 -2.5 μM was found to induce GILZ in H9C2 cells. Time course analysis with 1 μM CT indicated induction of GILZ at 6 hr with peak expression at 18 hr. Pharmacological inhibitors were used to determine the signaling pathways mediating GILZ expression. Inhibition of the GR by mifepristone led to blunting of GILZ induction by GC. Our data demonstrate GILZ induction in cardiomyocytes both *in vivo* and *in vitro by GCs* and point to H9C2 cell line as a valid model for studying the biological function of GILZ in cardiomyocytes.

Introduction:

Glucocorticoids (GCs) are among the most commonly prescribed pharmaceutical agents. Therapeutic actions of GCs are largely attributed to their anti-inflammatory and immunosuppressant properties. At the cellular level, GCs are best known for inducing apoptosis in lymphocytes and the molecular mechanism of such action has been extensively studied. GILZ was originally discovered in studies aimed at characterizing genes induced by dexamethasone (Dex) using a thymus subtraction library [73]. GILZ has been shown to mediate apoptosis of lymphocytes, T-helper cell differentiation, function of dendritic cells, increasing Na^+ transport in the kidney and inhibition of Ras driven tumorigenesis [73, 74, 76-78].

It was originally proposed that GILZ functions as a transcription factor. Lack of a canonical DNA binding domain in GILZ has ruled out direct transcriptional regulation. Amino acid analysis of GILZ revealed a central leucine zipper domain (LZ; 76-97 aa) with the ability to dimerize with other leucine zipper containing proteins [129, 130]. The N-terminal domain (N-ter; 1-75 aa) of GILZ has been shown to bind and inhibit proto-oncogene RAF, a serine/threonine-kinase [131]. The Tuberous Sclerosis Complex box (TSC box; 61-75 aa) located in the N-terminal domain of GILZ has been reported to bind Ras [81]. GILZ also contains a C-terminal proline and glutamic acid rich region that has been reported to interact with and inhibit NF-κB function. Therefore GILZ may regulate

transcription through interaction with signaling molecules and transcription factors.

GILZ is expressed at a high basal level in the lung, brain and skeletal muscle [84]. The gene encoding GILZ contains multiple Glucocorticoid Response Elements (GRE) in the promoter, which also contains putative binding sites for a number of transcription factors, including STAT6, Nucleofactor of Activated T Cells (NFAT), Oct-1, c-myc, Forkhead, cyclic AMP Response Element-Binding protein (CREB), and Estrogen Receptor [82, 83]. To date, there is no literature describing the regulation and function of GILZ gene in the heart.

In cardiomyocytes, GCs elicit a cytoprotective effect that protects against damaging agents from inducing apoptosis [35]. Identifying genes induced by GCs in cardiomyocytes allows the understanding of underlying mechanism of GC induced cytoprotection. Affymetrix microarray experiments analyzing mRNA levels in cardiomyocytes exposed to CT identified 140 genes up regulated and 108 genes down regulated by CT treatment. As expected, GILZ expression was found to be induced in primary cultured rat cardiomyocytes by CT [104].

H9C2 cardiomyocytes were derived from embryonic BD1X rat heart tissue and express biomarkers of cardiac muscle, such as rapidly activating cardiac L-type Ca^{2+} channel [132] and Small Nuclear Ribonucleoprotein-associated Protein N (SmN), a cardiac specific splicing protein [133]. This cell line has been widely used as an *in vitro* model for studying molecular events of cardiomyocytes [134, 135]. Unlike primary cultured cardiomyocytes, H9C2 can be subcultured and

maintained under tissue culture condition, providing the convenience for studying the regulation and function of GILZ gene. In this study, we determine GILZ expression between primary cultured cardiomyocytes and H9C2 cells, and address whether H9C2 cells can serve as an experimental model for studying the regulation and function of GILZ in cardiomyocytes.

Results

Dexamethasone Induces GILZ in Mouse Myocardium.

To address whether GCs induce GILZ in the myocardium, C57BL6 mice were treated with 20 mg/kg Dex (i.p.) with an equal volume of vegetable oil as a vehicle control. This dose has been shown previously from our laboratory to protect the heart from myocardial injury [70]. The hearts were collected at 24 hr after for measurements of GILZ protein by Western blot. The results indicated that mice dosed with Dex showed elevated GILZ protein levels compared to the control group (Figure 2).

Dexamethasone Induces GILZ in Neonatal Rat Cardiomyocytes and H9C2 Cells.

Primary cultured rat neonatal cardiomyocytes are commonly used *in vitro* model for studying cellular and molecular events induced by pharmacological agents. To characterize GILZ expression, we performed dose response and time course studies. Within 8 hrs of Dex treatment at various doses, 0.1 μM Dex treatment caused significant induction of GILZ protein while 1.0 μM Dex caused the highest level of GILZ protein induction in primary neonatal cardiomyocytes (Figure 3A). Time course analysis of GILZ expression was performed using 1 μM Dex. The results indicate the peak level of GILZ protein expression at 48 hours with Dex treatment in primary neonatal cardiomyocytes (Figure 3B).

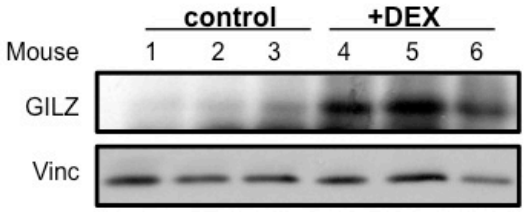


Figure 2: Dex induces GILZ expression *in vivo* in a time- and dose-dependent manner. Western Blot analysis measuring GILZ expression in whole hearts taken from mice dosed with 20 mg/kg Dex for 24 hours

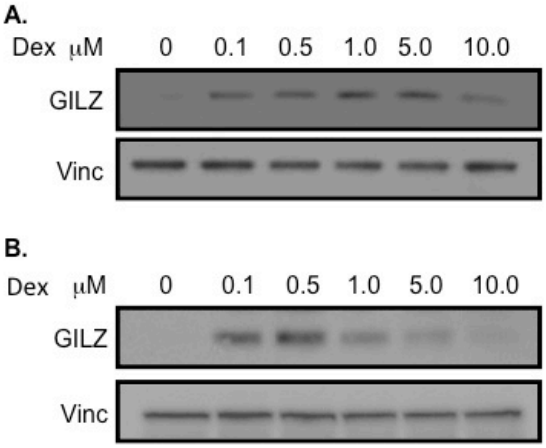


Figure 3: Dex induces GILZ expression in a dose-dependent manner. Dex induced expression of GILZ in a dose dependent manner over 24 hours in primary cultured neonatal rat cardiomyocytes (A) and H9C2 rat cardiomyocytes (B).

With H9C2 cells, a dose response similar to primary cultured cardiomyocytes was observed, with GILZ being induced at 0.1 μM Dex and 0.5 μM Dex caused the highest level of GILZ protein induction (Fig 2B). The time course study showed GILZ induction at 8 hrs, similar to primary cultured cardiomyocytes, but peak GILZ induction at 24 hr (Fig 3B). These data suggest that H9C2 cells can replace primary cultured cardiomyocytes for studying GILZ expression.

Corticosterone Induces GILZ in H9C2 Cells.

Corticosterone (CT) is the form of endogenous GCs in rodents and has been used to study the protective effect against apoptosis in cardiomyocytes [35]. The dose response experiments were performed over an 8 hr period with 0.25 to 2.5 μM corticosterone (CT), a form of endogenous GCs in rodents. The highest dose used, 2.5 μM, caused a maximal GILZ induction (Figure 5A). Time course analysis was performed using 1 μM CT and results indicated GILZ expression peaked at 18 hr (Figure 5A). qRT-PCR was performed to measure GILZ mRNA levels in cells responding to CT (Figures 5B,6B). GILZ transcript elevation was consistent with protein induction in both dose response and time course.

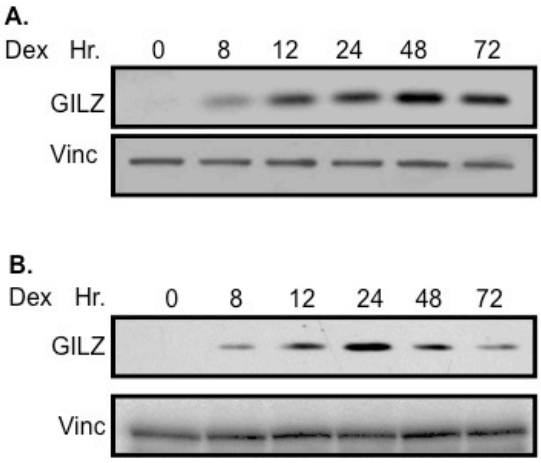


Figure 4: Time course of Dex induced GILZ expression. Western blot analysis measuring the timecourse of GILZ expression following 1μM Dex treatment in primary neonatal cardiomyocytes (A) or H9C2 cardiomyocytes (B)

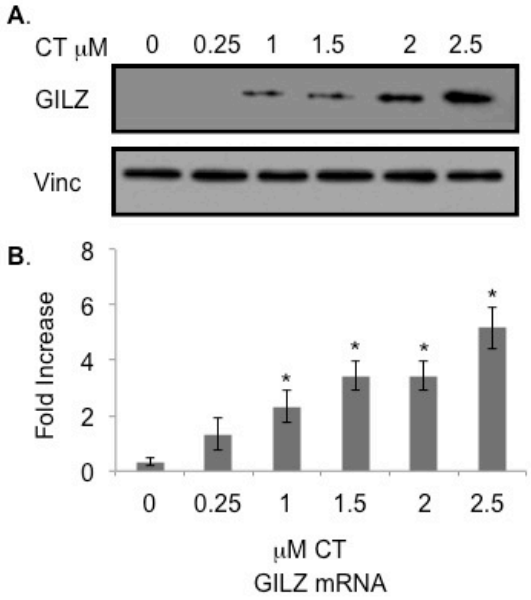


Figure 5: CT induces GILZ expression in H9C2 cardiomyocytes in a dose-dependent manner. Western blot analysis measuring GILZ protein 8 hr after treatment with varying CT doses in H9C2 cardiomyocytes (A). qRT-PCR measuring GILZ mRNA levels in H9C2 cardiomyocytes treated with increasing CT over 8 hr (B). (*) Indicates significant increases in mRNA as compared to control with $p<.05$ for three independent experiments.

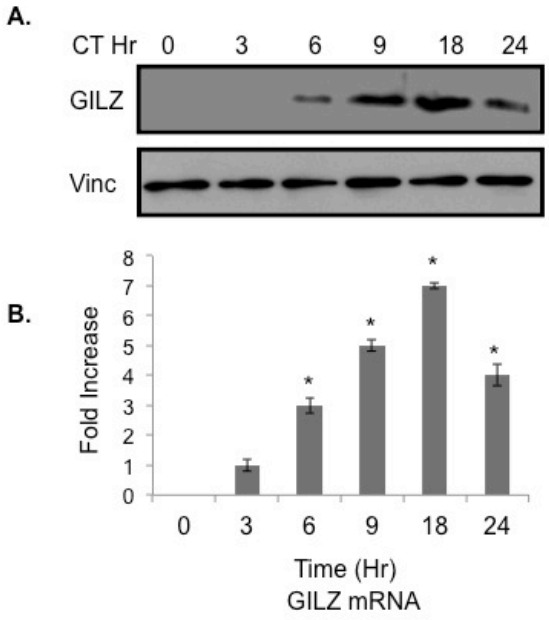


Figure 6: Time course of CT induced GILZ expression in H9C2 cardiomyocytes. Western blot analysis measuring GILZ protein at various time points after 1μM CT treatment in H9C2 cardiomyocytes (A). qRT-PCR measuring GILZ mRNA levels at various time points following 1μM CT treatment in H9C2 cardiomyocytes (B). (*) Indicates significant increases in mRNA as compared to control with $p<.05$ for three independent experiments.

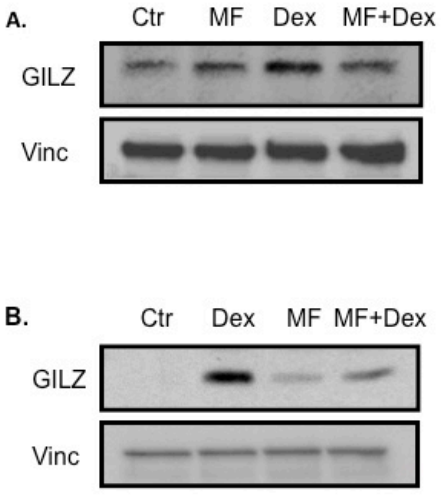


Figure 7: Glucocorticoid receptor activation is required for Dex induced GILZ expression. Western blot analysis of GILZ in primary neonatal cardiomyocytes (A) and in H9C2 cardiomyocytes (B) in the presence of 1μM Dex or mifepristone (MF) or both.

GILZ Expression Is Glucocorticoid Receptor Dependent

GILZ gene contains multiple GREs in the promoter, suggesting a role of Glucocorticoid Receptor (GR) in GILZ expression. To test whether GILZ induction by GCs is mediated through GR, we pre-treated primary neonatal cardiomyocytes or H9C2 cells with mifepristone (MF), a GC receptor antagonist, prior to exposing cells to 1μM Dex or CT for 8 hr. MF blocked the induction of GILZ in primary neonatal cardiomyocytes and H9C2 cardiomyocytes (Fig. 7A, 8B), suggesting that GC induced GILZ expression is indeed dependent on the GR.

Previous work from our laboratory and others indicate that GCs can activate phosphatidylinositol 3-kinase (PI3 kinase) [136-138]. Whether GCs activate or inhibit MAP kinases is controversial, since both activation and inhibition have been reported [139-143]. To address whether these signaling pathways are involved in the induction of GILZ by GCs, pharmacological inhibitors were utilized. Analyses of GILZ mRNA or protein levels suggest that GILZ is primarily regulated by the GR (Figure 7A, 7B). The PI3 Kinase inhibitor, Wortmannin as well as the mTOR inhibitor rapamycin failed to inhibit GILZ expression. Interestingly, H89, a PKA inhibitor, as well as the p38 inhibitor SB202190 induced GILZ expression when given alone and enhanced GILZ induction by CT. These findings suggest that GILZ

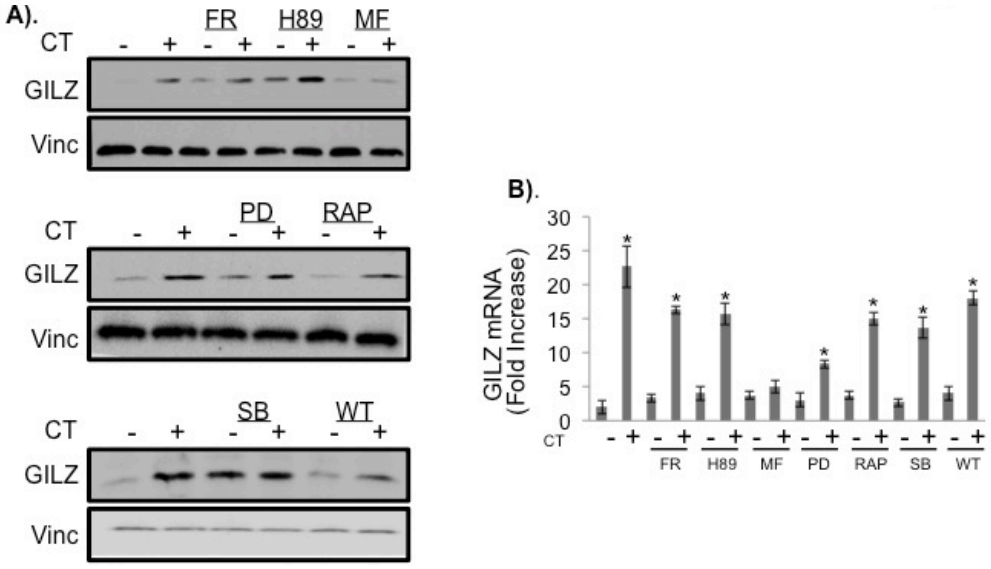


Figure 8: Western blot (A) and qRT-PCR (B) analysis of GILZ expression in H9C2 cardiomyocytes with or without CT in the presence of ERK inhibitor (FR), PKA inhibitor (H89), GR inhibitor (MF), MEK inhibitor (PD), mTOR inhibitor (RAP), p38 inhibitor (SB), or PI3 Kinase inhibitor (WT).

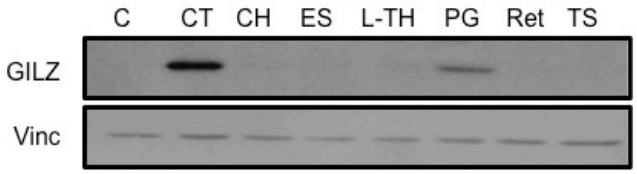


Figure 9: Western blot analysis of GILZ in the presence of corticosterone (CT), cholesterol (CH), estrogen (ES), L-Thyroxine (L-TH), progesterone (PG), retinoic acid (Ret) or testosterone (TS).

expression is coupled to GR activation by GCs and that PI3K pathway does not play a role in GILZ induction.

Progesterone Induces GILZ in H9C2 Cells.

GILZ has been reported to be induced by steroid hormones other than glucocorticoids in selective tissues [65]. To determine if steroids other than GCs induce GILZ in cardiomyocytes, we treated H9C2 cells with 1 μM cholesterol, estrogen, L-Thyroxen, progesterone, retinoic acid or testosterone with corticosterone as a positive control. Estrogen, testosterone, thyroxine, retinoic acid, or cholesterol did not cause GILZ elevation (Fig 9). Progesterone increased GILZ expression in H9C2 cardiomyocytes (Fig 9).

Discussion

In this report, we describe the ability of GCs to induce GILZ in the myocardium, primary cultured cardiomyocytes and H9C2 cardiomyocyte cell line. GILZ induction was dose and time dependent in both H9C2 cells as well as primary neonatal cardiomyocytes, suggesting that H9C2 cells serve as a valid model for studying molecular mechanism underlying GILZ induction. The hormone receptor (HR) inhibitor, mifeprestone, blocks GILZ expression in both primary cardiomyocytes and H9C2 cells suggesting GILZ expression is dependent on GR activation. In addition to GCs, progesterone was found to induce GILZ expression in H9C2 cardiomyocytes confirming the necessity of hormone response element activation in the expression of GILZ.

The identification and characterization of GILZ in T-lymphocytes has suggested GILZ can contribute to signaling events regulating survival [73]. Given our previous reports suggesting GCs contribute to cardiomyocyte cytoprotection [35] and GILZ potential to mediate survival, we determined it necessary to identify a valid cardiomyocyte cell model to study the role of GILZ in survival elicited by GC in cardiomyocytes. Cardiomyocyte cell lines provide powerful tools for investigating mechanisms underlying pathologies associated with heart failure. Established cardiomyocyte cell lines include HL-1 [144] and H9C2 cells [145]. The HL-1 cell line was derived from mouse atrial tumors and retains contractility in culture. In contrast, H9C2 cells were derived from the embryonic rat myocardium. Although H9C2 cells do not contract, they retain many

molecular and biochemical characteristics of primary cardiomyocytes, such as membrane morphology, G-protein signaling and electrophysiological properties [132, 146]. Like primary cultured neonatal cardiomyocytes, H9C2 cells also demonstrate similar characteristics during hypertrophy [147, 148] suggesting H9C2 cells are a valid cardiomyocyte cell model.

We show similar Dex induced GILZ induction between the two cell lines however some differences should be noted. Peak levels of GILZ expression were observed at a slightly lower concentration in H9C2 cells and began decreasing above 0.5μM Dex (Fig 3B). Primary cells induced peak levels of GILZ at 1.0μM Dex and a high level of GILZ expression was maintained at higher concentrations of Dex suggesting a more robust capacity of expression in primary cells (Fig 3A,B). Both H9C2 and primary neonatal cardiomyocytes have sustained GILZ elevation over an extended period of time. Furthermore both cell lines induce peak level of GILZ expression at 24 Hr. however primary cells are able to maintain a high level of expression up to 48 Hr. after Dex stimulation. Again this implies a more robust capacity of expression in primary cells (Fig 4A,4B).

Corticosterone, the primary GC in rodents, induced GILZ in a time and dose dependent manner in H9C2 cardiomyocytes (Fig 5,6). Although peak levels of expression were observed at higher concentrations as compared to Dex induction (1.0μM Dex vs 2.5μM CT), 1.0μM GC is sufficient to induce GILZ both cell systems. Furthermore, GILZ expression is detected at earlier time points

and is maintained for a longer time in the presence of Dex as compared to CT. Dex is reported to be a more stable synthetic GC and it is therefore expected stimulation would be maintained over longer periods. Differences in GC and Dex stimulation have been reported in other cell systems [149], here we report CT and Dex are both able to induce GILZ in cardiomyocytes in a both time and dose responsive manner.

GCs induce transcription of target genes through activating GR, which allows for nuclear translocation of GR where it can bind to HREs in target genes. The mechanism involves GR homodimerization or heterodimerization with other transcription factors followed by DNA binding to induce or repress genes regulated by GC signaling. In addition to this well-known genomic effect, GCs can also elicit non-genomic actions through various protein-protein interactions. In this manner, GR can activate or suppress gene expression independent of DNA binding. For instance, GCs can activate kinase-mediated signaling pathways within minutes through nongenomic GR mediated signaling to regulate gene expression [137, 150] [151].

Pharmacologic inhibitors of pathways influenced by GC stimulation were screened to determine the mechanism of GILZ induction. Of the inhibitors used, only the GR inhibitor Mifepristone attenuated GILZ expression indicating that GR is responsible for GILZ induction (Fig 8). This is logical because the gene encoding GILZ contains multiple Glucocorticoid Response Elements (GRE) in the promoter, which also contains putative binding sites for a number of transcription

factors, including STAT6, Nucleofactor of Activated T Cells (NFAT), Oct-1, c-myc, Forkhead, cyclic AMP Response Element-Binding protein (CREB), and Estrogen Receptor [82, 83].

Interestingly, p38 inhibitor as well as pKA inhibitor alone induced the expression of GILZ. These findings suggest that cAMP-PKA and p38 MAPK signaling pathways serve to negatively regulate GILZ expression. Both p38 and PKA inhibitor induce GILZ protein independent of increases in mRNA (FIG 8B). Both pathways have also been reported to mediate protein stability in cardiomyocytes. For example, p38 has been reported to directly phosphorylate p300 in cardiomyocytes which lead to the subsequent degradation of p300 by the proteasome [152]. Furthermore, PKA, activity has been reported to induce proteasome activation in cardiomyocytes [153, 154]. It is therefore reasonable to predict both PKA and p38 pathways regulate GILZ protein levels in cardiomyocytes.

In addition to GCs, progesterone was also observed to increase GILZ expression. Progesterone receptor (PR) is expressed in mammalian myocardium [155-159]. PR belongs to the same family of nuclear receptor as GR, and binds to a common HRE in the promoter of targeted genes. Additionally, progesterone has been reported to induce transcription of genes containing HREs [160-163]. HREs are often referred to as GREs when glucocorticoids are the activating factor. At equivalent dose of 1 μM, progesterone is less potent than CT in inducing GILZ protein levels (Fig 9). This

suggests that GILZ expression is more specifically coupled to GR binding to its cognate HRE. Additionally GRE's have been reported to be promiscuous and retain an ability to be activated by PR [164, 165]. Moreover, high concentrations of PG has been reported to induce GRE activity [166].

. Nevertheless, GILZ induction correlates with cytoprotection, since both GCs and PG exhibit cytoprotective effect in cardiomyocytes [2, 35, 166]. Further studies will examine whether GILZ indeed functions as a cytoprotective gene in cardiomyocytes.

CHAPTER 4: GILZ IS CYTOPROTECTIVE IN CARDIOMYOCYTES

Abstract

Doxorubicin (Dox) is an indispensable chemotherapeutic agent for treatment of neoplasia such as lung, breast and liver cancers. Cardiotoxicity is a major concern in patients receiving Dox therapy. Previous work from our laboratory has demonstrated the potential of glucocorticoids (GCs) in alleviating Dox-induced cardiac toxicity. Here, we have identified Glucocorticoid-Induced Leucine Zipper (GILZ) as a mediator of GC-induced cytoprotection. GILZ was found to be induced in cardiomyocytes due to GC treatment. Knocking down GILZ using siRNA resulted in cancelation of GC-induced cytoprotection against apoptosis by Dox treatment. Overexpressing GILZ by transfection was able to protect cells from apoptosis induced by Dox as measured by caspase activation, Annexin V binding and morphologic changes. Western blot analyses indicate that GILZ overexpression prevented cytochrome c release from mitochondria and cleavage of caspase-3. When bcl-2 family proteins were examined, we found that GILZ overexpression causes induction of the pro-survival protein Bcl-xL. Since siRNA against Bcl-xL reverses GC induced cytoprotection, Bcl-xL induction represents an important mediator of GILZ-induced cytoprotection. Our data suggest that GILZ functions as a cytoprotective gene in cardiomyocytes.

Introduction

Doxorubicin (Dox) is an anthracyclin often used for cancer chemotherapy. Treatment of breast cancer, solid tumors, tissue sarcoma and aggressive lymphoma requires Dox[167]. A major side effect of Dox treatment is cardiac toxicity. Acute cardiac toxicity is shown as arrhythmias. The regiment and administering protocol have been improved to reduce acute cardiac toxicity. However certain patients develop cardiomyopathy months or years after Dox treatment [95, 168-172]. On average, about 48% adult patients develop congestive heart failure at a cumulative dose of 700 mg/m^2 and above. Children and elderly patients are at a higher risk for Dox-induced cardiomyopathy [170]. Reducing Dox-induced cardiotoxicity is important for successful treatment of cancer and extension of the lifespan.

Glucocorticoids (GCs) are used as an anti-emetic agent for patients receiving chemotherapy and are also a key component of combination therapy for lymphoma, leukemia and myeloma. Treatment of lymphoid cancer using GCs is due to the fact that GCs are potent apoptotic inducers for lymphoid-derived cells. Additional uses of GCs during cancer therapies include anti-inflammatory response for cranial metastasis, anti-hypercalcemic effect and the ability to suppress tumor related fevers [173].

Our works with isolated cardiomyocytes suggest that GCs are protective against Dox-induced apoptosis [35]. GC-induced cytoprotection was attributed in part to the induction of pro-survival Bcl2 family member, Bcl-xL. Preconditioning

of cardiomyocytes with GC protected cardiomyocytes for at least 72 hours of Dox exposure [35]. This discovery prompted us to investigate mechanisms by which GC elicits cytoprotection. Affymetrix microarray analysis of GC treated cardiomyocytes revealed that 140 genes were up-regulated and 108 genes were down regulated [35]. Among the genes upregulated is Glucocorticoid Induced Leucine Zipper (GILZ).

GILZ was first identified in a thymus subtraction library during investigation of glucocorticoid induced genes [73]. In the same study GILZ was described as playing a role in mediating TCR/CD3 induced cell death in lymphocytes. Further studies investigating GILZ significance in cellular processes revealed roles of GILZ in T-lymphocyte activation, regulation of T-helper cell differentiation and dendritic cell function, IL-2 production, apoptosis and cell proliferation [74-76]. Since glucocorticoids induce cytoprotection in cardiomyocytes, we ask whether GILZ serves as a mediator for such cytoprotection.

Results:

GILZ Is Essential for Corticosteroid-Induced Cytoprotection

We investigated the role of GILZ in GC-induced cytoprotection by knocking down GILZ expression with siRNA. Following siRNA-mediated knockdown, H9C2 cardiomyocytes were treated with 1 μM CT 24 hours prior to Dox treatment. The negative control, i.e. scrambled siRNA, had no effect on CT's ability to protect against Dox-induced apoptosis, while H9C2 cells transfected with GILZ siRNA had slightly higher basal caspase activity compared to control cells (Figure 10A). Furthermore, siRNA knockdown of GILZ abolished CT's potential to attenuate Dox induced apoptosis, implying GILZ is essential for CT-mediated cytoprotection. We have previously shown Bcl-xL is necessary for GC-induced cytoprotection [35]. As expected, siRNA against Bcl-xL also led to decreased cell viability, serving well as a positive control (Figure 10A). Western blot analysis of GILZ demonstrated siRNA against GILZ was effective in knocking down the protein in H9C2 cardiomyocytes (Figure 10B).

GILZ Protects Against Doxorubicin Induced Apoptosis

To determine the potential of GILZ to alleviate Dox toxicity in cardiomyocytes, a stable transfectant of H9C2 cells was established with constitutively elevated expression of GILZ. H9C2 cells undergoing apoptosis show morphological changes including rounding up and detaching (Fig 11A).

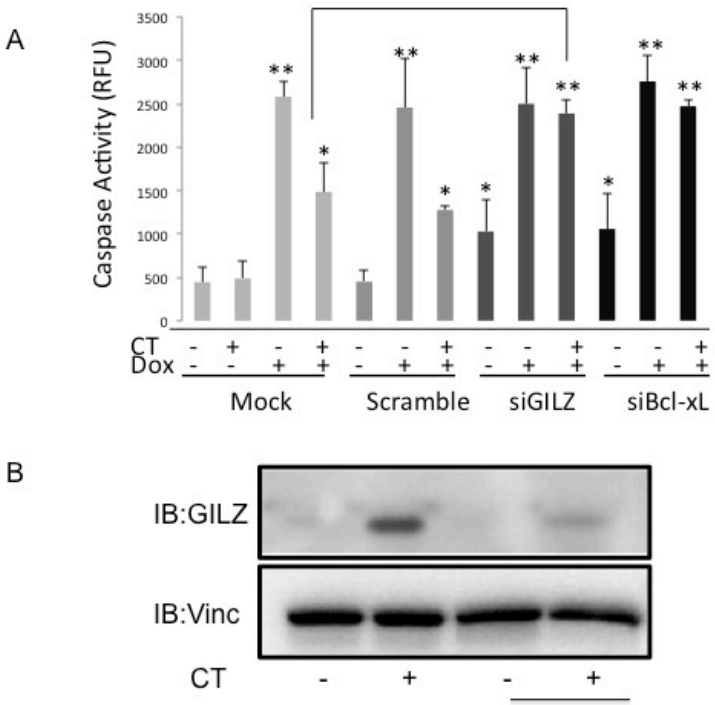


Figure 10: GILZ is necessary for CT induced protection against Dox in cardiomyocytes. Control scrambled siRNA or siRNA against GILZ and Bcl-xL (positive control) was transfected into H9C2 cardiomyocytes. 24 hours after transfection cells were treated with 1mM CT and then challenged with 0.75mM Dox for 24 hours. Caspase activity assay was performed to determine role of GILZ in CT induced cardioprotection. (A). Western blot analysis showing siRNA effectively knocked down GILZ in H9C2 cardiomyocytes (B). (*) or (**) indicate a significant difference ($P<0.05$) between groups as determined by ANNOVA.

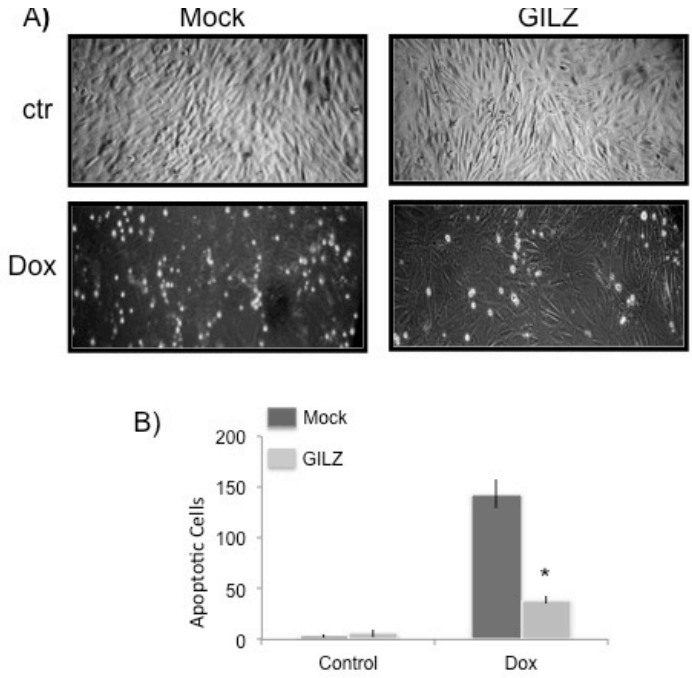


Figure 11: GILZ protects H9C2 cardiomyocytes from apoptosis. Phase contrast microscopy indicate H9C2 cardiomyocytes stably transfected with GILZ is sufficient to protect against 0.75μM Dox toxicity over 24 Hr (A). Three individual fields were quantified under a light microscope and (*) indicate a significant difference ($P<0.05$) (B).

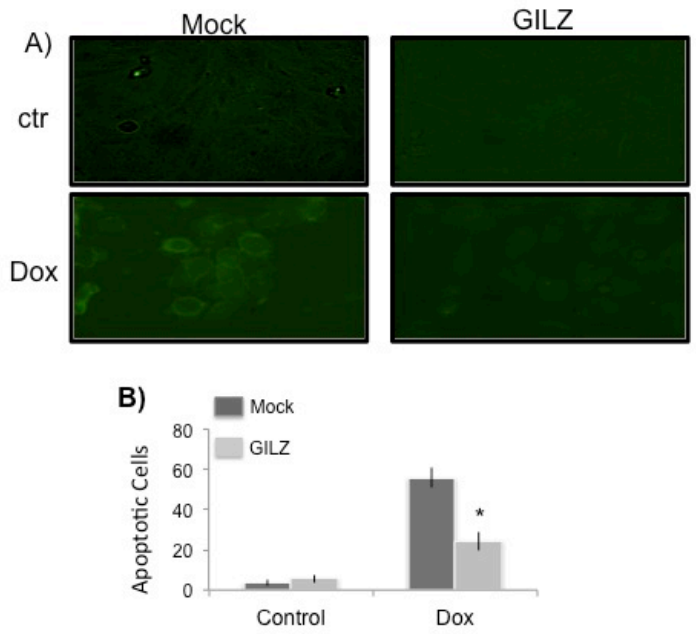


Figure 12: GILZ is cytoprotective in H9C2 cardiomyocytes Fluorescent microscopy indicating Annexin V positive staining in H9C2 cardiomyocytes stably transfected with GILZ expressing construct in the presence of .075μM Dox over 24 Hr. Three individual fields were quantified under a light microscope and (*) indicate a significant difference ($P<0.05$) (B).

When apoptotic cells were quantified under a microscope, GILZ overexpression significantly protected cells against Dox induced apoptosis (Fig 11B). Annexin V binding assay has been used to quantify the fraction of apoptotic cells. Compared to mock or empty vector transfected controls, cells stably transfected with GILZ exhibited a decrease in Annexin V fluorescence in response to Dox treatment (Fig 12A). Cells quantified under fluorescent microscopy indicated a significant reduction in Annexin V stained H9C2 cardiomyocytes stably expressing GILZ (Fig 12B).

The caspase 3 activity was measured to further demonstrate the protective effect of GILZ overexpression. As shown in Fig 13A, mock or empty vector transfected cells show a Dox dose dependent increase of caspase activity. Cells stably transfected with GILZ were resistant to Dox induced caspase 3 activation. Time course analysis of caspase activation by Dox treatment over 48 hours in cells stably transfected with GILZ or empty vector indicate GILZ cytoprotection lasted for 36 and more hours (Figure 13B).

We further verified an inhibition of Dox induced apoptosis by measuring cleaved caspase 3 via Western blot analysis. H9C2 cardiomyocytes overexpressing GILZ exhibited reduced caspase 3 cleavage in response to Dox treatment, confirming the results of Caspase 3 activity assay (Figure 14). Additionally, cytochrome C release into the cytosol as a result of Dox treatment was measured in H9C2 cardiomyocytes overexpressing GILZ. Cells expressing

GILZ released cytochrome C into the cytosol to a much lesser extent as compared to mock transfected cells (Figure 14).

The cytotoxic effects of Dox include inhibition of metabolism, and induction of apoptosis or necrosis. Reduction of mitochondrial succinate dehydrogenase activity is a general measurement of inhibition of metabolism, which usually occurs in parallel or prior to necrotic cell death. To address whether GILZ protects cardiomyocytes against cytotoxicity of Dox in general, we performed MTT assay. Although an inhibition was observed in GILZ transfected cells, GILZ overexpression did not completely prevent the loss of MTT by Dox treatment (Fig 15A). MTT time course analysis indicated GILZ does not protect against Dox induced necrosis (Fig 15B). These data suggest that the observed protective effect of GILZ is limited to apoptosis, not overall cytotoxicity of Dox.

GILZ May Protect H9C2 Cells From Apoptosis by Increasing Protein Levels of Bcl-xL Pro Survival Protein.

GILZ effect on pro-survival or pro-apoptotic proteins were measured in H9C2 cells transiently transfected with GILZ vector. Cells transfected with GILZ have elevated levels of Bcl-xL protein compared to mock treated or H9C2 cells transfected with empty vector (EV)(Figure 16). Another pro-survival protein, Bcl-2, is somewhat decreased in GILZ transfected cells compared to mock or EV transfected cells. Pro-apoptotic Bax and Bak protein levels remained unchanged in our results. P21 is a cyclin dependent kinase inhibitor (CDKN1A) and

responses to stress by elevating the level of protein. Elevating p21 protein may result in a reduction in apoptosis in some cells [174]. P21 is slightly decreased in GILZ transfected cells. These data suggest that GILZ specifically induces Bcl-xL. GILZ-induced increases in Bcl-xL protein provide a plausible mechanism of protection from Dox-induced apoptosis.

Bcl-xL mRNA Does Not Increase in the Presence of GILZ

To demonstrate Bcl-xL induction in response to GILZ expression is independent of transcription, we measured mRNA levels of Bcl-xL using quantitative RT-PCR. GILZ transfected cells did not contain increased levels of Bcl-xL mRNA compared to cells transfected with empty vector (Figure 17).

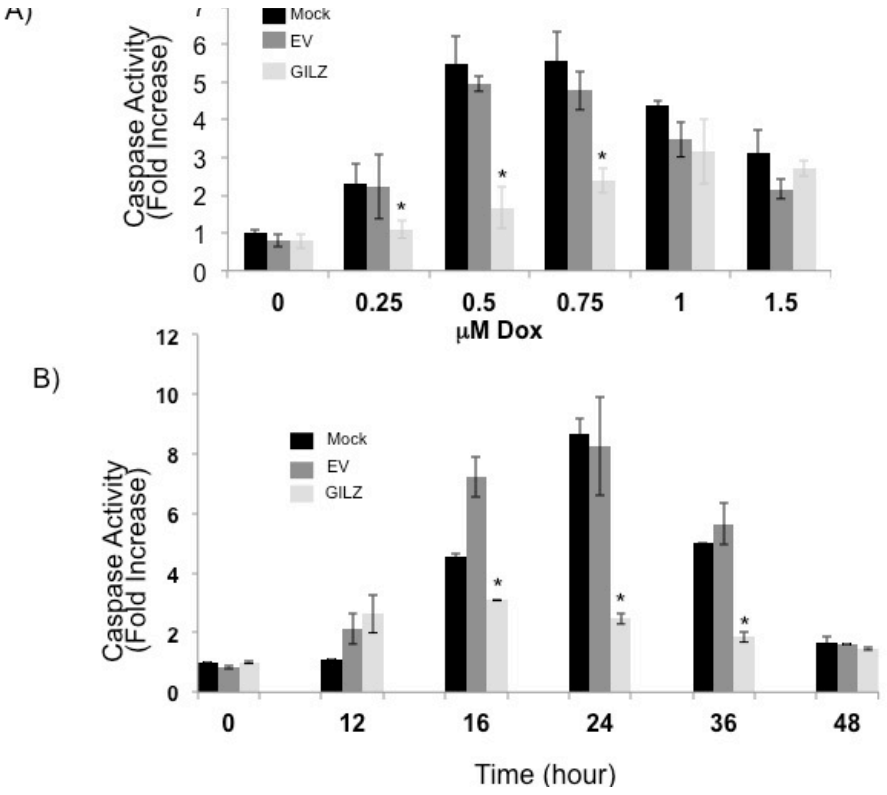


Figure 13: GILZ protects H9C2 cardiomyocytes from Dox toxicity. Dox dose (A) and time (B) response caspase activity assay in H9C2 cardiomyocytes stably transfected with GILZ or empty vector construct. Dose response was performed over 24 hours while time course assay was performed using 0.75 μM Dox. (*) indicate a significant difference ($p<0.05$) between groups as determined by ANNOVA.

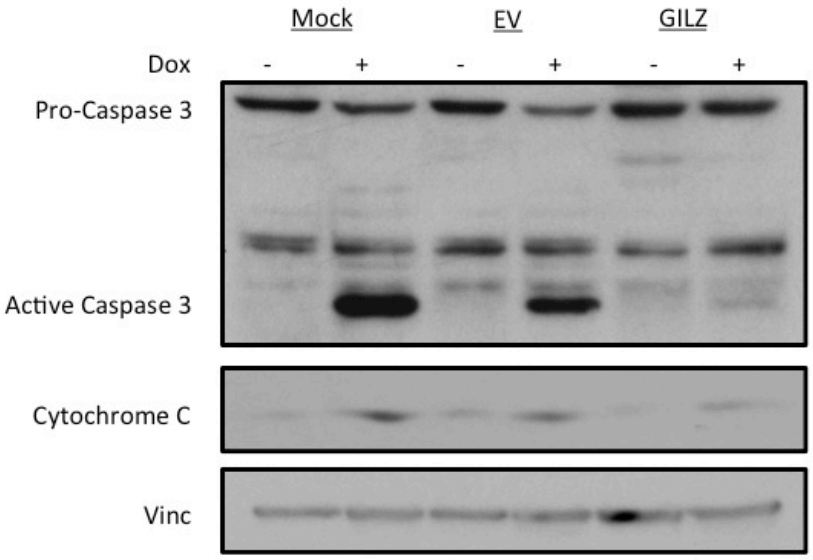


Figure 14: GILZ protects H9C2 cardiomyocytes from Dox induced apoptosis. Western blot analysis of cleaved caspase 3 or cytosolic cytochrome C in H9C2 cardiomyocytes stably transfected with GILZ or empty vector and treated with 0.75 μM Dox for 24 hours.

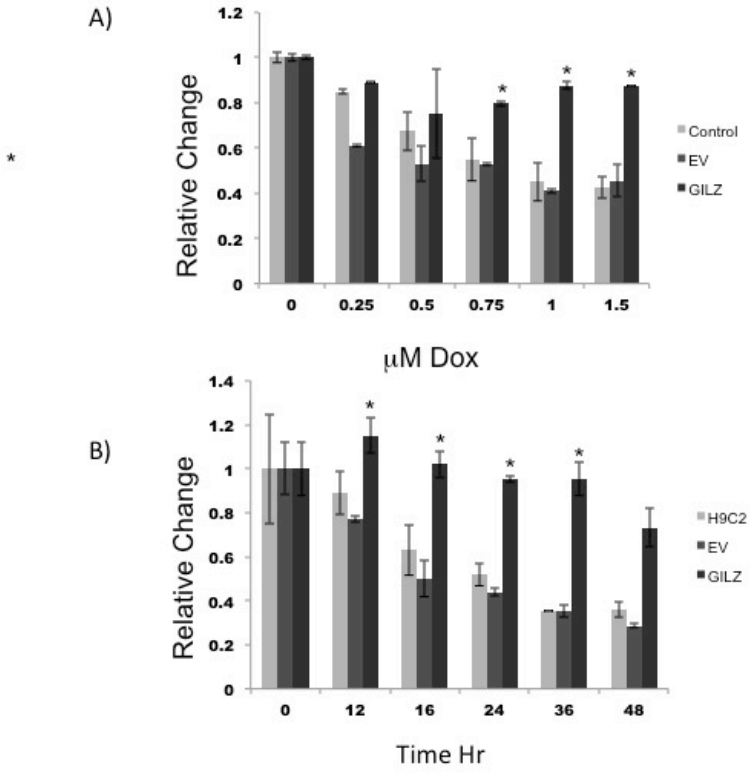


Figure 15: GILZ does not protect from Dox induced necrosis. MTT dose response (A) or time course (B) assay in H9C2 cardiomyocytes stably transfected with GILZ or empty vector construct. Dose response was performed over 24 hours and time course assay was performed using 0.75mM Dox. (*) indicates a significant difference ($P<0.05$) between groups as determined by Annova

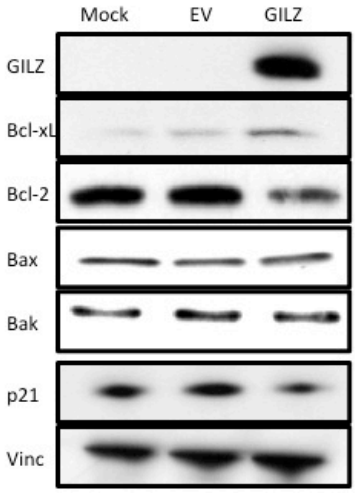


Figure 16: Western blot analysis determining changes in proteins regulating cell survival or death in GILZ transiently transfected H9C2 cardiomyocytes

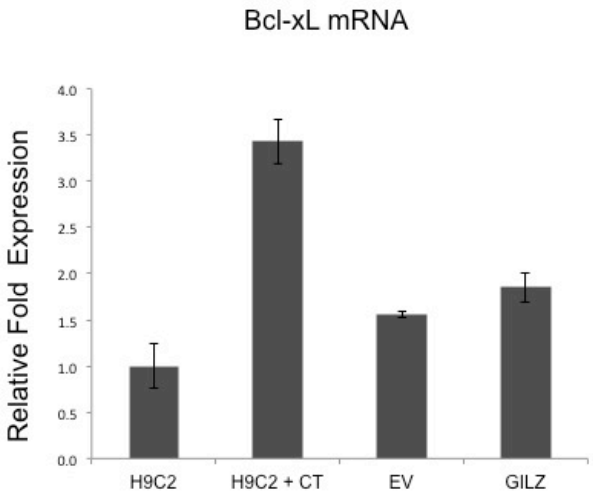


Figure 17: GILZ does not induce Bcl-xL mRNA. qRT-PCR analysis determining changes in Bcl-xL mRNA in H9C2 cardiomyocytes expressing GILZ.

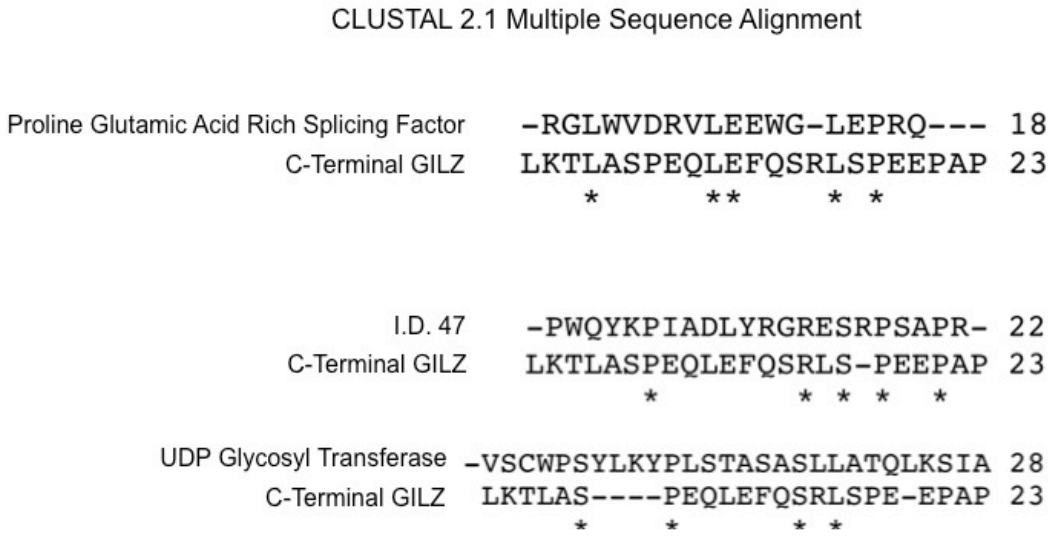


Figure 18: Protein sequence alignment of GILZ proline and glutamic acid rich C-terminal domain with reported Bcl-xL interacting proteins.

Discussion

The data presented describes GILZ as a GC-inducible inhibitor of apoptosis in cardiomyocytes. GILZ protects H9C2 cardiomyocytes from Dox toxicity as measured by changes in morphology, caspase assay and Annexin V staining (Figures 10-13). The results from caspase activity assays were confirmed by Western blot analysis measuring cleaved caspase 3 (Figure 14). siRNA knockdown of GILZ in H9C2 cardiomyocytes prevented the cytoprotective effects of GC against apoptosis due to Dox toxicity. When GILZ was overexpressed, the cells were protected against Dox induced apoptosis, indicating that GILZ alone is sufficient to elicit cytoprotection. We have preliminary evidence suggesting that Bcl-xL protein elevation mediates cytoprotection by GILZ.

Most assays measuring apoptosis were performed in GILZ stably transfected H9C2 cells and support GILZ as a cytoprotective protein in cardiomyocytes. GILZ is a GC induced protein GCs have been previously reported to play a dual role in apoptosis in the immune system by both inducing apoptosis in thymocytes and rescuing them from T-cell receptor (TCR) induced cell death [66]. GILZ mimics GC effects as evidenced in transgenic mice overexpressing GILZ. T-lineage cells in transgenic animals were reported to undergo apoptosis [75] and TCR-induced cell death is inhibited [175]. It is

therefore reasonable to predict GILZ has the potential to serve as GC induced cytoprotective protein in cell types where GILZ is induced.

Bcl-xL is a well known mediator of apoptosis and as mentioned previously has been reported to be regulated by GILZ [75]. Bcl-xL normally resides on the outer membrane of the mitochondria and helps to maintain mitochondrial membrane integrity, therefore preventing the release of cytochrome C for formation of the apoptosome. Interestingly apoptosis observed in lymphocytes is believed to be due to GILZ induced decrease in Bcl-xL, whereas the protection observed in H9C2 cardiomyocytes is at least partly attributed to an increase in Bcl-xL (Figure 16). These apparently contradictory findings may be due to the difference in cell types. It is not understood why GILZ suppresses Bcl-xL in lymphocytes while increases Bcl-xL in cardiomyocytes. This ambiguity reflects the feature of glucocorticoids, which can act as an inducer of cell survival or cell death depending on the cell type.

We have previously reported Bcl-xL plays a critical role in GC cytoprotection in cardiomyocytes [35]. The data of GILZ overexpression causing elevated levels of Bcl-xL protein in H9C2 cardiomyocytes is consistent with cell survival by Bcl-xL (Figure 16). Since glucocorticoids causes elevated expression of Bcl-xL independent of increases in Bcl-xL mRNA nor does the overexpression of GILZ induce Bcl-xL promoter activity (Figure 17) it is likely GILZ induces Bcl-xL protein via a post-transcriptional mechanism.,

GILZ contains several domains reported to interact directly with other proteins. The central Leucine zipper can interact with other Leucine zipper proteins [129, 130]. GILZ can bind to and inhibit NF-κB activity through the C-terminal proline and glutamic acid rich domain [176]. Additionally, Ras as well as Raf have been reported to bind to GILZ through the N-terminal and Ras binding domains respectively [76, 131]. A GILZ/Bcl-xL protein interaction may offer a potential mechanism as to how GILZ may be regulating Bcl-xL protein. Bcl-xL has been previously reported to interact with proline and glutamic acid rich proteins via mRNA display libraries [177]. Sequence alignment of GILZ C-terminal proline and glutamic acid rich domain and reported binding partners suggest a potential interaction with GILZ and Bcl-xL (Figure 18)

Necrotic cell death can result from damage to the plasma membrane due to increased reactive oxygen species, causing plasma membrane leakage and cell death independent of caspase activation [178]. The data from MTT assay suggest that GILZ is unable to protect H9C2 cells from necrotic cell death (Figure 6). GILZ therefore is primarily mediating cytoprotection via the apoptosis pathway.

The utilization of GCs as a pharmacological agent for cardiac protection remains controversial. Prolonged GC administration has been reported to cause cardiac hypertrophy, myocardial fibrosis, hypoxia, and ventricular dysfunction [179]. Furthermore disruption in calcium kinetics has been reported to contribute to the deleterious effects of prolonged GC treatment [2]. Additionally in heart

failure patients, GC administration is not recommended due to increased sodium and fluid retention. However a recent case report of a patient suffering from acute decompensated heart failure (ADHF) provided evidence suggesting GC treatment is safe and associated with improvement in congestion, neurohormonal status and renal function [108]. Our report suggests GC pre-conditioning for short periods of time rather than pro-longed administration protects cardiomyocytes from Dox induced toxicity. The finding of GILZ is sufficient for protecting cardiomyocytes provides a hope for designing new therapy utilizing GILZ as a pharmacological agent. The GILZ based therapy may circumvent issues with GCs.

CHAPTER 5: MECHANISM OF GILZ INDUCED CYTOPROTECTION

Abstract

We have previously described glucocorticoid induced leucine zipper (GILZ) as a novel cytoprotective protein that is required for GC elicited cytoprotection in cardiomyocytes. Induction of GILZ correlates with increased protein levels of Bcl-xL, a pro-survival Bcl-2 family member. Two-D gel electrophoresis revealed GILZ overexpressing resulted in a distinct protein, correlating with the approximate size of Bcl-xL. Western blot analysis for Bcl-xL in transiently transfected H9C2 cardiomyocytes with increasing concentrations of GILZ-pcDNA3 plasmid demonstrates a direct relationship between GILZ and Bcl-xL protein levels. GILZ overexpression did not result in an increase of Bcl-xL in HEK293, suggesting cardiomyocyte specific induction of Bcl-xL by GILZ. When Bcl-xL protein stability was determined, we found that GILZ overexpression extends the half life of Bcl-xL in cardiomyocytes. With inhibitors of calpain and proteasomes, we found that Bcl-xL is primarily degraded via the proteasomes in cardiomyocytes. GILZ co-localizes and physically associates with Bcl-xL as demonstrated by immunocytochemistry and immunoprecipitation. These data

suggest that physical interaction of GILZ with Bcl-xL results in an increased stability and therefore accumulation of Bcl-xL protein.

Introduction

The discovery that Bcl-2 allowed cytokine dependent hematopoietic cells to survive in the absence of cytokines paved the path for understanding the mechanisms of apoptosis [180]. .Since the discovery of Bcl-2 in 1985 [180] at least 15 members of the Bcl-2 family have been identified in mammalian cells [181]. Many of these Bcl-2 family members are constitutively expressed in the heart. Pathological states often induce a shift in the ratio of pro-survival versus pro-apoptotic Bcl-2 family member, contributing to an increase in apoptosis of cardiomyocytes [182-184]. Among the pro-survival members, Bcl-xL has been shown to be cardiac protective [43] [185]. Therefore inducers of Bcl-xL provide a hope for new therapies against heart diseases involving apoptosis.

GILZ was originally identified as a dexamethasone-induced gene in the thymus [73]. Since its discovery, GILZ has been described to play roles in T-lymphocyte activation, IL-2 production, apoptosis, and cell proliferation. GILZ has been described to mimic GC effects by inhibiting IFN-γ induced expression of molecules, such as CD80 and CD86, and inhibits the production of chemokines, such as RANTES and MIP-1 [81]. Since GILZ is a GC-inducible protein, its expression may be an important mediator of GC action [75].

GILZ has emerged as an interesting regulator of Bcl-xL protein levels. Transgenic mice overexpressing GILZ in the T-Cell lineage have an increased

caspase activity and a decreased level of Bcl-xL. A decrease in the transcription of Bcl-xL gene was suggested to be primarily responsible for decreases in Bcl-xL protein levels in T-lymphocytes [75].

GILZ protein has been shown to interact with a number of signaling molecules and transcription factors. As a leucine zipper, GILZ lacks nuclear localization domain therefore is mainly located in the cytosol. GILZ has been reported to hetero and homodimerize with other Leucine zipper proteins however because of it lacks a canonical DNA binding domain it does not act as a transcription factor.[73, 84, 130]. Other direct protein-protein interactions include NFκB binding to the C-terminal domain containing a proline and glutamic acid rich region (PER) [81], Raf 1 binding to N-terminal and Ras binding to Ras-binding domain [131]. We have found that GILZ induces an elevation of Bcl-xL protein through a transcription independent manner in cardiomyocytes. Here we address the mechanism of Bcl-xL protein elevation by GILZ expression.

Results:

Increases in Bcl-xL Protein Correlates with Increases in GILZ Protein

GILZ overexpression causes an increased resistance against apoptosis and induction of Bcl-xL protein. Two dimensional gel electrophoresis was used to assess global changes in proteins as a result of GILZ overexpression. As shown in Figure 19, GILZ transfected cells show a spot at 32 kDa and PI of 7. Further proteomic analysis will identify this protein and offer insight into post-translational modifications of other altered proteins. This molecular weight and PI corresponding to that of Bcl-xL protein.

GILZ Causes Increases of Bcl-xL in a Tissue Specific manner

To test whether Bcl-xL is stabilized in the presence of GILZ in cells other than H9C2 cardiomyocytes, human embryonic kidney cells (HEK) were used for GILZ plasmid transfection. Changes in Bcl-xL protein levels were measured after 24 hours. Our results indicate that GILZ overexpression does not induce protein levels of Bcl-xL in HEK cells (Figure 21). This finding supports cell specific functions of GILZ.

To determine if increases in GILZ protein correlated to increases in Bcl-xL, we transfected GILZ plasmid at different concentrations and measured Bcl-xL protein levels via western blot analysis. With an increasing level of GILZ expression, Bcl-xL protein levels increased (Figure 20), suggesting a cause-effect relationship between GILZ expression and levels of Bcl-xL protein.

GILZ Stabilizes Bcl-xL Protein Levels

To determine whether GILZ contributes to the stability of Bcl-xL protein, we transfected H9C2 cardiomyocytes with GILZ expression vector or empty vector and determined the half-life of Bcl-xL by blocking new Bcl-xL protein synthesis using cycloheximide, an inhibitor of protein synthesis. In empty vector transfected cells, Bcl-xL has a half-life about 3 hrs. With GILZ overexpression, Bcl-xL half-life was increased to over 6 hours (Figure 22). This result suggests that GILZ affects mechanisms controlling the stability of Bcl-xL in H9C2 cardiomyocytes.

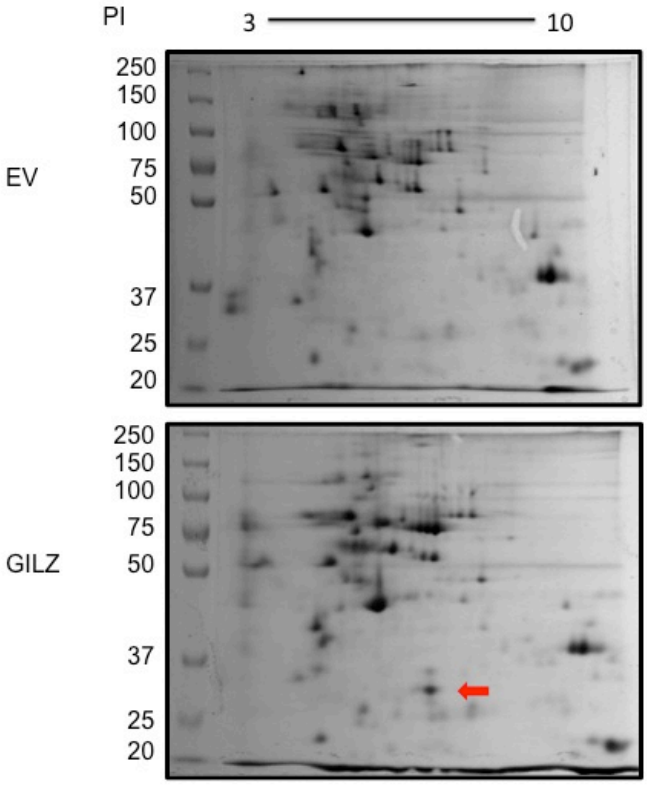


Figure 19: Coomassie blue stain of 2D Gel s containing GILZ or empty vector lysates from transiently transfect H9C2 cardiomyocytes. Red arrow indicates predicted spot correlating to Bcl-xL

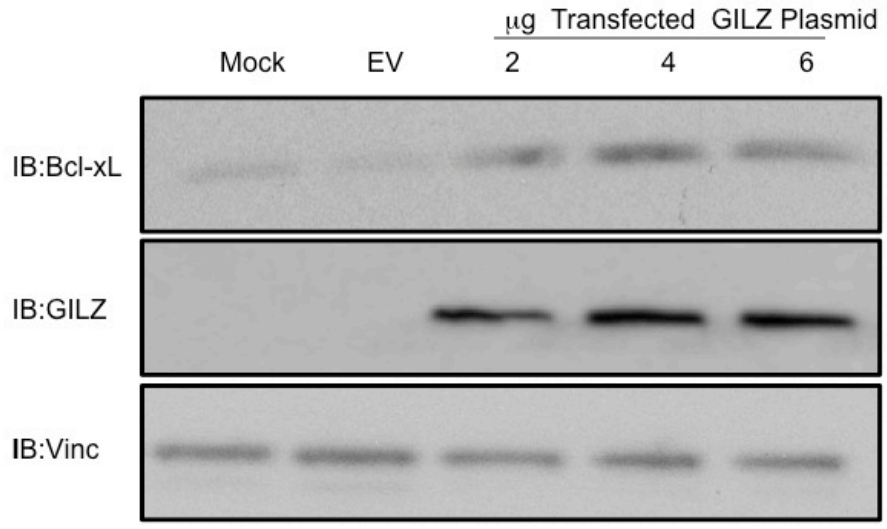


Figure 10: Bcl-xL protein levels correlate to levels of GILZ expression. H9C2 cardiomyocytes transfection using Amaxa nucleofection method with increasing concentration of pcDNA3 plasmid containing GILZ expression vetor.

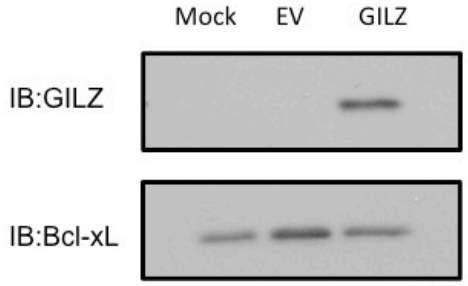


Figure 21: GILZ does not induce Bcl-xL protein in human embryonic kidney (HEK) cells. Western blot analysis of Bcl-xL and GILZ in HEK cells transiently transfected with empty vector (EV) or GILZ plasmid

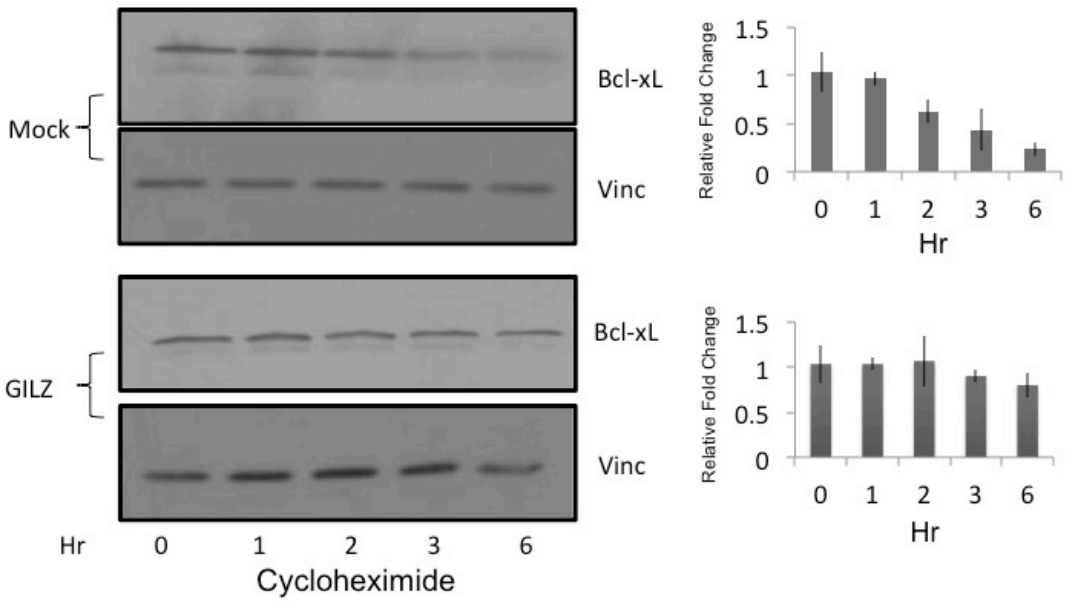


Figure 22: GILZ stabilizes Bcl-xL protein levels. Western blot analysis of Bcl-xL or GILZ in transiently transfected H9C2 cardiomyocytes in the presence of protein synthesis inhibitor cycloheximide. Bar graphs indicate changes in band intensity of 3 independent experiments determined by densitometry using Image J software.

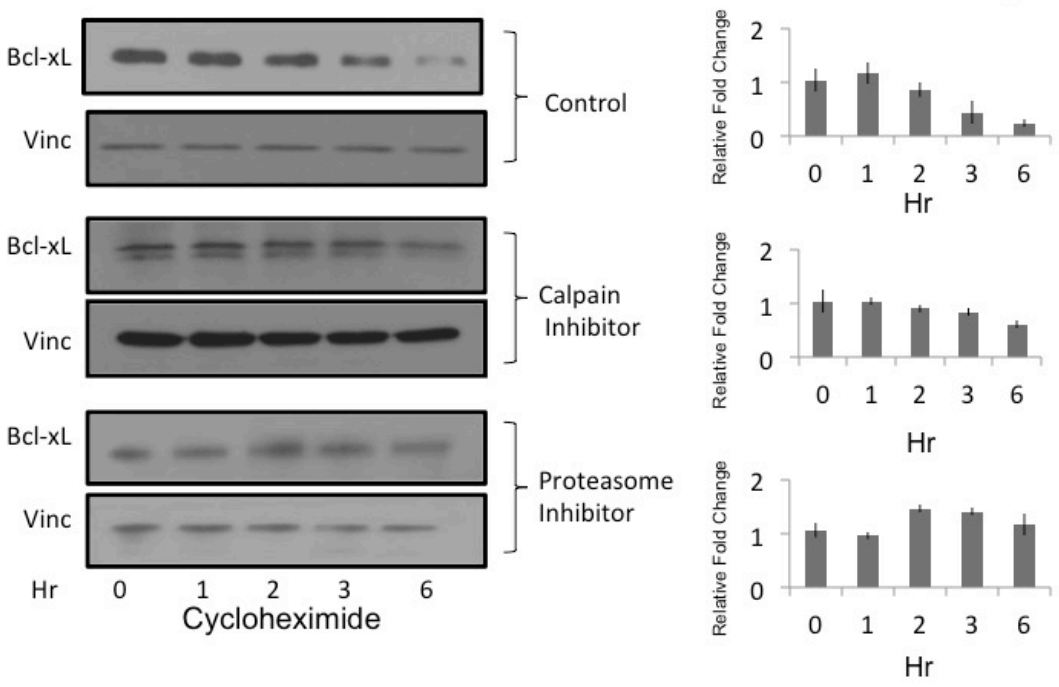


Figure 23: Protein levels of Bcl-xL are primarily regulated by the proteasome in H9C2 cardiomyocytes. Western blot analysis of Bcl-xL in H9C2 cells treated with cycloheximide and cysteine protease calpain inhibitor or proteasome inhibitor. Bar graphs represent densitometry of 3 independent experiments using Image J software.

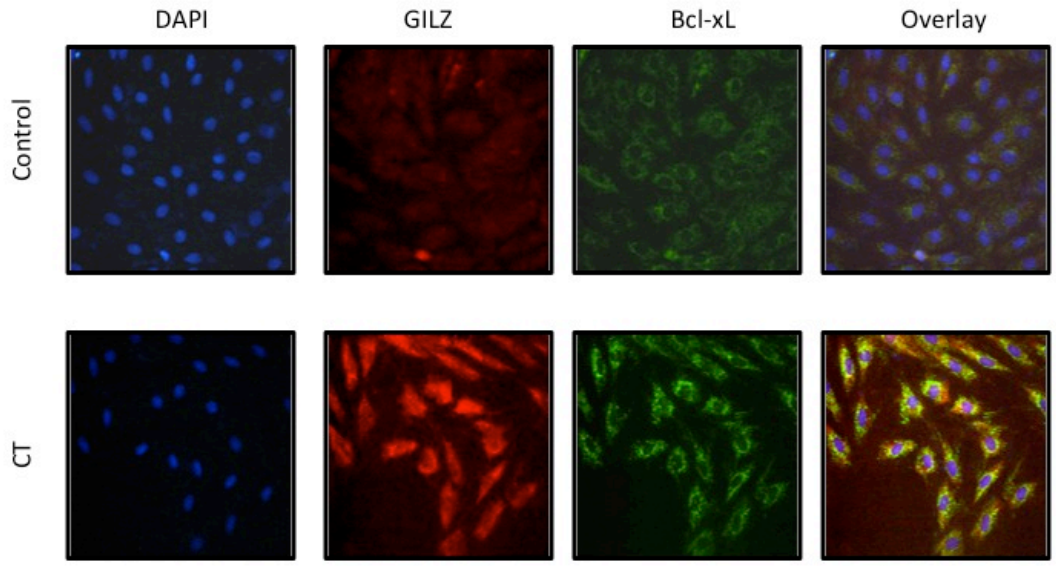


Figure 24: GILZ interacts with Bcl-xL in H9C2 cardiomyocytes. Immunocytochemistry in H9C2 cells treated with 1μM CT for 6 Hr andfixed with ice cold MeOH on coverslips in 6 well plates. Overlay of DAPI nuclear stain with GILZ(Red) and Bcl-xL(Green) indicates GILZ and Bcl-xL interact with a perinuclear manner.

Bcl-xL Protein Is Degraded via Proteasomes

A large portion of cellular proteins are degraded by the proteasomes following ubiquitination. Inhibitors of proteasomes will allow us to test whether Bcl-xL is degraded by the proteasomes. In addition to the proteasomes, there is also evidence that Bcl-xL can be degraded by the cysteine protease Calpain in the cytosol [114]. To test for the stability of Bcl-xL protein, we measured the degradation of Bcl-xL over time when new protein synthesis was blocked using cycloheximide (CXM). The Western blot analyses indicate the half-life of Bcl-xL protein in H9C2 cardiomyocytes was about 3 hours (Figure 23).

The calpain inhibitor ALLM was used to determine whether Bcl-xL protein is degraded by calpain in H9C2 cardiomyocytes. This inhibitor increased the half-life of Bcl-xL protein from 3 to 6 hours. When MG132, a proteasome inhibitor, was used to determine the half-life of Bcl-xL, the inhibitor extended the half-life of Bcl-xL protein beyond 6 hours. These results suggest that Bcl-xL can be degraded by Calpain or Proteasomes, whereas the proteasomes are the major player of Bcl-xL protein stability (Figure 23).

GILZ and Bcl-xL Localize to the Perinuclear Space in the Presence of CT

Bcl-xL normally resides outside of the mitochondrial membrane and helps to maintain mitochondrial membrane integrity and protect against apoptosis. To test whether GILZ physically associates with Bcl-xL, we performed immunocytochemistry following the stimulation of CT, which induces GILZ

Antibodies against Bcl-xL (Cell Signaling) and GILZ (Santa Cruz) were incubated with H9C2 cardiomyocytes treated with 1 μM CT. Fluorescently labeled probes against rabbit or mouse antibody were used to detect localization of Bcl-xL and GILZ. Both GILZ and Bcl-xL were detected in the perinuclear region after CT treatment (Figure 6). The colocalization of GILZ and Bcl-xL to perinuclear region suggest that these two proteins may physically interact.

GILZ Physically Associates with Bcl-xL

In order to demonstrate a physical interaction between GILZ and Bcl-xL, H9C2 or primary neonatal rat cardiomyocytes were treated with 1 μM corticosterone (CT) or 1 μM dexamethasone (Dex), respectively, for immunoprecipitation (IP) of GILZ to detect Bcl-xL. Western blot analyses revealed the presence of Bcl-xL in GILZ immunocomplex, supporting an association between GILZ and Bcl-xL. Such interaction was observed in both H9C2 cells and primary cultured neonatal rat cardiomyocytes after GC treatment (Figure 25).

GILZ Binds to $PGAM_5$ in the Presence of CT

Bcl-xL degradation by the proteasomes is mediated by its interaction with Phosphoglycerate Mutase 5 ($PGAM_5$) [186]. $PGAM_5$ forms a redox regulated KEAP1 dependent ubiquitin ligase complex, providing a mechanism for the degradation of Bcl-xL in redox sensitive manner. Under this scenario, Bcl-xL is

targeted for proteasomal degradation following formation of a Bcl-xL/$PGAM_5$/KEAP1 complex. We tested the potential for GILZ in disrupting PGAM5 and Bcl-xL interaction. If so, GILZ may interact with PGAM5 to displace the interaction of PGAM5 with Bcl-xL. Immunoprecipitation of GILZ was performed using H9C2 cells collected after treatment of CT. Western blot analyses were performed with the immunocomplex of GILZ. Preliminary data suggests that GILZ and $PGAM_5$ indeed interact in the presence of CT (Figure 26).

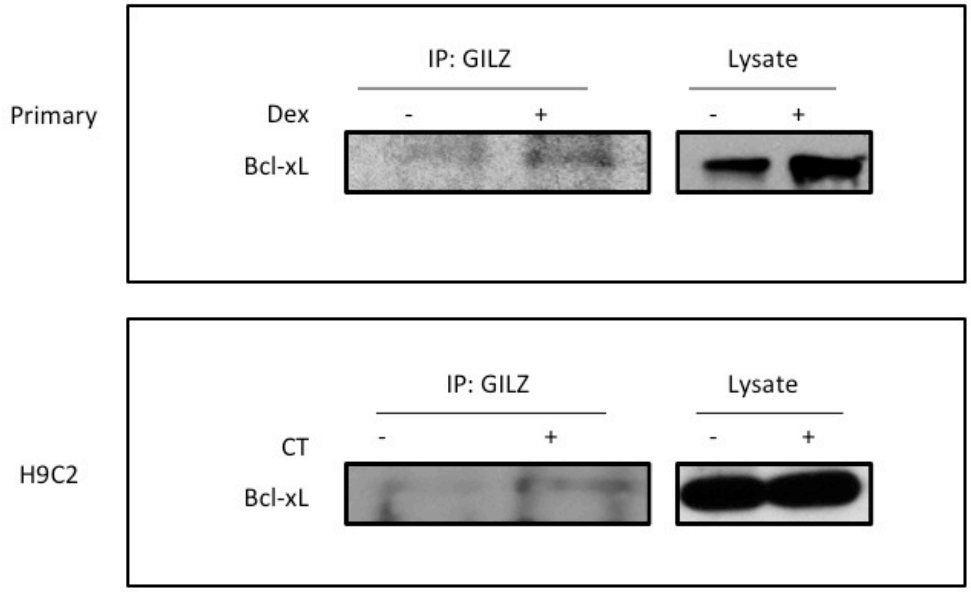


Figure 25: GILZ interacts with Bcl-xL directly *in vivo* and *in vitro*. Immunoprecipitation of GILZ and western blot for Bcl-xL in primary neonatal cardiomyocytes dosed with dexamethasone for 8 hours (A). Immunoprecipitation of GILZ and western blot analysis of Bcl-xL in H9C2 cardiomyocytes dosed with CT for 8 hours (B).

Figure 26: Immunoprecipitation of GILZ and western blot analysis for PGAM5 and H9C2 cardiomyocytes dosed with CT for 8 hours.

Discussion

GILZ is a novel GC-induced protein mediating cytoprotection in cardiomyocytes. In this study, we found that Bcl-xL protein level increases with an increased dosage of GILZ plasmid by transfection in H9c2 cardiomyocytes (Figure 20). Data with inhibitors of calpain or proteasomes support that Bcl-xL protein is primarily degraded by the proteasome in H9C2 cardiomyocytes (Figure 23). We have identified GILZ as a novel binding partner of Bcl-xL and this interaction occurs in the perinuclear region of the cell (Figure 24). Such interaction may displace Bcl-xL's interaction with PGAM5, which mediates Bcl-xL degradation by the proteasomes. The protein-protein interaction of GILZ with Bcl-xL described here offers a novel insight of GC-induced protection in cardiomyocytes.

GILZ interaction with Bcl-xL may disrupt Bcl-xL shuttling to the proteasome. Hannink et al [186] reported that Bcl-xL interacts with the C-terminus of $PGAM_5$. $PGAM_5$ forms a complex with KEAP1, which recruits ubiquitin ligase and facilitates the shuttling of substrate proteins to the proteasome. We report an interaction between GILZ and $PGAM_5$ (Figure 26). GILZ physical association with Bcl-xL may potentially impede formation of a Bcl-xL/$PGAM_5$/KEAP1 complex, thereby preventing proteasomal degradation of Bcl-xL.

Bcl-xL is known to localize to the mitochondria. Immunocytochemistry studies indicate the colocalization or GILZ with Bcl-xL. The interaction of Bcl-xL with GILZ suggests the possibility that GILZ may enhance the role of Bcl-xL in

protecting against mitochondrial release of cytochrome c, which triggers formation of apoptosomes and initiation of apoptosis.

GCs have been reported to produce diverse tissue specific effects. It is reasonable to predict proteins induced by GCs, such as GILZ, would also exhibit tissue specific effects. GILZ has been reported to decrease levels of Bcl-xL in transgenic mice overexpressing GILZ in T-cell lineage. In human embryonic kidney 293 (HEK 293) cells, Bcl-xL did not increase due to GILZ overexpression, implying that the observed induction of Bcl-xL is a cardiomyocyte specific phenomenon with GILZ overexpression.

Identifying and mapping the GILZ domain responsible for the GILZ/Bcl-xL interaction will be essential for characterizing how this interaction occurs. GILZ protein has four well defined domains: N-terminal domain (1-60aa), TSC box (61-75aa), central leucine zipper (76-97aa) and C-terminal proline and glutamic acid rich region (98-137aa). Ras, Raf and NFκB interact with GILZ through N-terminal domain, Raf binding domain and C-terminal (proline and glutamic acid rich region) respectively [81]. Future studies targeting these domains will determine how the GILZ/Bcl-xL interaction occurs in cardiomyocytes to promote Bcl-xL stability.

Currently, there is no literature describing the stabilization of Bcl-xL by GILZ. Our finding is significant because it offers new insight into the regulation of Bcl-xL, a pro-survival protein frequently studied in heart disease, cancer and other maladies. Identification of the GILZ domain responsible for the stabilization of

Bcl-xL offers clinical importance in that it provides a template for generating molecules that mimic the action of GILZ for stabilizing Bcl-xL. Such approach can bypass the deleterious effects of GC-treatment in patients.

CHAPTER 6: SUMMARY AND PERSPECTIVES

I have established that 1) H9C2 cardiomyocytes are a valid cell model to study the significance of GILZ (Chapter 3); 2) GILZ encodes a cytoprotective gene in cardiomyocytes (Chapter 4); 3) GILZ induces elevation of Bcl-xL protein through protein-protein interaction (Chapter 5). The data presented in Chapter 3 include characterization of GILZ expression in cardiomyocytes *in vivo* and *in vitro.* Chapter 4 has identified GILZ as a necessary mediator of cytoprotection elicited by GCs against doxorubicin-induced apoptosis in cardiac cells. Additionally, the work demonstrates that GILZ alone is sufficient to induce protection against Dox exposure in cardiomyocytes through the stabilization of Bcl-xL protein. We have identified a novel mechanism of GILZ-mediated cytoprotection whereby GILZ stabilizes Bcl-xL through protein-protein interaction that prevents Bcl-xL degradation by the proteasomes.

GILZ has previously been reported to mediate a number of cellular responses in different tissue types. Since its discovery, GILZ has been shown to modulate T-Cell activation by inhibiting NF-κB [74], decrease Bcl-xL transcription in thymocytes of transgenic mice overexpressing GILZ in T-Cell lineage [75], and diminish proliferative activity mediated by Ras signaling [76]. Much of the work investigating the significance of GILZ in cellular physiology has been done in lymphocytes. There has been no research investigating GILZ function in the cardiomyocytes. This is the first study to date investigating the expression

patterns and functional significance of GILZ in cellular model relevant to the heart.

GILZ induction *in vivo* and *in vitro* follows similar expression patterns in primary cultured rat cardiomyocytes and H9C2 rat cardiomyocyte cell line In addition to cardiomyocytes in culture, we established that GC administration *in vivo* induces GILZ expression in mice hearts (Chapter 3). These data support our previous finding that GILZ mRNA is induced by GCs in cardiomyocytes as determined by gene chip microarray [35]. Here we have established H9C2 as a valid *in vitro* model system to study GILZ significance, since the response of GILZ induction by GCs is comparable with primary neonatal cardiomyocyte cultures. Several cardiomyocytes cellular model systems exist. For example, cardiomyocytes derived from inducible pluripotent stem cells (iPS) have been shown to beat spontaneously in vitro [187]. This model has a particular advantage over non-human model systems, retaining genetic similarity and maintaining a consistent functional phenotype *in vitro*. Another commonly used cardiomyocyte cell line is the HL-1 cell line derived from mice. This cell line also maintains the ability to contract [144]. Although these cell lines are powerful tools to study GILZ regulation and function, they are expensive to obtain and maintain in culture. Rat neonatal cardiomyocytes are another model that contracts in culture and are commonly used by laboratories. They are also powerful tools in cardiac studies, but preparing cardiomyocytes from neonatal rats is an expensive, time and labor consuming process. The preparation often

contains fibroblasts. H9C2 cells are easily maintained and retain many characteristics of cardiomyocytes. Once significance has been established in H9C2 cells, findings would then be further validated in an *in vivo* system. We found that GILZ is induced in hearts of mice administered with 20 mg/kg dexamethasone. This concentration is consistent with our previous study reporting a protective role of dexamethasone in myocardial injury [70]. Since such dose is quite high, varying concentrations of dexamethasone can determine the dosing range of dexamethasone necessary to induce GILZ *in vivo*, and defining the minimal necessary dose for cardiac protection Because GC serum concentrations have been shown to vary during seasonal changes as well as under stress [188], it would be necessary to address whether the myocardial level of GILZ protein changes according to variations of GC concentration in the blood.

Upon establishing H9C2 cardiomyocytes as a valid in vitro model to study GILZ, we aimed to determine its functional significance. As stated previously, most research involving GILZ has been limited to lymphocytes and there is currently no research investigating GILZ function in the heart. Previous reports indicate GILZ contributes to several GC actions including lymphocyte apoptosis and anti-inflammatory effects. Given GILZ potential to mediate GC-stimulated effects, we investigated a role of GILZ in mediating GC-induced cytoprotection in cardiomyocytes. We have shown that GILZ indeed elicits cytoprotection against the apoptotic effect of the known cardiac toxicant Dox (Chapter 4). This was

determined by challenging H9C2 cardiomyocytes over-expressing GILZ either stably or transiently with Dox and measuring apoptosis or necrosis by several methods including changes in morphology, caspase activity assay or MTT assay (Chapter 4). Our findings suggest for the first time that GILZ contributes to GC-mediated cytoprotection in cardiomyocytes.

GILZ is necessary for GC induced cytoprotection. This was concluded based on the data from knocking down GILZ by siRNA and subsequently challenging H9C2 cardiomyocytes with Dox. A similar experiment was performed by our lab where the pro-survival protein Bcl-xL was knocked and Dox was administered to primary neonatal cardiomyocytes under similar conditions [35]. GILZ is necessary for GC-induced cytoprotection much like Bcl-xL in that study (Chapter4). Interestingly, this finding is contrary to what was observed in transgenic mice overexpressing GILZ in T-cell lineage [75]. There was marked decrease in Bcl-xL observed and that decrease was suggested to contribute to apoptosis in lymphocytes. Overexpression of GILZ in thymic hybridoma cells selectively protects from activation-induced cell death, as triggered by anti-CD3 monoclonal antibody (mAB) but not from apoptosis induced by other stimuli. This further supports that GILZ has cell-dependent effects as seen by other research groups. T-cell specific adaptor protein (TSAd) promotes chemokine-induced migration and cytoskeletal rearrangements of human and murine T cells [189]. Tissue specific adaptor proteins such as TSad are capable of shifting protein localization within a cell by rearranging the cytoskeleton and this may partly

explain tissue specific effects elicited by GILZ. In our studies, we demonstrate that in another cell type, HEK293 cells, GILZ does not induce Bcl-xL. The diverse role of GILZ in regulating apoptosis reflects the same diversity GC elicits in regulating apoptosis, since GCs have been reported to promote survival as well as inhibit apoptosis among various cell types [69].

Based on my research, it is reasonable to conclude that GILZ alone would promote cytoprotection in cardiomyocytes in a way similar to GCs. Future animal studies would verify a cytoprotective role of GILZ in the heart. This can be accomplished by pre-conditioning mice with GCs or vehicle control for 24 hours and subsequently challenging the mice with a high dose Dox over a short period of time to induce acute cardiotoxicity. Animals would then be sacrificed and hearts harvested to determine the degree of Dox-induced pathology. Levels of GILZ can be determined by Western blot analyses in GC-treated or vehicle control treated mice, which can then be used to correlate cytoprotection with GILZ protein levels.

Additionally, transgenic mice can be constructed to express GILZ specifically in the heart by placing the GILZ coding sequence downstream of cardiac αMHC promoter. These animal models will provide powerful tools for *in vivo* studies investigating GILZ role in mediating stressful events such as ischemia-reperfusion injury and myocardial infarction, in addition to cardiomyopathy induced by Dox. In addition to end-point pathology, echocardiography could be used to monitor the hearts of mice. Furthermore, the

transgenic model will be useful in determining GILZ function in the developing heart. We would predict, based on our data, that the GILZ transgenic mice will be protected from Dox-induced cardiotoxicity.

Additional survival-related proteins, including other Bcl-2 family members, were screened for changes by western blot analysis. Most of the screened proteins, including the pro-apoptotic BH3-only family members Bax and Bak, showed no expression changes in cardiomyocytes over-expressing GILZ. We did observe a decrease in p21, a potent cyclin dependent kinase inhibitor known to contribute to apoptosis (Chapter 5). Interestingly, a decrease in the pro-survival protein, Bcl-2, protein was observed. This would suggest increased sensitivity to apoptosis, however, the upregulation of Bcl-xL protein is up to ten times more potent in maintaining cell survival than Bcl-2 [181]. It is, therefore, probable a certain ratio of Bcl-xL to Bcl-2 favors survival in cardiomyocytes. Regardless, the mechanism for the decrease in Bcl-2 needs to be investigated further.

GILZ mediates cell survival in H9C2 cardiomyocytes by preventing Bcl-xL degradation. In order to determine the mechanism behind the increase in Bcl-xL levels, we next investigated whether induction of Bcl-xL protein is the result of transcriptional regulation. qRT- PCR experiments of GILZ confirmed the increase in Bcl-xL protein was independent of transcription (chapter 5). Bcl-xL promoter activity was also similar between empty vector and GILZ-transfected cells (Chapter 5). NFκB transcriptional activity has been reported to regulate Bcl-

xL expression in H9C2 cardiomyocytes [145, 190]. Since GILZ has been shown to bind and inhibit NFκB activity, we would expect a repressed transcriptional activity of Bcl-xL gene in cells over-expressing GILZ. Increased levels in Bcl-xL protein by GILZ are, therefore, due to post-transcriptional processes. GILZ regulation of Bcl-xL protein in cardiomyocytes is novel mechanism, and prompted us to investigate mechanisms by which this occurs.

Protein levels of Bcl-xL in cells can be reduced via cytosolic proteolysis or by shuttling to the proteasomes for degradation with adaptor proteins. Data presented in this study suggests Bcl-xL half-life is primarily mediated by proteasomal degradation in H9C2 cardiomyocytes (Chapter 5). Bcl-xL half life in H9C2 cardiomyocytes over-expressing GILZ was increased, suggesting GIlZ may inhibit proteasomal degradation of Bcl-xL (Chapter 5). Inhibiting degradation may result from direct binding of GILZ to Bcl-xL or GILZ effect on carriers shuttling Bcl-xL to the proteasome. $PGAM_5$ is a substrate for Cul3 dependent E3 ubiquitin ligase and has been identified as a Bcl-xL interacting protein [186]. Preliminary data suggests GILZ may directly bind to both $PGAM_5$ and Bcl-xL (Chapter 5). The presence of GILZ in the complex may disrupt shuttling of Bcl-xL to the proteasomes (Figure 27).

GILZ contains several domains known to interact with a variety of partners, which prompted us to investigate whether a direct protein/protein interaction between GILZ and Bcl-xL was responsible for the decrease in Bcl-xL turnover. Immunocytochemistry revealed GILZ and Bcl-xL co-localization in the

perinuclear region of H9C2 cardiomyocytes (Chapter 5). This region is where Bcl-xL normally resides on the outer mitochondrial membrane [145]. The colocalization suggests that stabilization of Bcl-xL by GILZ potentially occurs on or in proximity to the mitochondrial membrane, and thus, would be ideally situated to maintain mitochondrial function and prevent mitochondrial outer membrane permeabilization (MOMP) for cytochrome c release into the cytosol. Further experiments using mitochondrial markers, such as mito-ds-red or mitotracker red, will verify the localization of GILZ with the mitochondria.

To determine overall changes attributed to GILZ over-expression in the proteome, 2D gel electrophoresis was employed and followed by Coomassie blue staining to detect changes in the array of proteins. A unique spot corresponding to the approximate size of Bcl-xL was observed around 32 kD (Chapter 5). Mass spectrometric analysis of peptides extracted from the spot would determine the protein's identity. If the spot is Bcl-xL, determination of unique post-translational modifications on Bcl-xL in cardiomyocytes may shed light into the mechanism of GILZ-mediated stabilization of Bcl-xL.

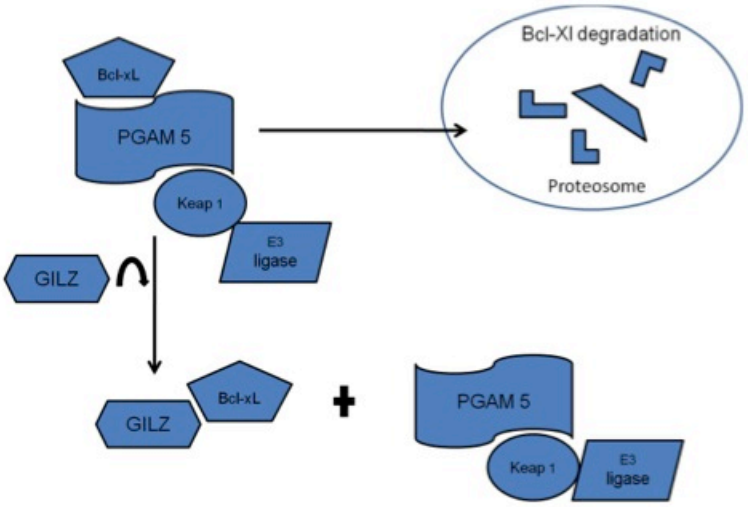


Figure 27: Proposed mechanism of GILZ mediated inhibition of Bcl-xL degradation. We hypothesize GILZ interacts with PGAM5, a substrate for keap 1 adaptor protein and prevents ubiquitination of Bcl-xL by the proteasome

The domain of GILZ responsible for the binding of Bcl-xL may provide a novel tool for controlling Bcl-xL stability. Increases in Bcl-xL and other pro-survival Bcl-2 family members have been well described in cancer where cells have mechanisms to increase survival and evade apoptosis. There is a potential for similar binding motifs, such as a specific domain of GILZ, to be used for screenings for proteins of similar action or as a target for novel anti-cancer therapies.

Mutational analysis by deleting various GILZ domains will provide insight as to which domains are critical for changes observed in Bcl-xL stabilization in cardiomyocytes. Such study has a profound implication in medicine to prevent cardiac injury, especially cardiotoxicity resulting from many chemotherapeutic drugs. Concurrent GC treatment with one of these cardiotoxic agents, especially in cancer treatment, may reduce incidence of heart failure and allow longer life spans for those patients. A limitation to this concurrent treatment would be the immunosuppressant action of GCs. In a condition such as cancer where a patient's immune system may be important in fighting the disease, this could potentially worsen the cancer prognosis. However, knowledge of the molecular mechanism involving GILZ may lead to the availability of better targeted therapeutics that do not have immunosuppressant activity of GCs while allowing

for increased cardiomyocyte survival. Alternatively, this could also be utilized in improved treatments for cancer that target to cell survival pathways.

Heart disease continues to be the leading cause of death in the United States. Investigating novel glucocorticoid-induced cytoprotective molecules that are able to promote increased viability of heart cells will be critical for creating new treatments for heart disease. This study describes a novel GC-induced cytoprotective protein, GILZ, which alleviates Dox-induced toxicity in cardiomyocytes. The studies also show that the effect is due to increased stability of Bcl-xL offering more insight into mechanisms of cell survival, which may be used for treatments for a wide variety of diseases including heart disease and cancer.

In conclusion, GILZ is a novel GC induced cytoprotective protein in the heart. Findings from these studies provide for the first time a descriptive analysis of GC-induced GILZ expression in both *in vivo* and *in vitro* cardiomyocytes. Furthermore, we have described remarkable similarities in GILZ expression between H9C2 cardiomyocytes and primary neonatal cardiomyocytes. Our findings suggest H9C2 cardiomyocytes are a valid *in vitro* model to study the significance of GILZ in in vivo cardiomyocytes. Additionally, the findings presented highlight GILZ's cytoprotective role in cardiomyocytes through alleviating doxorubicin-induced apoptosis. Additionally, we have shown GILZ not only contributes to GC-induced cytoprotection, but is a necessary component of the process. Finally, we have described a mechanism by which GILZ elicits

cytoprotection in where Bcl-xL protein levels are increased in the presence of GILZ. We show that the increase in Bcl-xL is due to GILZ preventing Bcl-xL degradation by the proteasome. Additional work will address the mechanism of Bcl-xL stability by GILZ and other critical proteins involved in the cytoprotection in the heart.

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