UNIVERSITY OF CALIFORNIA, SAN DIEGO

The role of CaMKII-delta subtypes

in myocardial ischemia/reperfusion injury

A dissertation submitted in partial satisfaction of the requirements of the degree Doctor of Philosophy

in

Biomedical Sciences

by

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The dissertation of Charles Burdis Burns Gray is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2016

DEDICATION

I dedicate this dissertation to John Edward Gray

EPIGRAPH

"Don't feed the plants, feed the soil"

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AMVM	Adult mouse ventricular myocytes
BMS	BMS-345541
Ca^{2+}/CaM	Calcified calmodulin
CaMKII	Ca^{2+}/CaM-dependent protein kinase II
CaSpF	Ca^{2+} spark frequency
CD68	Cluster of differentiation 68
CNS	Central nervous system
CsA	Cyclosporine-A
FDAR	Frequency dependent acceleration of relaxation
GPCRs	G protein–coupled receptors
G_qTG	G_q transgenic mice
HRP	Horseradish peroxidase
I/R	Ischemia/reperfusion
IKK	IκB kinase
IL-6	Interleukin-6
ISO	isoproterenol
KO	CaMKII-delta knockout
LVEDP	Left ventricular end-diastolic pressure
MI	Myocardial infarction
NF-κB	nuclear factor-κB
NLS	Nuclear localization signal
NRVMs	Neonatal rat ventricular myocytes
NRVMs	Neonatal rat ventricular myocytes
PE	Phenylephrine
PKA	Protein kinase A
PLN	Phospholamban
PPARα	Peroxisome proliferator–activated receptor α
PTMs	Posttranslational modifications
Q209L	Active G_q
RIP3	Receptor-interacting protein kinase 3
ROS	Reactive oxygen species
RyR2	The cardiac ryanodine receptor
SR	Sarcoplasmic reticulum
TAC	Trans-aortic constriction
UCP3	Uncoupling protein 3
WT	Wild-type
α-MHC	α-myosin heavy chain

LIST OF FIGURES

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The background information presented in Chapter 1 contains excerpts from a review article published in the journal *Frontiers in Pharmacology* of which I was the primary author along with co-author Joan Heller Brown.

Work presented in Chapter 2 is reprinted with minor modifications from *Circulation Research*. Haiyun Ling, Shikha Mishra were the primary authors of this work, to which the dissertation author made significant contributions along with co-authors Shigeki Miyamoto, Alexander Zambon, Nancy Dalton, Kirk Peterson, Yusu Gu, Don Bers, and Nicole Purcell. Additional work presented in Chapter 2 will be submitted to *Circulation Research* following its ongoing revision with co-authors B Daan Westenbrink, Sunny Xiang, Shikha Mishra, Shigeki Miyamoto, and Joan Heller Brown. The dissertation author was the primary author of this work.

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Charles B. B. Gray B.S., B. Daan Westenbrink PhD, Sunny Xiang PhD, Shikha Mishra PhD, Shigeki Miyamoto DVM PhD, and Joan Heller Brown PhD. CaMKIIδ subtypes differentially regulate infarct formation following *ex vivo* myocardial ichemia/reperfusion through NF-κB and TNF-α. *Circulation Research* 2016 (*in revision)*

ABSTRACT OF THE DISSERTATION

The role of CaMKII-delta subtypes

in myocardial ischemia/reperfusion injury

by

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Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2016

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Ca^{2+} dysregulation underlies many forms of cardiac stress and contributes significantly to arrhythmia and dysfunction during heart disease. Ca^{2+}/CaM-dependent protein kinase II (CaMKII) is a multifunctional kinase that is activated by intracellular Ca^{2+}, and signals through a variety of substrates to

regulate cardiomyocyte biology. Elevated CaMKII activity has been associated with a range of pathologies including Ca^{2+} leak from the sarcoplasmic reticulum, mitochondrial dysfunction, and cell death. The predominant form of CaMKII in the heart is CaMKIIδ, which is alternatively spliced to yield the $CaMKII\delta_B$ and $CaMKII\delta_C$ subtypes. This dissertation describes the role of CaMKIIδ in cardiac stress generally, and specifically the role of the $CaMKII\delta_B$ and $CaMKII\delta_C$ subtypes in ischemia/reperfusion injury. Chapter 1 provides background information on the role of CaMKIIδ in cardiovascular disease and the structure and function of $CaMKII\delta_B$ and $CaMKII\delta_C$. Chapter 2 describes the majority of the dissertation, focusing on studies on the subcellular distribution of CaMKIIδ subtypes and their role in ischemia/reperfusion injury and inflammatory signaling downstream of NF-κB. Chapter 3 contains studies on the involvement of CaMKIIδ in pathological signaling during cardiac stress elicited by chronic β-adrenergic stimulation and G_q overexpression. Chapter 4 describes ongoing and future studies and the relevance of these findings to human disease.

Chapter 1

CaMKII subtype localization and location-specific activation

1.1 Summary

CaMKIIδ is the predominant cardiac isoform of CaMKII and is alternatively spliced to generate the CaMKIIδ$_B$ and CaMKIIδ$_C$ subtypes. CaMKIIδ$_B$ and δ$_C$ possess similar catalytic activity and sensitivity to Ca^{2+}/CaM. Furthermore, both subtypes can undergo autophosphorylation and acquire a similar degree of Ca^{2+}-independent or autonomous activity[1-3]. Despite the broad similarities between the two subtypes, transgenic mice that express CaMKIIδ$_B$ or CaMKIIδ$_C$ in cardiomyocytes display markedly distinct phenotpyes. CaMKIIδ$_C$-expressing animals rapidly develop heart failure and exhibit premature death, while CaMKIIδ$_B$-expressing animals have a mild hypertrophic phenotype[4-6]. Based on the inclusion of a nuclear localization signal (NLS) in CaMKIIδ$_B$, the different phenotypes of CaMKIIδ$_B$- and CaMKIIδ$_C$-expressing animals were initially thought to primarily relate to differential localization of the two subtypes. The goal of the thesis research presented here is to extend knowledge regarding localization and function of the CaMKIIδ$_B$ and CaMKIIδ$_C$ subtypes and to provide insight into regarding the mechanisms by which CaMKIIδ$_B$ and CaMKIIδ$_C$ elicit their distinct effects.

1.2 CaMKIIδ structure and function

Calcium/calmodulin dependent protein kinase II (CaMKII) isoforms are multifunctional, in that they have many targets and can serve as nodal regulators of a diverse range of biological processes. CaMKII exists as a multimeric enzyme consisting of distinct subunits which can be encoded by any of four different genes, CaMKIIα, β, γ, and δ. These genes have a high degree of sequence homology but show differential tissue expression. CaMKIIα and β are predominantly expressed in neuronal tissue while γ and δ are present throughout the body, including the heart[7-9]. CaMKII substrates include but are not limited to molecules involved in cell survival, contractile function, regulation of Ca^{2+} homeostasis, inflammation, and gene transcription. In the central nervous system (CNS), CaMKIIα and β regulate various aspects of learning and memory as well as neuronal action potentials. In the heart, CaMKIIδ has been shown to participate in processes central to both physiological and pathophysiological regulation, such as frequency dependent acceleration of relaxation (FDAR) and diastolic sarcoplasmic reticular (SR) Ca^{2+} leak[10-12]. All of the isoforms of CaMKII have a catalytic domain, a regulatory domain, a variable segment, and an association domain. The regulatory domain features a pseudosubstrate site which binds to the catalytic domain and blocks its ability to phosphorylate proteins, while the catalytic domain has several binding sites for ATP and other substrate anchor proteins and is responsible for the transfer of phosphate from ATP to serine or

threonine residues within CaMKII substrates. The association domain assembles single CaMKII proteins into dodecamers (Figure 1-1), while the variable segments of CaMKII genes are inserted or deleted during splicing. Differences in these variable domains accounts for the distinct amino acid sequences of the CaMKII subtypes[3, 11-35].

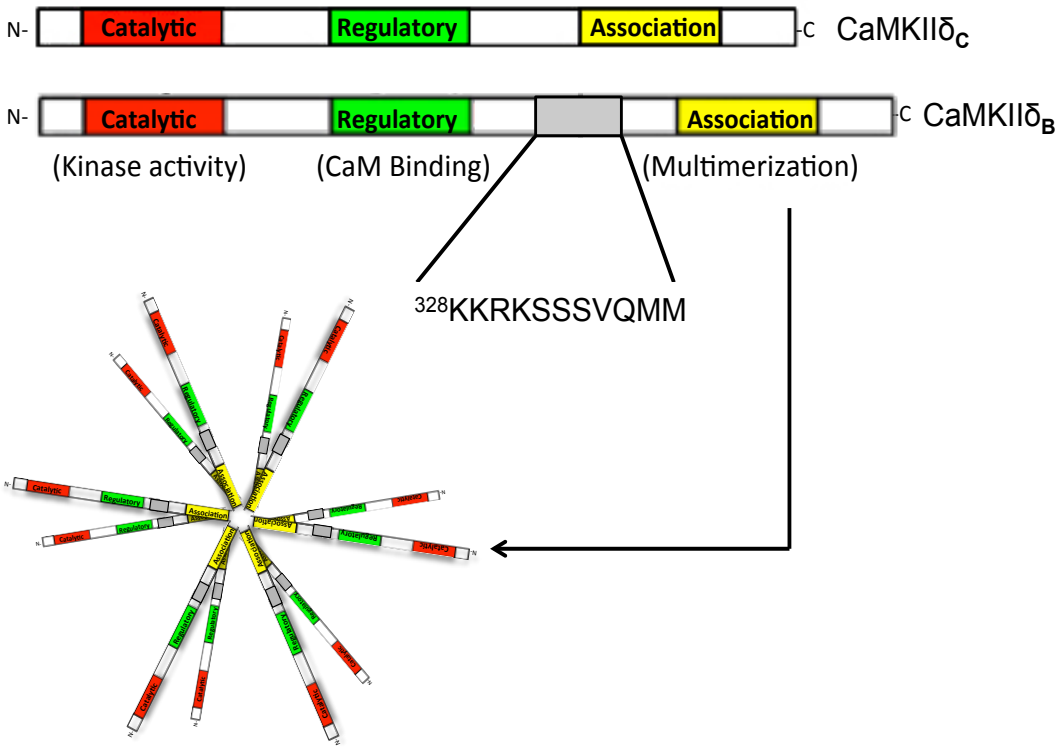

Figure 1-1. Structure of CaMKIIδ subtypes. The kinase domain of CaMKIIδ$_B$ and CaMKIIδ$_C$ is depicted in red, the regulatory domains of the two splice variants are depicted in green. In gray, the 11-amino acid insert that contains an NLS that is present in CaMKIIδ$_B$ is depicted. The mulitermization domain of the kinase is depicted in yellow, and the structure of the CaMKII dodecameric holoenzyme is indicated with an arrow.

In the absence of any activating stimuli, the catalytic domain and regulatory domain associate with one another and prevent CaMKII substrate phosphorylation. A number of different mechanisms collaborate to regulate the activity of CaMKII. Following binding of calcified calmodulin (Ca^{2+}/CaM), autoinhibition is relieved by the dissociation of the catalytic and regulatory domains and subsequently CaMKII autophosphorylation of threonine 286 can occur. Phosphorylation of this site sterically inhibits binding of the autoinhibitory domain to the catalytic domain. This enables CaMKII to be active, even in the absence of elevated calcium-liganded calmodulin (Figure 1-2). In addition to phosphorylation, other activating posttranslational modifications of CaMKII have been described including oxidation, nitrosylation, and O-GlcNAcylation[16-20]. These additional posttranslational modifications alter CaMKII in a similar fashion to auto phosphorylation, in that they prevent the reassociation of the catalytic and regulatory domains following Ca/CaM binding. Increases in reactive oxygen species (ROS) that occur in the heart for a variety of reasons lead to methionine oxidation and a similar degree of activation of CaMKII as would occur via autophosphorylation. More recently, modifications such as O-GlcNAcylation and s-nitrosylation have been identified and shown to serve as activating modifications of CaMKII. Thus, CaMKII is not only able to phosphorylate and functionally regulate a dizzying number of substrates when active, it is also able to receive signaling input from a wide variety of cellular processes that increase CaMKII

posttranslational modification. Emerging research has implicated these modifications in multiple physiological and pathophysiological settings.

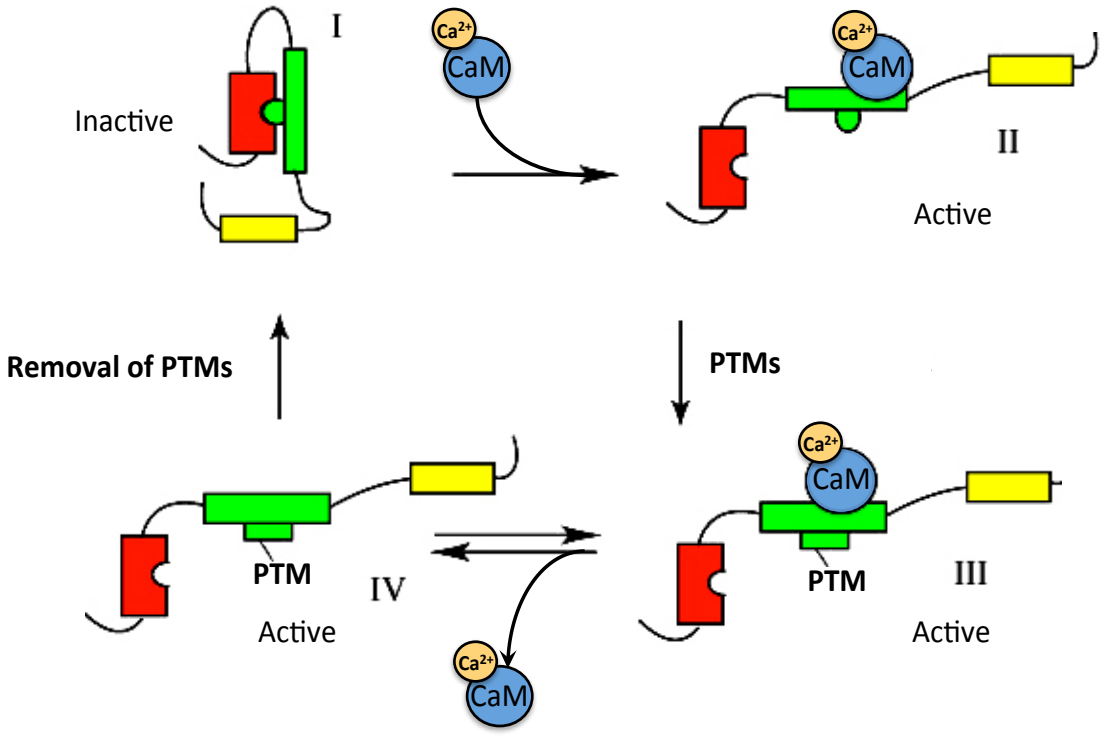

Figure 1-2. Mechanism of CaMKII activation. Upon binding of Ca^{2+}-liganded calmodulin (Ca^{2+}/CaM), CaMKII is activated. Subsequently, a number of posttranslational modifications (PTMs) can occur in the regulatory domain of the kinase which prevent re-association of the catalytic and regulatory domains, rendering the kinase active even in the absence of Ca^{2+}/CaM. CaMKII only returns to an inactive state upon removal of these PTMs.

In addition to activating modifications of CaMKII, there are several posttranslational modifications that are inhibitory. For example, an inhibitory s-nitrosylation site has recently been characterized. Both activating and

inhibitory autophosphorylation sites are present in the same region of the kinase, however inhibition is largely understudied in the heart. In the brain however, CaMKIIα inhibitory autophosphorylation has been shown to regulate localization of the kinase[17, 19].

1.3 CaMKIIδ subtypes: localization and function

CaMKIIδ, the predominant cardiac isoform, is alternatively spliced to generate multiple subtypes. Initial reports described distinct mRNAs with differential expression patterns and termed them CaMKIIδ$_B$ and CaMKIIδ$_C$[1]. These splice variants were shown to be identical except for the insertion of an 11-amino acid sequence in the variable domain of CaMKIIδ$_B$ (Figure 1-1), the slightly more abundant of the two subtypes in the heart. Subsequently, their catalytic activity and regulation by calcium-liganded calmodulin (Ca^{2+}/CaM) was characterized. CaMKIIδ$_B$ and δ$_C$ possess similar catalytic activity and sensitivity to Ca^{2+}/CaM. Furthermore, both subtypes can undergo autophosphorylation and acquire a similar degree of Ca^{2+} independent or autonomous activity [2].

The 11-amino acid insert in CaMKIIδ$_B$ (^{328}KKRKSSSSVQMM) is also present in some splice variants of CaMKIIα and γ; this conservation suggests an important function[21]. When constructs of CaMKIIδ$_B$ are transfected into fibroblasts the expressed protein is localized to the nucleus. This is not the

case for constructs of CaMKIIδ$_C$, implying that that the additional amino acid sequence present in CaMKIIδ$_B$ is responsible for nuclear localization. A similar differential localization pattern was also observed when CaMKIIδ subtypes were expressed neonatal rat ventricular myocytes (NRVMs)[22]. Further studies showed that the 11-amino acid insert in CaMKIIδ$_B$ can confer nuclear localization when inserted into the variable domain of CaMKIIα and that mutagenesis of the first two lysines in the insert abrogates the nuclear localization of these constructs. Thus it is widely accepted that the CaMKIIδ$_B$ variable domain contains a nuclear localization signal (NLS).

CaMKII heteromultimerization is permissive in that the CaMKII holoenzyme can include multiple CaMKII isoforms and multiple splice variants of those genes[7, 9]. It seems likely that, for the predominant cardiac isoform CaMKIIδ, more than a single CaMKIIδ subtype is present in a single multimer and accordingly the ratio of δ$_B$ to δ$_C$ in a multimer could regulate the localization of the holoenzyme. This has been demonstrated experimentally. When CaMKIIδ$_B$ and δ$_C$ are cotransfected into fibroblasts or NRVMs, the localization of the expressed protein can be shifted in accordance with the ratio of the expressed CaMKIIδ subtypes, i.e., high expression of δ$_C$ sequesters δ$_B$ in the cytosol and blocks its nuclear localization. The opposite is also true: high relative expression of δ$_B$ can localize δ$_C$ to the nucleus.

Transgenic animals expressing δ$_B$ or δ$_C$ exhibit strikingly different phenotypes. Animals expressing δ$_B$ develop a mild hypertrophic phenotype

associated with increases in cardiac fetal gene expression while those expressing δ_C spontaneously develop heart failure and premature death[5]. A number of studies in our lab and others have established that expression of CaMKIIδ_C is associated with deleterious changes in Ca^{2+}-handling and inflammatory signaling which can predispose to cardiomyocyte cell death[4, 23-30](Figure 1-3). The main focus of my thesis was to elucidate mechanisms by which δ_B and δ_C elicit such distinct phenotypes in the context of cardiac stress or injury.

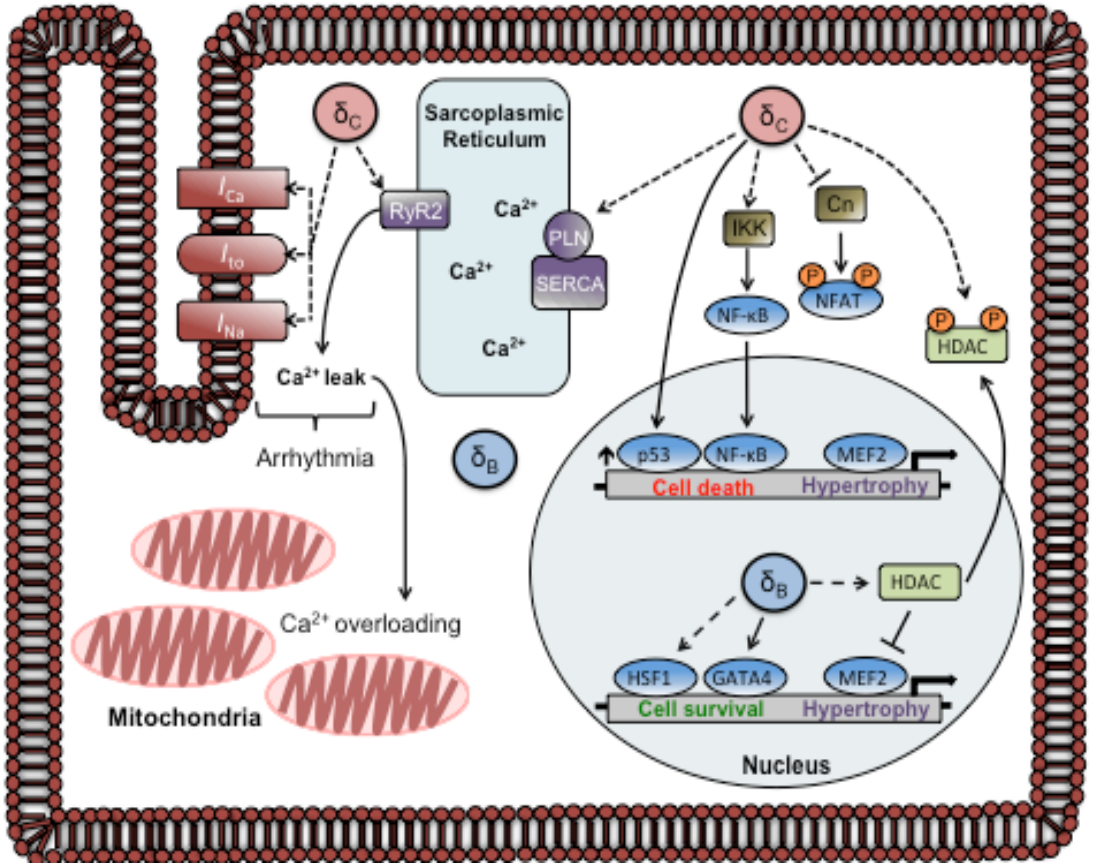

Figure 1-3. Regulation of Ca^{2+} and gene transcription by CaMKIIδ subtypes. CaMKIIδ$_C$ phosphorylates and regulates a number of channels at the plasma membrane resulting in Ca^{2+} leak, arrhythmia, and mitochondrial Ca^{2+} overloading. CaMKIIδ$_C$ also regulates gene transcription primarily through substrate phosphorylation outside of the nucleus, while CaMKIIδ$_B$ is thought to regulate nuclear events in CaMKIIδ-mediated gene transcription.

1.4 CaMKIIδ in myocardial disease

During cardiac stress induced by pressure overload or ischemia/reperfusion, alterations in Ca^{2+} handling are known to occur as are increases in the levels of cellular ROS[31, 32]. These changes would seem likely to modulate CaMKII activity, and it has been proposed that altered activation

of CaMKII is an essential part of the deleterious phenotype that results from increases in intracellular [Ca^{2+}] and ROS, which typically precede the development of myocardial dysfunction and/or disease. CaMKIIδ has been implicated in the development of heart failure through a variety of observations. First, upregulation of CaMKIIδ expression and activity have been reported to be a general feature of heart failure in humans and in animal models[14, 33, 34]. Further evidence linking CaMKIIδ and heart failure comes from studies demonstrating that blocking CaMKIIδ by either pharmacological inhibition or genetic approaches prevents progression to heart failure-associated phenotypes in animal models of structural heart disease[35-40].

Myocardial hypertrophy is the evolutionarily conserved cardiac reaction to hemodynamic overload or injury. This genetically programmed response improves ventricular ejection performance by restoring a more normal ratio of left ventricular wall thickness to intracavitary pressure. While initially compensatory, reactive hypertrophy inevitably “decompensates” and fails. Thus, cardiac hypertrophy is an independent risk factor for both heart failure and death[41-43]. Mice in which CaMKIIδ is genetically deleted show no gross baseline changes in ventricular structure or function. However, when subjected to chronic cardiac stress attained by trans-aortic constriction (TAC), significant differences are observed. Remarkably, TAC induced similar levels of cardiac hypertrophy in WT and CaMKIIδKO (KO) mice, however while KO mice show preserved hypertrophy after 6-week TAC, WT mice transition to

heart failure. The fraction of the cardiac ryanodine receptor (RyR2) phosphorylated at the CaMKII site increased significantly during development of heart failure in WT mice, but not KO mice, and this was associated with enhanced Ca2+ leak from the SR only in WT mice [38]. Using KO mice or those expressing CaMKII inhibitory peptides, the pathogenic effects of a variety of additional stressors including pathological stimulation of the alpha subunit of the heterotrimeric G protein G_q, ischemia/reperfusion (I/R), and chronic β-adrenergic stimulation[27, 44, 45] have been attributed to activation of CaMKIIδ,

1.5 Conclusions

CaMKIIδ is an important nodal regulator of a variety of signaling processes in the heart, many of which are of central importance in the context of disease. Use of KO mice and pharmacological or genetic inhibition of CaMKII has yielded vital information about the properties of the CaMKIIδ writ large, but relatively few studies have focused on determining the properties of individual CaMKIIδ splice variants. The main focus of my thesis work, presented in chapter 2, has been to investigate how $CaMKII\delta_B$ and $CaMKII\delta_C$ differentially regulate cardiovascular disease. Additional research contributions to work published during my training are presented in chapter 3 and include investigations of the role of the CaMKIIδ in cardiomyopathy induced by G_q and β-adrenergic stimulation. Overall my thesis research and publications have

broadened the body of knowledge about how CaMKIIδ regulates pathophysiological processes and specifically provides novel and unique insights into the distinct properties of the predominant CaMKIIδ subtypes in the context of I/R.

1.6 Acknowledgements

The background information presented in Chapter 1 contains excerpts from a review article published in the journal *Frontiers in Pharmacology* of which I was the primary author along with co-author Joan Heller Brown.

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Chapter 2

The role of CaMKIIδ subtypes in ischemia/reperfusion injury

2.1 Summary

Several studies from our laboratory and others have demonstrated that inhibition or abrogation of CaMKIIδ has a beneficial effect in the context of chronic cardiac stress, but CaMKIIδ is crucially important in the development of acute stress as well. Following myocardial infarction, reperfusion of the ischemic region of the heart results in a transient but robust increase in intracellular [Ca^{2+}] and reactive oxygen species (ROS), both of which can directly activate CaMKIIδ. Hearts from mice lacking CaMKIIδ expression (KO) in cardiomyocytes are protected from *in vivo* ischemia/reperfusion (I/R) injury in that KO mice do not develop as large of an infarct as WT mice do in response to ischemia/reperfusion. Following I/R injury an inflammatory signaling cascade is activated, leading to infiltration of leukocytes into the heart. KO mice did not exhibit a similar degree of inflammatory cell infiltration as WT animals after *in vivo* I/R, and further experimentation revealed that the loss of CaMKIIδ in *in vivo* I/R was associated with a reduction in inflammatory signaling under the control of the NF-κB transcription factor. Chapter 2.2

describes these findings and includes excerpts from Ling et al. 2013[1] of which I was a co-author.

As discussed in Chapter 1.3 the predominant cardiac CaMKIIδ subtypes, CaMKIIδ$_B$ and CaMKIIδ$_C$, are not necessarily segregated into distinct subcellular compartments despite the inclusion of an NLS only in CaMKIIδ$_B$. Using animals that overexpress CaMKIIδ$_B$ and CaMKIIδ$_C$ in the KO background to prevent heteromultimerization, we demonstrated that CaMKIIδ$_B$ and CaMKIIδ$_C$ are not restricted to distinct sub-cellular locales but in fact are similarly both present throughout the cell. Additionally, we determined that in response to pharmacological stimulation CaMKIIδ$_B$ and CaMKIIδ$_C$ can both be activated at a variety of locations within the cell. Chapter 2.3 describes these findings and includes excerpts from Mishra et al. 2011[2] of which I was a co-author.

In my most recent body of work I discovered, however, that in response to *ex vivo* I/R injury CaMKIIδ$_B$ and CaMKIIδ$_C$ are differently activated within the cytosol. Using the aforementioned mice, which express only CaMKIIδ$_B$ or CaMKIIδ$_C$, we were able to associate the selective activation of CaMKIIδ$_C$ with deleterious signaling events culminating in increased infarct formation. Specifically we demonstrated that, compared to animals expressing CaMKIIδ$_B$, mice expressing CaMKIIδ$_C$ exhibit larger infarcts after *ex vivo* I/R. Additionally, CaMKIIδ$_C$ mice displayed robust activation of NF-κB and TNF-α mRNA upregulation. Finally we show that inhibition of NF-κB or TNF-α blocked the

deleterious effects of CaMKIIδ$_C$ overexpression. These studies represent unique contribution to the existing body of knowledge regarding CaMKIIδ subtypes and provide novel mechanistic insight into the differing properties of CaMKIIδ$_B$ and CaMKIIδ$_C$. The work establishes for the first time that a single splice variant of the CaMKIIδ gene, CaMKIIδ$_C$, mediates the deleterious effects of CaMKIIδ gene expression in *ex vivo* I/R. Chapter 2.3 describes these findings which comprise the main focus of my thesis research.

2.2 Ca^{2+}/Calmodulin-Dependent Protein Kinase II δ Mediates Myocardial Ischemia/Reperfusion Injury Through Nuclear Factor-κB

2.2.1 Introduction

Ischemia/reperfusion (I/R) injury is a significant and common mechanism by which cardiac stress and injury can occur in humans. I/R injury occurs following the occlusion of a coronary artery, a condition known as myocardial infarction (MI). Typically, a blot clot will become lodged in a coronary artery that has already been partially occluded by atherosclerotic plaque formation. In order to restore normal blood flow to the occluded region of the myocardium, the occlusion is removed, leading to a sudden influx of blood to the previously ischemic area. Associated with the reperfusion of the myocardium is an acceleration of cell death and myocardial damage which,

while less than the damage that would occur were the occlusion to remain, promotes further damage above that which occurs during the ischemic period. No therapies in clinical use specifically target reperfusion damage, and so further investigations into the molecular events that underlie reperfusion are ongoing [3].

Cardiomyocyte cell death in response to I/R results from increases in Ca^{2+} and reactive oxygen species and is mediated through opening of the mitochondrial permeability transition pore. Concurrently, an inflammatory cascade that perpetuates further damage to cardiac tissue is activated[4-7]. One of the central players in inflammatory signaling is the transcription factor nuclear factor-κB (NF-κB). Numerous reports have shown that NF-κB is activated after myocardial I/R[8, 9]. It has been demonstrated that cardiac-specific blockade of NF-κB activation reduces infarct size and preserves heart function in response to I/R indicates that the activation of NF-κB within the cardiomyocyte is critical to this series of events[10]. Pharmacological inhibitors of NF-κB activation also provide protection against cardiac I/R injury, attenuate the release of TNFα and interleukin-6 (IL-6), inhibit inflammation and apoptosis, reduce infarct size, and improve functional recovery[11-13]. Thus, inflammation mediated through NF-κB plays a critical role in infarct development after I/R.

Whereas a maladaptive role of NF-κB in I/R injury is well documented, the initiating signals have not been fully elucidated. CaMKII has been

demonstrated to mediate I/R injury in the isolated perfused heart and cardiac remodeling after MI based on experiments using pharmacological inhibitors or transgenic mice overexpressing a CaMKII inhibitory peptide[14-16]. CaM kinase has also been demonstrated to play a role in inflammatory gene expression in macrophages[17, 18]. The studies presented here use CaMKIIδ KO mice to test the hypothesis that CaMKII is required for the initiation of I/R-induced NF-κB activation in cardiomyocytes and plays a critical role in the subsequent inflammatory responses that contribute to myocardial I/R damage.

2.2.2 Results

2.2.2.1 CaMKIIδ is activated during *in vivo* I/R and regulates infarct formation and cell death

To test the role of CaMKIIδ in myocardial tolerance to I/R injury, CaMKIIδ KO and WT mice were subjected to left anterior descending coronary artery ligation for 1 hour followed by reperfusion for various times. Using a direct kinase activity assay, we demonstrated that CaMKII is activated in WT mice early during reperfusion after *in vivo* ischemia (Figure 2-1A). Susceptibility to and recovery from I/R injury were then assessed. The area of the myocardium subjected to ischemia (area at risk) was not different in CaMKIIδ KO and WT littermates, but infarct size after I/R was significantly

reduced in the KO hearts (Figure 2-1B). It is well accepted that cardiomyocyte cell death contributes to I/R injury. We examined the extent of apoptosis at 24 hours of reperfusion after 1-hour ischemia. Apoptosis (assessed by TUNEL staining (Figure 2-1C) and other methods) was significantly greater in WT than in KO mice. Inflammation, a recognized consequence of myocardial ischemic insult, was evidenced by enhanced mononuclear cell infiltration as assessed by CD68 staining. The increase in CD68 staining observed in WT hearts at 24 hours reperfusion was significantly diminished by CaMKIIδ deletion (Figure 2-1D).

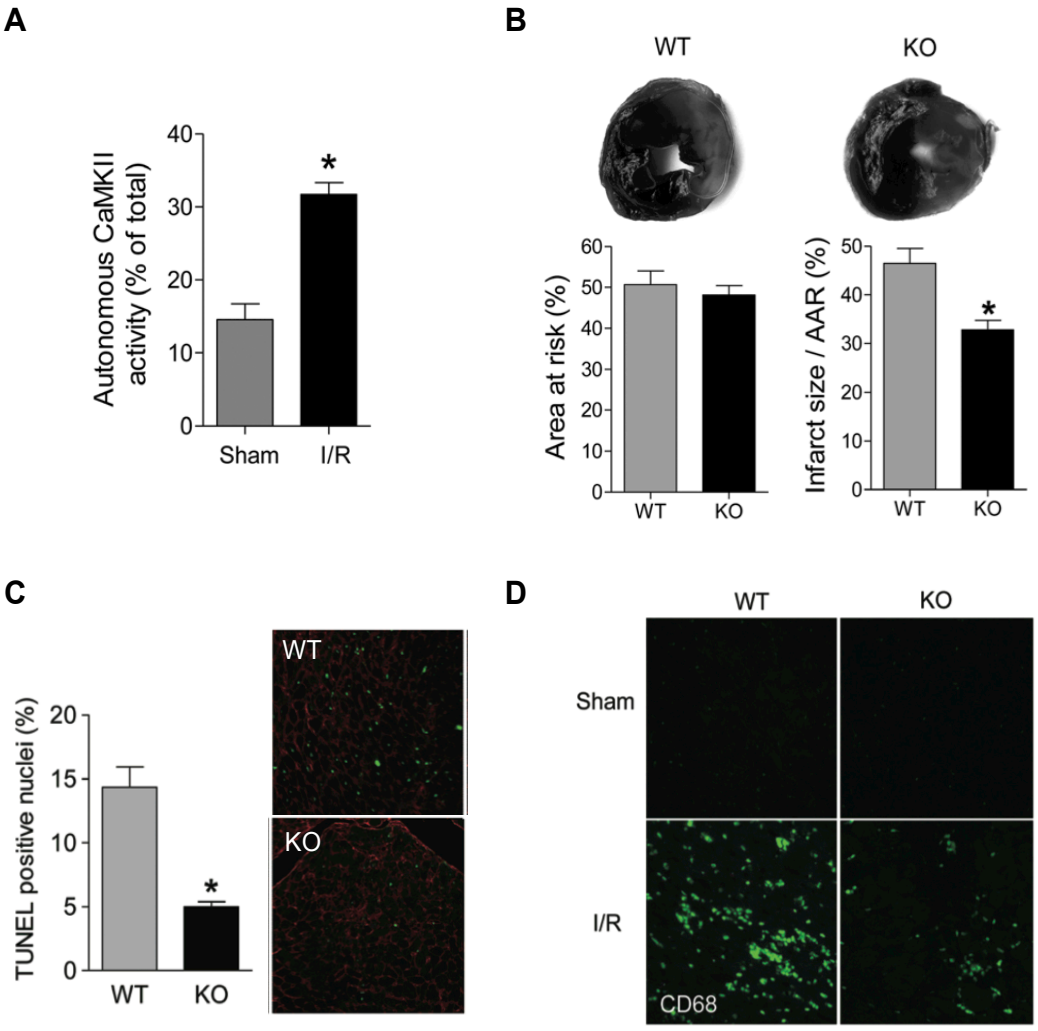

Figure 2-1. CaMKIIδ deletion attenuates *in vivo* I/R injury and subsequent inflammation. Animals were subjected to 1-hour ischemia followed by various times of reperfusion. (A) Increases in autonomous CaMKII activity were measured at 3-minute reperfusion. Data are mean±SEM of values from 3 mice. **P*<0.05 vs Sham. (B) Area at risk (AAR) and the ratio of infarct size to AAR were determined in WT and KO mice at 24-hour reperfusion. Data are mean±SEM of values from 6 mice. (C) TUNEL staining; apoptotic nuclei were stained (green), and cardiomyocytes were detected by wheat germ agglutinin staining (red). Original magnification is ×20. Data are mean±SEM of values from 3 hearts per group, with at least 5000 nuclei examined per heart. **P*<0.05 vs wild-type (WT) ischemia/reperfusion (I/R). (D), Inflammatory cells were identified by CD68 staining. Original magnification is ×20.

2.2.2.2 CaMKIIδ regulates inflammatory responses during I/R that culminate in infarct formation and cell death through activation of NF-κB in the cardiomyocyte.

The NF-κB family of transcription factors plays a central role in regulating the expression of inflammatory and cell death genes linked to cardiovascular pathology[5, 10, 12, 19]. We tested the hypothesis that NF-κB activation in response to I/R is regulated through CaMKIIδ. An early event in NF-κB activation is the degradation of IκBα. In WT mice, reperfusion after 1-hour ischemia was accompanied by a rapid and transient decrease in IκBα expression; this response was seen as early as 5 minutes after reperfusion and prevented by CaMKIIδ deletion. Subcellular fractionation of hearts isolated at various times after I/R demonstrated increases in the p65 subunit of NF-κB in nuclear fractions from WT but not from KO mice (Figure 2A). Finally, we assessed NF-κB activity in ventricular lysates through binding to its consensus sequence. NF-κB binding was not changed during ischemia, but quickly increased in WT hearts at 5 minutes and at 2 hours of reperfusion; this increase was sustained throughout 24 hours of reperfusion. In contrast, activation of NF-κB was not significantly increased in KO hearts at any time after reperfusion (Figure 2A).

To explore the dependence of NF-κB activation on opening of the mitochondrial permeability transition pore and development of necrosis, mice were treated with cyclosporin A[20]. Cyclosporin A treatment reduced

cytochrome C release into cytosolic fractions after I/R, indicating effective permeability transition pore inhibition (Figure 2B). Nuclear p65 translocation in response to I/R was, however, unchanged by cyclosporin A treatment (Figure 2C). This observation suggests that I/R can induce NF-κB activation independent of necrotic damage to mitochondria, consistent with the very rapid onset of NF-κB activation.

We used Affymetrix Gene arrays to compare changes in mRNA levels at 24-hour reperfusion after 1-hour ischemia in WT and KO mouse hearts. There were 3129 differentially expressed transcripts in WT versus CaMKIIδ KO after I/R. The 5-kb promoters of these differentially expressed genes were scanned for enrichment of transcription factor–binding sites. Transcription factor binding sites enriched in the differentially regulated genes included those for the known CaMKIIδ target MEF2c and those for NF-κB. There were 313 predicted NF-κB target genes upregulated in WT hearts that were significantly suppressed or downregulated in CaMKIIδ KO hearts. Many of these genes clustered by Gene Ontology analysis into categories related to apoptosis, cytokine signaling, and regulation of NF-κB signaling.

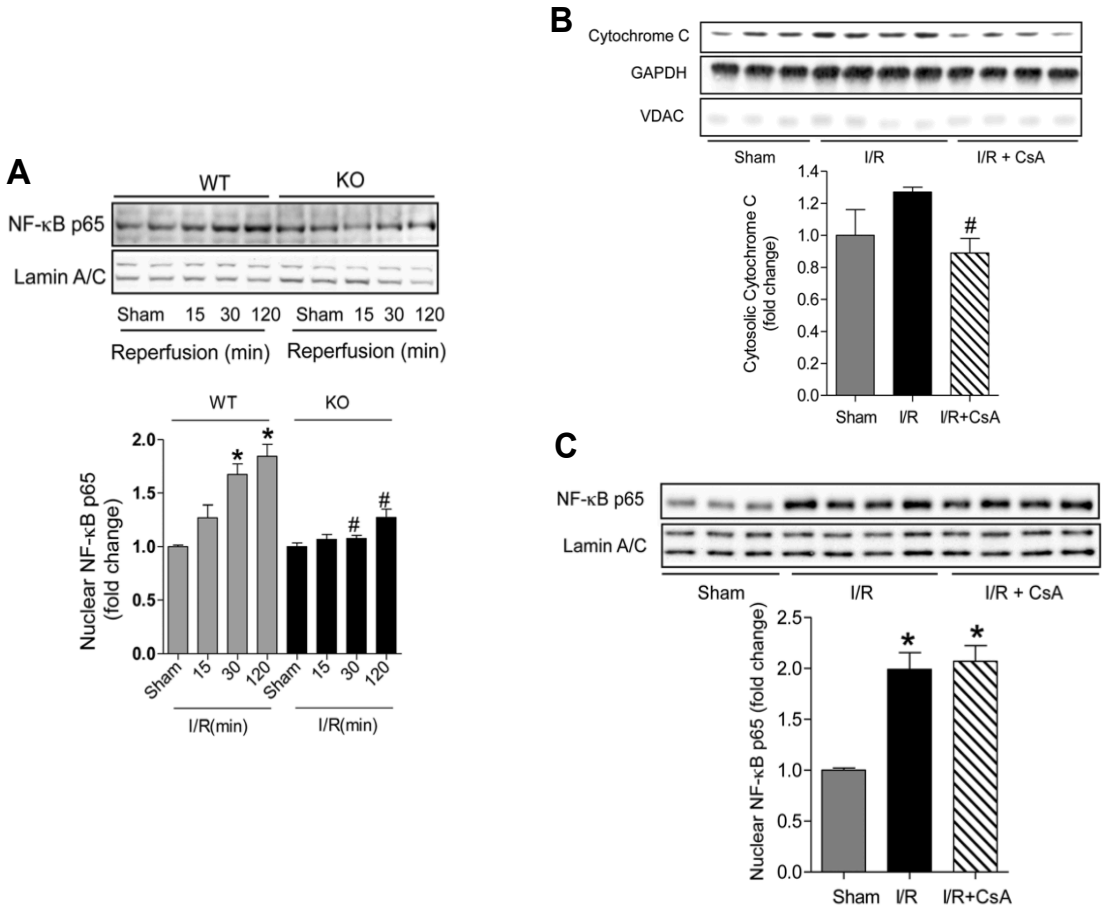

Figure 2-2. CaMKIIδ deletion inhibits NF-κB nuclear localization in response to *in vivo* I/R. Animals were subjected to 1-hour ischemia followed by various times of reperfusion. (A) Translocation of NF-**κ**B p65 to the nucleus was detected by Western blotting after nuclear fractionation. Lamin A/C was used as a loading control. Data are mean±SEM of values from 3 to 6 determinations. **P*<0.05 vs Sham; #*P*<0.05 vs WT I/R. (B) Hearts were collected from mice treated ± CsA at 15-minute reperfusion after 1-hour ischemia. Cytochrome C expression detected by Western blotting in mouse heart cytosolic fractions. GAPDH and VDAC were used as cytosolic and mitochondrial loading controls to assess purity of cytosolic fractions. Data are mean±SEM of values from 3 to 4 determinations. #*P*<0.05 vs I/R. **(**C**)** Translocation of NF-**κ**B p65 to the nucleus was detected by Western blotting in nuclear fractions from the same hearts. Lamin A/C was used as a loading control. Data are mean±SEM of values from 3 to 4 mice. **P*<0.05 vs Sham.

To directly demonstrate and determine the mechanism for CaMKII effects on NF-κB activation, we expressed constitutively active CaMKIIδ (the

$CaMKII\delta_C$ subtype was used in these studies) in neonatal rat cardiomyocytes. The amount of nuclear p65 was increased in cells expressing CaMKIIδ (Figure 2-3A). In addition, we determined that IκB kinase (IKK), a proximal kinase in the NF-κB signaling cascade, was phosphorylated when CaMKII activity was increased (Figure 2-3B). Pharmacological inhibition of IKK with BMS-345541 (BMS)[21] blocked the ability of CaMKIIδ to increase nuclear p65 (Figure 2-3A). These data suggest that CaMKIIδ activates IKK to initiate NF-κB signaling. To test the importance of IKK activation and NF-κB signaling using the *in vivo* I/R injury model, BMS was administrated to mice intravenously 10 minutes before reperfusion. The efficacy of IKK inhibition was evidenced by the ability of this drug to significantly decrease I/R-induced NF-κB activation in WT mice (Figure 2-3C). Treatment of WT mice with BMS led to a decrease in infarct size (Figure 2-3D). In contrast, the inhibitor did not further reduce I/R-induced NF-κB activation or infarct size in KO mice (Figure 2-3C and 2-3D). The lack of additive protection by CaMKIIδ deletion plus NF-κB inhibition is consistent with there being a common mechanism underlying the benefit of deleting CaMKIIδ and of inhibiting NF-κB signaling. The finding that there is no additional benefit to CaMKIIδ deletion when IKK is inhibited supports the cell-based studies showing that CaMKIIδ regulates NF-κB signaling in cardiomyocytes through IKK, rather than by regulating a more distal process in the NF-κB activation cascade.

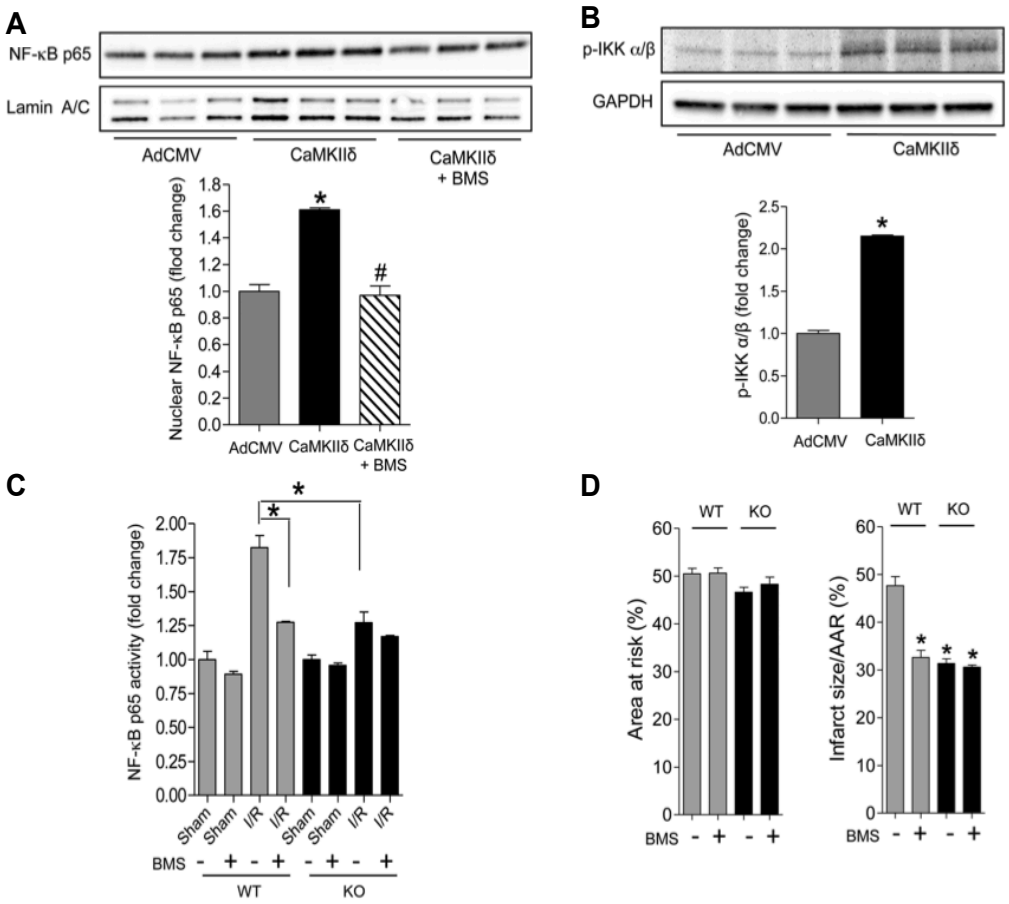

Figure 2-3 CaMKIIδ induces IKK phosphorylation, and inhibition of IKK reduces NF-κB activation and infarct development in response to *in vivo* I/R. For (A**)** and (B**)**, neonatal rat ventricular myocytes were infected with adenovirus expressing GFP or constitutively active CaMKIIδ$_C$ at 40 MOI and harvested 24-hour after infection. (A) Translocation of NF-κB to the nucleus was detected by Western blotting after nuclear fractionation of cells treated ± 15 μmol/L BMS-345541. Lamin A/C was used as a loading control. Data are mean±SEM of values from 3 samples. **P*<0.05 vs AdCMV; #*P*<0.05 vs CaMKIIδ. (B) Phosphorylated IKK was detected by Western blot. Data are mean±SEM of values from 3 samples. **P*<0.05 vs AdCMV. For (C) and (D), hearts were collected at 24-hour reperfusion after 1-hour ischemia. (C), NF-κB activation by *in vivo* I/R measured by the TransAM oligonucleotide binding assay in wild-type (WT) and knockout (KO) mice pretreated for 10 minutes with 2 mg/kg BMS-345541 or vehicle. Data are mean±SEM of values from 3 mice. **P*<0.05 vs WT without BMS treatment. (D) Area at risk (AAR) and infarct size to AAR ratio (infarction size/AAR) after in vivo I/R ± BMS treatment. Data are mean±SEM of values from 4 to 6 determinations. **P*<0.05 vs WT without BMS treatment.

2.2.3 Discussion

The studies presented here provide evidence that CaMKIIδ is a mediator of the effects of I/R on IκBα degradation, NF-κB activation, and upregulation of putative NF-κB target genes and that its actions are mediated through IKK phosphorylation. Studies using these mice provide evidence that deletion of CaMKIIδ results in no overt basal phenotype but markedly limits the ability of I/R to increase NF-κB activation. Concomitantly, CaMKIIδ gene deletion attenuates I/R-induced cardiac inflammation, cardiomyocyte death, and infarct development.

Previous studies used the *ex vivo* isolated perfused heart to examine the involvement of CaMKII in global I/R injury[15, 16]. These studies concluded that the salutary effects of pharmacological CaMKII inhibition result from preventing the dysregulation of Ca^{2+} homeostasis that is thought to contribute to cell death after I/R. I/R-induced activation of CaMKII and phosphorylation of cytosolic Ca^{2+} handling proteins could contribute to necrotic cell death through effects on mitochondrial Ca^{2+} and ROS generation. Importantly, however, our data demonstrate that there is also a rapid CaMKII–mediated activation of NF-κB that is not secondary to necrosis and which sets into motion a subsequent series of proinflammatory processes. Reperfusion after ischemia *in vivo* is associated with influx of leukocytes and activation of endogenous inflammatory cells that contribute to development of apoptosis and cardiac inflammation. The studies presented here suggest that averting the early and

proximal cardiomyocyte autonomous activation of NF-κB by CaMKIIδ deletion significantly attenuates these more chronic inflammatory responses observed 24 hours after reperfusion.

NF-κB has been extensively implicated in myocardial I/R injury. There is abundant evidence that blocking NF-κB signaling limits infarct size, reduces neutrophil invasion, and decreases inflammatory gene expression[10, 22]. Activation of NF-κB proceeds through a number of distinct pathways that converge on the phosphorylation and subsequent proteasomal degradation of the IκB inhibitor protein. Degradation of this protein facilitates the translocation of NF-κB dimers to the nucleus, where they bind target genes and activate transcription. In our studies comparing WT and CaMKIIδ KO mice we report that I/R leads to a rapid decrease in IκBα levels in WT mice. We demonstrate that IκBα degradation is attenuated by CaMKIIδ deletion, suggesting that CaMKII either affects steps upstream of its phosphorylation or alters its proteasomal processing. We further show that both NF-κB nuclear translocation and it transcriptional activity are regulated through CaMKIIδ after I/R.

Our studies using BMS-345541, an inhibitor of IKK, the kinase responsible for IκB phosphorylation and its subsequent degradation, confirm the important role of NF-κB activation in infarct development after I/R. The data showing that pharmacological blockade of IκB kinase has no additional ameliorative effect when CaMKIIδ is deleted further imply that a common

mechanism underlies the beneficial effects of blocking CaMKII and NF-κB signaling and suggest that NF-κB activation is directly downstream of CaMKII. We show here that a proximal event, the activation of IKK, is regulated through CaMKIIδ in NRVMs. Whether IKK is a direct target of CaMKII-mediated phosphorylation remains to be determined. Nonetheless the finding that inhibiting IKK blocks the ability of CaMKII to elicit NF-κB activation in NRVMs and prevents further effects of CaMKIIδ deletion on infarct development in the *in vivo* I/R model supports the conclusion that IKK is the proximal site of CaMKII action.

In summary, we demonstrate here that CaMKIIδ mediates rapid NF-κB activation and the development of apoptosis and inflammation in response to I/R in the *in vivo* mouse heart. The activation of NF-κB signaling is independent of necrosis and results in regulation of multiple NF-κB target genes. We suggest that rapid CaMKIIδ activation during I/R leads to IKK phosphorylation and NF-κB activation. Inhibiting CaMKII during reperfusion could provide significant therapeutic advantage by targeting an early causal event in this cascade.

2.3 Location Matters: Clarifying the Concept of Nuclear and Cytosolic CaMKII Subtypes

2.3.1 Introduction

Transgenic overexpression of the predominant cardiac isoform of CaMKIIδ elicits hypertrophy and heart failure, whereas genetic deletion or inhibition of CaMKIIδ prevents HF development[17, 18, 23-25]. Two splice variants, CaMKIIδ$_B$ and CaMKIIδ$_C$, are known to be present in cardiac myocytes[26]. The CaMKIIδ$_B$ and CaMKIIδ$_C$ subtypes have been implicated in distinct cardiomyocyte functions, but the exclusivity of their localization, potential selectivity in activation mechanisms, and relationship of localization and subtype to functional outcomes have not been well defined.

We have determined in *in vitro* systems that heterologously expressed CaMKIIδ$_B$ primarily localizes to the nucleus, whereas δ$_C$ is found primarily in the cytosol. Accordingly, we postulated different functions of the two subtypes, with nuclear δ$_B$ involved in hypertrophic gene regulation and cytosolic δ$_C$ in the regulation of Ca^{2+} handling and ion channels. This was supported by prior studies using isolated neonatal rat ventricular myocytes and by the differential phenotypes that we observed in the CaMKIIδ$_B$ and δ$_C$ mice[27, 28].

Transgenic animals overexpressing the CaMKIIδ$_C$ subtype exhibit marked features of myocardial stress and disease. Mice expressing δ$_C$ rapidly progress to heart failure and premature death. By 6 weeks of age CaMKIIδ$_C$

transgenic mice display marked changes in cardiac morphology and by 12 weeks these animals display severe cardiac dysfunction and upregulation of hypertrophic genes[29]. CaMKIIδ$_C$ transgenic mice show hyperphosphorylation of PLN at Thr17 and of the cardiac ryanodine receptor (RyR2), the channel through which Ca^{2+} exits the SR[30]. Taken together, these changes would predict dysregulation of SR Ca^{2+} cycling and excitation-contraction coupling.

In contrast, transgenic mice expressing CaMKIIδ$_B$ under the control of the cardiac-specific α-myosin heavy chain (α-MHC) promoter develop mild cardiac hypertrophy, without the rapid progression to heart failure and premature death observed in CaMKIIδ$_C$. Reinforcing the conclusion that CaMKIIδ$_B$ and CaMKIIδ$_C$ expression confers distinct phenotypes, none of the defects in SR substrate phosphorylation and Ca^{2+} handling are present in CaMKIIδ$_B$ animals[30]. These observations taken together led to the notion that the harmful targets of CaMKIIδ are present in the cytosol and at the SR, where CaMKIIδ$_C$ is localized, and thus the catastrophic phenotype seen in CaMKIIδ$_C$ transgenic animals was simply a result of enhanced cytosolic CaMKII activity. Correspondingly CaMKIIδ$_B$ transgenic animals do not display cardiac dysfunction because CaMKIIδ$_B$ is restricted to the nucleus where it can't phosphorylate SR substrates of CaMKII. The experiments described here test the assumption that CaMKIIδ subtypes are localized in distinct compartments, and demonstrate that CaMKIIδ subtype localization and activation is far more complex than had been predicted by *in vitro* studies.

2.3.2 Results

2.3.2.1 Distribution of endogenous CaMKIIδ subtypes

Ventricular tissue and AMVMs isolated from adult WT and KO mice were analyzed by immunoblot using an antibody that recognizes both CaMKIIδ_B and δ_C. Two bands were clearly evident in the WT and absent in the CaMKIIδ KO mouse heart. The difference in mobility of these bands is consistent with the inclusion of an 11–amino acid (2 kDa) NLS in CaMKIIδ_B. Quantification of the individual bands indicates that CaMKIIδ_B is the more predominant splice variant, with approximately 60% of the total endogenous CaMKII migrating as the δ_B subtype, and just under 40% as δ_C (Figure 2-4A). To determine which subcellular compartments contain endogenous CaMKIIδ, isolated left ventricle was fractionated into cytosolic, mitochondrial, SR/membrane, and nuclear fractions.

To compare CaMKII protein expression among these fractions, the entire volume of each fraction was loaded onto SDS gels. Immunoblotting for CaMKIIδ revealed that there were nearly equivalent amounts of CaMKIIδ in the SR/membrane and nuclear compartments of the cell (together accounting for approximately 75% of the total enzyme), whereas less than 20% was in the cytosolic fraction and a smaller percentage was associated with the mitochondrial fraction (Figure 2-4B). The subtype composition of CaMKIIδ in each subcellular fraction was then analyzed by separately quantifying the

individual δ_B and δ_C bands. This analysis revealed that both subtypes were present in every compartment examined (Figure 2-4C). Importantly, CaMKIIδ_B was detected not only in the nuclear compartment, but also in the SR/membrane compartment; conversely CaMKIIδ_C, though abundant in the SR/membrane, was also clearly present in the nuclear compartment (Figure 2-4C).

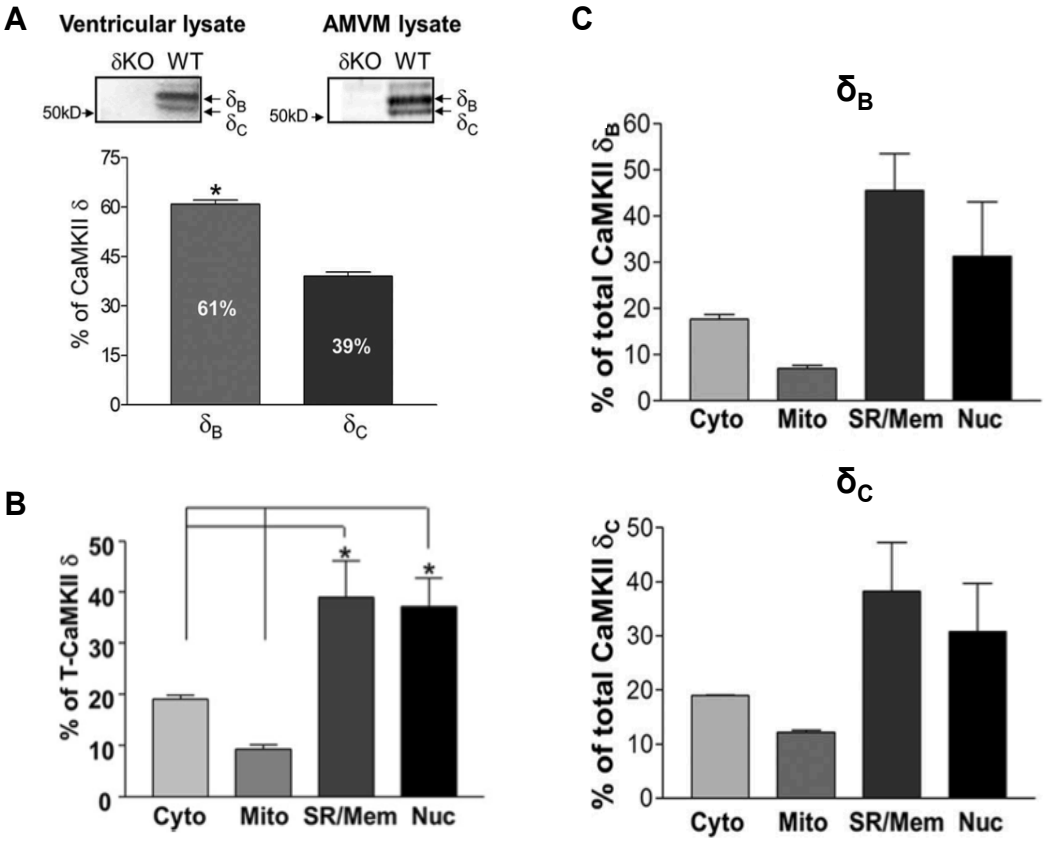

Figure 2-4. Subcellular distribution of endogenous CaMKIIδ subtypes. Ventricular tissue and adult mouse ventricular myocytes (AMVM) were isolated from WT and KO mice, lysed, and subjected to Western blotting. (A) Representative blots and quantitative analysis of CaMKIIδ subtype expression in ventricular lysate and AMVM lysate demonstrating two bands absent in KO mice. (B) Quantitative analysis of the percent of total CaMKIIδ in each subcellular compartment isolated from WT mouse ventricle. All fractions were suspended in the same volumes and equal portions loaded for Western blotting. n=6. **P*<0.01. (C) The relative distribution of CaMKIIδ subtypes in the cytosolic, mitochondrial, SR/membrane, and nuclear fractions. Equal portions of each fraction were loaded for Western blotting. Graphs show percent of total CaMKII δ_B or δ_C in each subcellular fraction from WT mice. n=6.

2.3.2.2 Distribution of CaMKIIδ subtypes in transgenic mice

We have previously assumed that the phenotypic differences observed in the CaMKIIδ subtype TG lines correlated with differential increases in expression of δ_B and δ_C in the nuclear versus SR compartments, respectively. To reevaluate this assumption in light of our findings on the distribution of endogenous CaMKII subtypes, we isolated and fractionated ventricular tissue from CaMKIIδ_B and δ_C TG mice. As in the experiments above, the entire volume of each fraction was loaded onto SDS gels to compare CaMKII protein expression among these fractions. The percentage of the total CaMKIIδ transgene in each subcellular compartment was quantified (Figure 2-5A). Remarkably, whereas CaMKIIδ_BTG mice show a high concentration of CaMKIIδ_B in cardiomyocyte nuclei, based on immunofluorescence staining, subcellular fractionation indicates that significant amounts of CaMKIIδ_B are also present outside of the nucleus in the cytosolic and SR/membrane fractions (Figure 2-5A). In the CaMKIIδ_C TG mice, immunostaining revealed relative exclusion of CaMKIIδ_C from the nucleus, but, whereas most CaMKIIδ_C is in the SR/membrane fraction, the CaMKIIδ_C subtype is clearly detectable in the nuclear fraction as well (Figure 2-5A). Thus distribution of the transgenes, like that of endogenous CaMKIIδ subtypes, is not exclusive.

CaMKIIδ is believed to exist as a multimer of 12 subunits. Nuclear versus cytosolic localization can be significantly affected by changing the expression ratio of δ_B and δ_C splice variants, consistent with

heteromultimerization of these subtypes[31]. Multimerization of transgenically expressed CaMKIIδ$_C$ with endogenous CaMKIIδ$_B$ could promote its localization to the nucleus, whereas multimerization of CaMKIIδ$_B$ with endogenous CaMKIIδ$_C$ could lead to its exclusion from the nuclear compartment. To test the hypothesis that the broad and relatively nonselective subcellular distribution of the CaMKIIδ$_B$ and δ$_C$ subtypes results from their heteromultimerization, we crossed the CaMKIIδ$_B$ and δ$_C$ TG mice with the CaMKIIδ KO mice previously developed in our laboratory. Progeny from these crosses were shown to express only a single CaMKIIδ subtype (δ$_B$ or δ$_C$) in the CaMKIIδ null background. Interestingly, the subcellular distribution of the CaMKIIδ$_C$ and δ$_B$ transgenes expressed in the CaMKIIδ null background (Figure 2-5B) was not appreciably different from that of the δ$_B$ and δ$_C$ transgenes expressed in the WT background (Figure 2-5A).

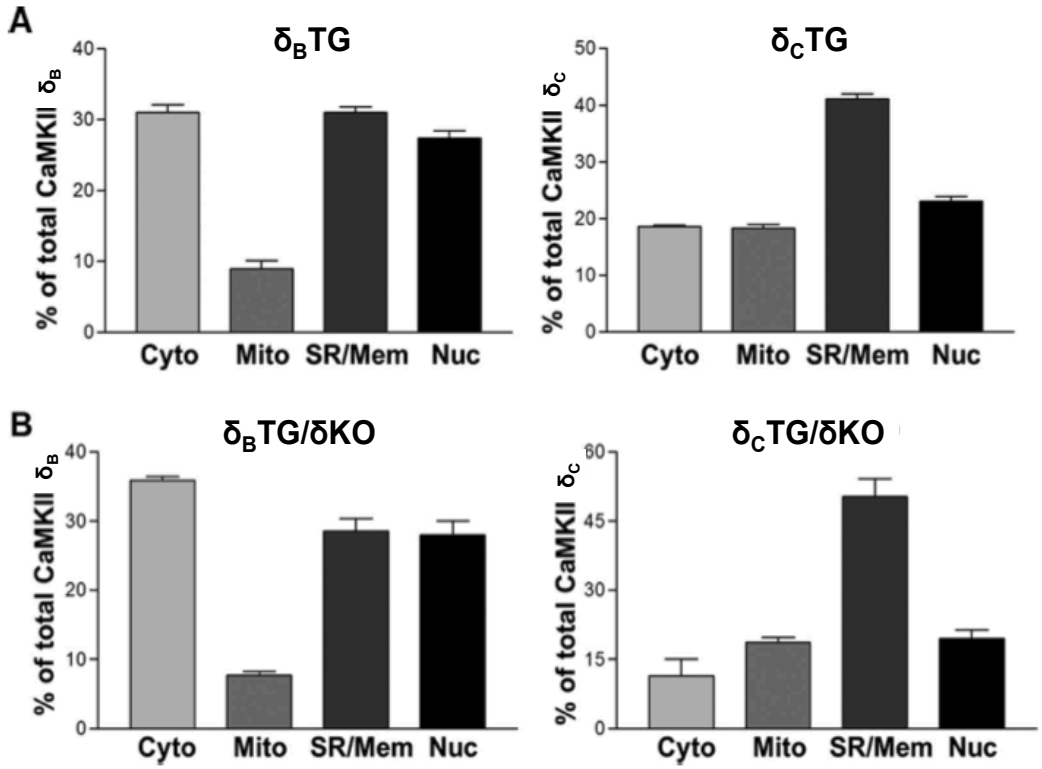

Figure 2-5. Comparative subcellular distribution of CaMKII δ_B and δ_C subtypes in transgenic versus transgenic in CaMKII δ null background. Ventricular tissue isolated from δ_BTG, δ_CTG, δ_B/δKO, and δ_C/δKO mice was harvested and fractionated into cytosolic, mitochondrial, SR/membrane and nuclear fractions and subjected to Western blotting. The distribution of transgenically expressed CaMKIIδ_B and δ_C is examined in the WT background (A) or in the CaMKIIδ null background (B). n=4.

2.3.2.3 Activation of CaMKIIδ subtypes by caffeine and phenylephrine at the SR and nucleus

The finding that CaMKIIδ_B and δ_C subtypes colocalize in the same subcellular compartment suggested that they might also be activated in parallel. To determine whether this is the case, we isolated hearts from WT

mice, perfused them in the Langendorff mode and then either treated them with a bolus injection of caffeine to release SR Ca^{2+}, or perfused them for 15 minutes with phenylephrine (PE) to increase nuclear Ca^{2+} levels. Hearts were then fractionated to obtain purified SR and nuclei and analyzed by immunoblot. Phosphorylation of CaMKII at Thr286, the site of enzyme autophosphorylation, was used as a read-out for CaMKII activation. Perfusion with caffeine increased CaMKII Thr286 phosphorylation in the SR fraction but not in the nuclear fraction. In contrast, PE treatment increased CaMKII Thr286 phosphorylation levels in the nuclear fraction but not in the SR fraction (Figure 2-6A).

We repeated these experiments using the δ_B and δ_C TG mice in the CaMKIIδ null background. The data shown in Figure 2-6B and 2-6C demonstrate that caffeine significantly increases phosphorylation of both CaMKIIδ_B and CaMKIIδ_C in the SR (but not in the nuclear fraction). In contrast PE treatment elicits increases in phosphorylation of both CaMKIIδ_B and CaMKIIδ_C in the nuclear fraction, but not in the SR. Thus caffeine selectively activates whichever CaMKIIδ subtype is located at the SR but not that located in the nucleus, whereas PE selectively activates whichever CaMKIIδ subtype is localized to the nucleus.

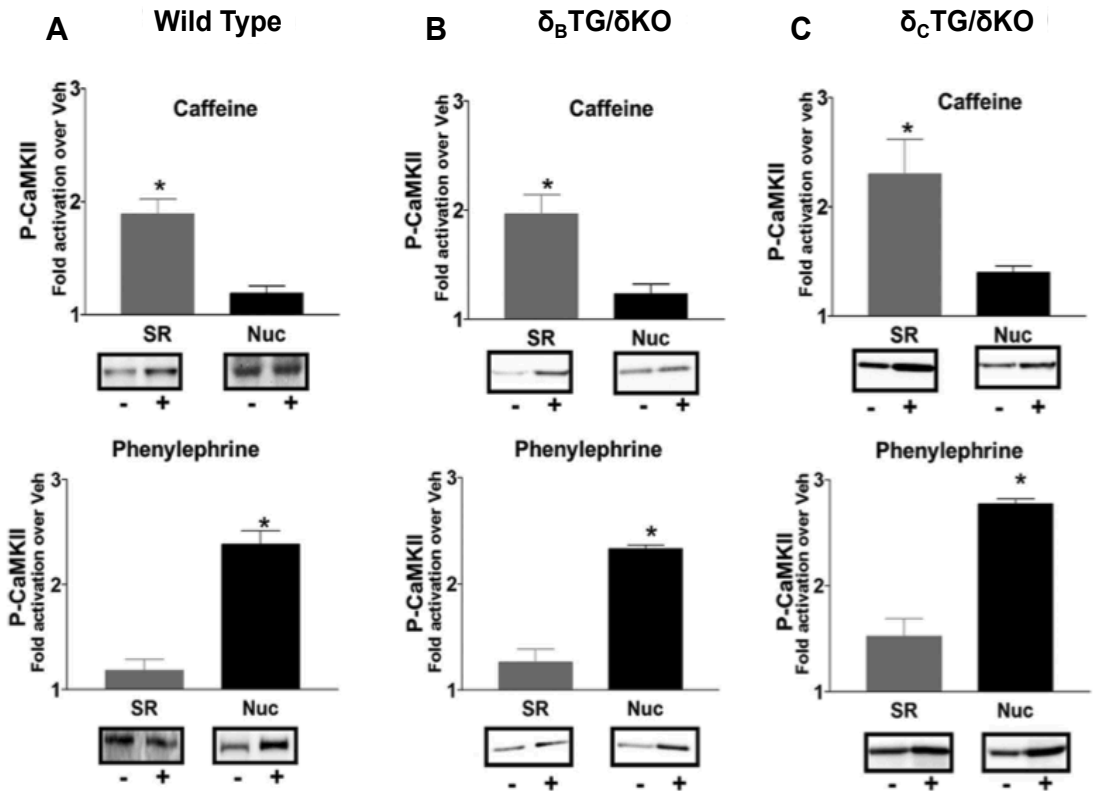

Figure 2-6. Caffeine activates CaMKII at the SR and phenylephrine activates CaMKII at the nucleus. SR or nuclei were isolated from ventricular tissue of WT, CaMKII δ_B, or CaMKII δ_C TG mice in the CaMKII δ null background after bolus injection of 10 mmol/L caffeine or 15 minutes of perfusion with 100nmol/L PE. P-CaMKII levels were measured by Western blotting, using an antibody directed against the CaMKII autophosphorylation site (Thr286). Data are shown for WT (endogenous CaMKII) (A) δ_B TG in the KO background (B) and δ_C TG in the KO background (C). **P*=0.01 for SR versus Nuc.

The experimental findings cited above suggest that CaMKIIδ_B or CaMKIIδ_C can be activated by the same agonists and could subserve similar functions. We examined functional consequences of compartmentalized CaMKII activation by measuring the phosphorylation of established CaMKII phosphorylation sites on two CaMKII targets, PLN localized to the SR and

HDAC5, largely localized to the nucleus. Perfused hearts were treated with PE or caffeine using the same protocol used to examine CaMKII activation and homogenized for analysis of CaMKII substrate phosphorylation. In WT mice treatment with caffeine lead to significant increases in phosphorylation of PLN at Thr17 while treatment with PE did not lead to PLN phosphorylation (Figure 7A). Conversely, treatment with PE increased phosphorylation of HDAC5 at Ser498, whereas caffeine did not increase phosphorylation (Figure 7B).

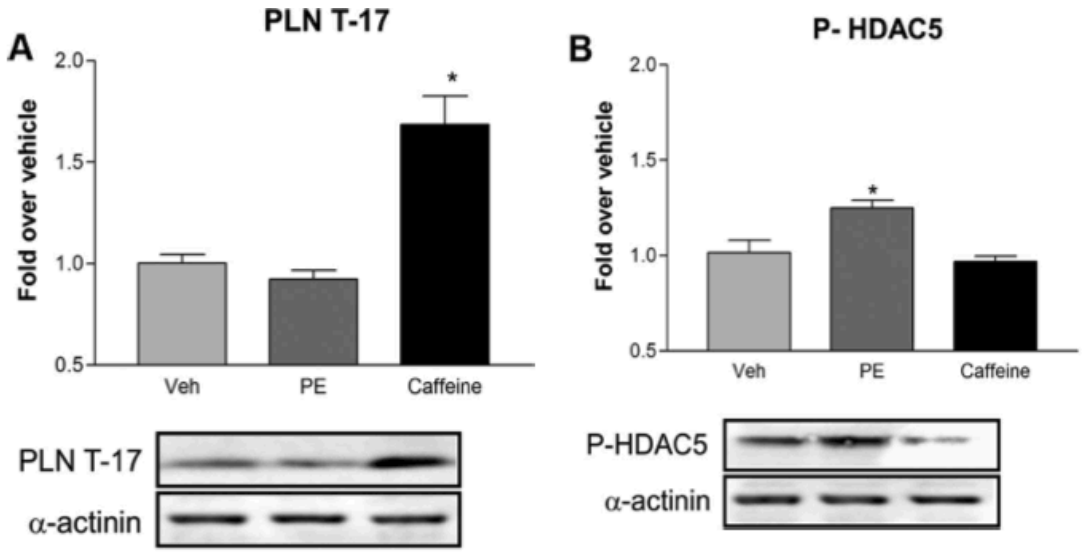

Figure 2-7. Caffeine preferentially increases phospholamban phosphorylation and PE preferentially increases HDAC5 phosphorylation in mouse heart. Hearts were isolated from mice perfused with 10mmol/L caffeine or 100 nmol/L phenylephrine. Ventricular homogenate was subjected to Western blotting for (A) PLN phosphorylation at the CaMKII phosphorylation site, threonine-17; and (B) HDAC5 phosphorylation using an antibody for the CaMKII- specific epitope. Quantified data are from n=5. *P<0.01.

2.3.3 Discussion

CaMKIIδ$_B$ and δ$_C$ subtypes, which differ only by the inclusion of a nuclear localization sequence, are present in the mouse heart ventricle at similar protein levels. Our early studies in which we expressed CaMKIIδ$_B$ and δ$_C$ in NRVMs supported the notion that CaMKIIδ$_B$ would be localized to and signal in the nucleus, whereas δ$_C$ would localize to and signal outside of the nucleus. Subsequently, we generated CaMKIIδ$_B$ or δ$_C$ TG mice. The concept that nuclear and cytosolic splice variants/subtypes subserved different functions was supported by the distinct phenotypes that we observed in the CaMKIIδ$_B$ and δ$_C$ TG mice[29, 30].

The more extensive analysis presented here demonstrates that endogenous CaMKIIδ is found in both the SR/membrane and nuclear compartments isolated from mouse ventricle and that there are two CaMKIIδ immunoreactive bands in both of these compartments that represent δ$_C$ and the 11-amino-acid-larger NLS-containing δ$_B$. The ability of various CaMKII isoforms and subtypes to form heteromultimers was suggested as a mechanism for the presence of either subtype in the nucleus or SR regardless of whether it possesses an NLS. Remarkably, however, the expression of CaMKIIδ$_B$ in the absence of CaMKIIδ$_C$ did not restrict its localization to the nucleus nor was δ$_C$ confined to the cytosolic/SR compartment when expressed in the absence of δ$_B$. Thus heteromultimers of CaMKIIδ$_B$ and δ$_C$ appear unlikely to account for the indiscriminate distribution of these subtypes.

The finding that both CaMKII subtypes are present throughout the cell raised the question of whether localization or subtype would determine when and how the enzyme was activated. We used interventions expected to mobilize Ca^{2+} from distinct cellular locations to examine CaMKIIδ activation in WT mouse hearts. Our findings clearly demonstrated that PE increases phosphorylation of CaMKIIδ_B or δ_C in the nuclear compartment with little change in activation of either subtype at the SR; conversely, caffeine activates both CaMKIIδ_B and δ_C in the SR, with little change in activation of either subtype in the nuclear compartment.

The functional relevance of compartmentalized CaMKIIδ activation was demonstrated by studies in which we examined substrate phosphorylation. Phosphorylation of the SR target, phospholamban at its well-documented CaMKII-specific phosphorylation site was shown to be selectively increased after addition of caffeine. Phosphorylation of the nuclear transcriptional regulator HDAC5 at a CaMKII phosphorylation site was selectively increased after PE treatment.

In conclusion, we demonstrate for the first time that CaMKIIδ_B and δ_C subtypes are not exclusively localized. We also present evidence that both subtypes can be activated at the same cellular locations and that the activation is stimulus and location dependent rather than subtype dependent. Phosphorylation of different CaMKIIδ substrates is also dependent on the nature of the stimulus. The evidence for nonselective CaMKIIδ subtype

localization is particularly interesting and challenging with regard to understanding mechanisms by which CaMKIIδ$_B$ and δ$_C$ confer different effects in cardiomyocyte survival and heart disease.

2.4 CaMKIIδ subtypes differentially regulate infarct formation following *ex vivo* myocardial ischemia/reperfusion through NF-κB and TNF-α

2.4.1 Introduction

We previously generated global and cardiac specific knockouts of CaMKIIδ and demonstrated that deletion of this protein ameliorates heart failure development in response to pressure overload, Gα$_q$ expression, and isoproterenol infusion. CaMKIIδ deletion also diminishes infarct development in response to *in vivo* ischemia/reperfusion[1, 24, 32, 33]. Other studies have used CaMKII inhibitory peptides to similarly conclude that CaMKII activation by a range of cardiac insults, including myocardial infarction, is deleterious[34-36]. In all of the aforementioned studies both the CaMKIIδ$_B$ and CaMKII δ$_C$ splice variants of CaMKIIδ are genetically deleted or inhibited. Accordingly it is not yet known which subtype is responsible for the protective effect of ablating CaMKIIδ activity in the heart. To elucidate the distinct roles of CaMKIIδ$_B$ and CaMKII δ$_C$ in cardiac injury we used mice in which the previously generated TG lines were crossed into the global CaMKIIδKO background, as discussed

in Chapter 2.3. Accordingly these mice, in which CaMKIIδ deletion has been restored by exclusive expression of either δ_B or δ_C, allowed us to further examine the unique roles of CaMKIIδ_B and CaMKIIδ_C in cardiomyocyte survival and infarct formation in response to I/R.

The findings presented here establish that CaMKIIδ_C expression reverses the diminished I/R damage observed in CaMKIIδKO mouse hearts and further exacerbates I/R injury. In contrast CaMKIIδ_B further attenuates I/R damage. We demonstrate involvement of inhibitor of kappa B kinase (IKK), NF- kB activation and increased TNF-α expression in the response to CaMKIIδc and in infarct formation in the isolated perfused heart. We conclude that activation of the CaMKIIδc subtype in cardiomyocytes selectively regulates cardiac autonomous pro-inflammatory signaling events that contribute to ischemia/reperfusion-induced cardiomyocyte death.

2.4.2 Results

2.4.2.1 Characterization of δ_BTG/δKO and δ_CTG/δKO mice

To examine the independent roles of the CaMKIIδ_B and δ_C subtypes in the heart we used cardiac transgenic lines that express δ_B (δ_BTG/δKO) or δ_C (δ_CTG/δKO) in a CaMKIIδ-null (KO) background and should contain only δ_B or only δ_C monomers as depicted in Figure 2-8A and as described in Chapter 2-3.

CaM kinase enzymatic activity was measured in heart lysates of δ_BTG/δKO and δ_CTG/δKO mice by assessing ^{32}P incorporation into the synthetic CaMKII substrate peptide syntide-2. We used a saturating concentration of Ca^{2+}-liganded calmodulin (Ca^{2+}/CaM) to measure total/maximal CaMKII enzymatic activity. Under these conditions δ_BTG/δKO animals displayed a 10.7-fold increase in enzymatic activity over wild type mice, while δ_CTG/δKO animals exhibit a 16.7-fold increase over wild type animals (Figure 2-8B). Additionally, in order to assess Ca^{2+}-autonomous CaMKII activity, which results from posttranslational modifications in the regulatory domain of the kinase, we assessed CaMKII activity in the absence of Ca^{2+}/CaM[26]. The level of autonomous activity in δ_BTG/δKO was similar to that observed in δ_CTG/δKO mice (6-fold vs 7-fold greater than WT, respectively) (Figure 2-8C). Thus phenotypic distinctions in the δ_BTG/δKO and δ_CTG/δKO transgenic lines are unlikely to result solely from differences in the levels of CaM kinase activity.

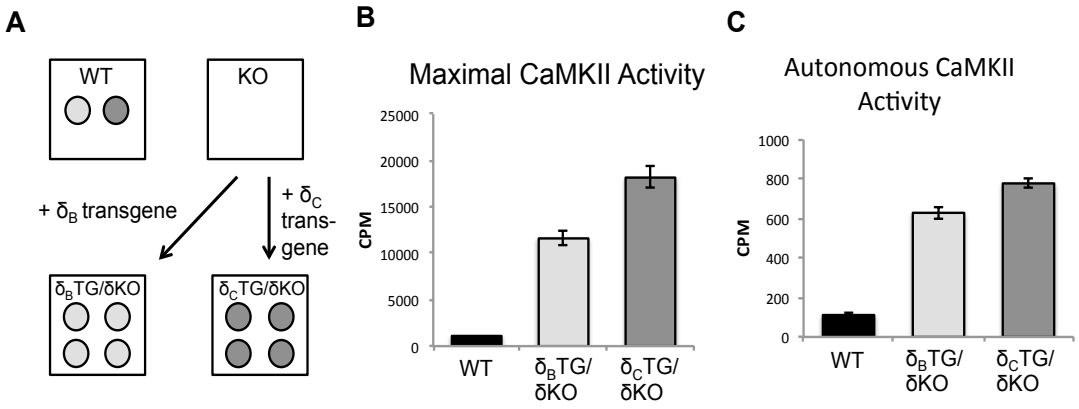

Figure 2-8. CaMKII activity in CaMKIIδ$_C$TG/δKO and CaMKIIδ$_B$TG/δKO mice. (A) Transgenic mice overexpressing CaMKIIδ$_C$ or CaMKIIδ$_B$ were crossed with mice in which the gene encoding CaMKIIδ has been deleted. The resulting animals express only the HA-tagged δ$_B$ or δ$_C$ transgenes with no expression of endogenous CaMKIIδ. (B) CaMKII activity was measured under maximally activating conditions, using syntide-2 as the substrate, in δ$_B$TG/δKO and δ$_C$TG/δKO animals (C) Autonomous CaMKII activity was measured under conditions in which the enzyme was not activated by exogenous CaM or Ca^{2+}. Data are mean ±SEM values from 4 mice.

Deletion of CaMKIIδ produced no overt phenotypic changes and did not affect survival relative to WT mice while δ$_B$TG/δKO animals also survived normally for at least 6 months (Figure 2-9A). In contrast the δ$_C$TG/δKO animals, like the previously studied δ$_C$TG[29], exhibited premature death with less than 20% survival by 21 weeks. Expression of the CaMKII δ$_B$ and δ$_C$ subtypes also had markedly different effects on *in vivo* cardiac function. Echocardiography on 6-8 week old mice revealed that fractional shortening (FS) was decreased by 63% in δ$_C$TG/δKO mice compared to WT mice, while δ$_B$TG/δKO animals did not display cardiac dysfunction (Figure 2-9B).

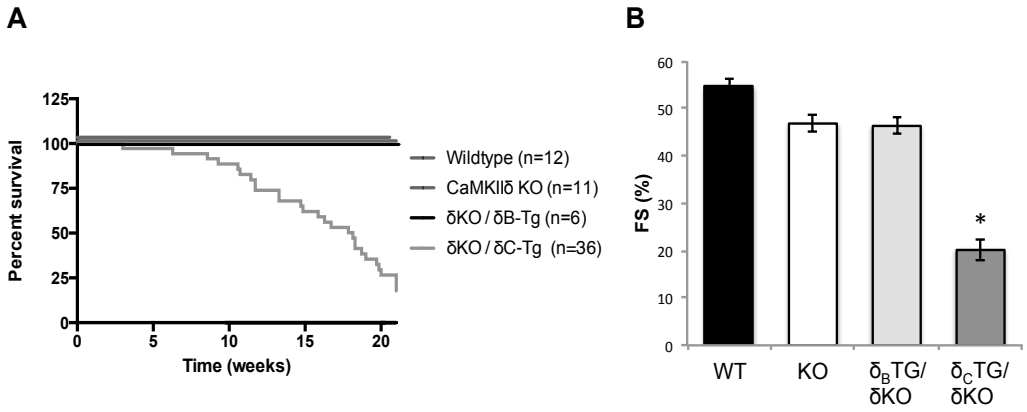

Figure 2-9. CaMKIIδ$_C$TG/δKO but not CaMKIIδ$_B$TG/δKO mice display diminished survival and cardiac function. (A) Kaplan-Meier analysis of survival of WT, CaMKIIδKO, CaMKIIδ$_B$TG/δKO, and CaMKIIδ$_C$TG/δKO mice. The first death was observed in CaMKIIδ$_C$TG/δKO mice at 3 weeks of age and at 22 weeks approximately 80% of the animals were deceased. (B) Fractional shortening (FS) was assessed following echocardiographic analysis of 6-8-week-old WT, CaMKIIδKO, CaMKIIδ$_B$TG/δKO, and CaMKIIδ$_C$TG/δKO mice. Data are mean ±SEM values from 4-6 mice. *P<0.05 vs WT.

2.4.2.2 CaMKIIδ subtypes differentially regulate I/R injury

We previously demonstrated that CaMKIIδ deletion attenuates I/R injury in response to *in vivo* left anterior descending coronary artery occlusion and 24 hours reperfusion. To determine which CaMKIIδ subtype or subtypes are responsible for CaMKIIδ-mediated infarct formation we performed *ex vivo* I/R experiments on isolated perfused hearts from 8-week-old WT and KO mice as well as on hearts from KO mice with forced expression of CaMKIIδ$_B$ or CaMKIIδ$_C$. Infarct formation following 25m ischemia and 1h reperfusion was determined by TTC staining of heart sections (Figure 2-10A). In WT animals,

ex vivo I/R induced infarcts comprising 36.2±2.5% of the cross-sectional area. In contrast infarcts were significantly smaller, only 24.1±1.4%, in CaMKIIδ KO mouse hearts. Thus regulation of infarct development by CaMKII is evident not only *in vivo* but also in an *ex vivo* I/R model.

When hearts from KO mice that re-express the δ_B isoform were examined they were found to be significantly protected against *ex vivo* I/R damage, with infarcts measuring only 12.2±1.9% of cross-sectional area. Conversely, in KO mouse hearts in which CaMKIIδ_C was re-expressed infarcts measured 45.2±1.8% of the cross-sectional area of the heart, significantly larger than those of WT, KO, and δ_BTG/δKO (Figure 2-10B).

To determine whether differences in susceptibility to I/R damage reflect intrinsic changes at the level of the cardiomyocyte, we isolated adult mouse ventricular myocytes (AMVM) from WT, KO, δ_BTG/δKO and δ_CTG/δKO animals. Cells were challenged for 16h with 15μmol/L H_2O_2 to mimic the oxidative environment found during I/R[37] and cell death was assessed by trypan blue exclusion. There was a significant reduction in cardiomyoycte cell death in AMVMs from CaMKIIδKO mice while cells isolated from animals in which CaMKIIδ_C was re-expressed died to the same extent as WT. In contrast, myocytes from mice expressing only CaMKIIδ_B were protected from H_20_2-induced cell death (Figure 2-10C). These data complement the findings in the intact heart demonstrating that expression of CaMKIIδ_C, but not of CaMKIIδ_B,

contributes to I/R-induced cardiac damage, diminished ventricular function, and shortened lifespan.

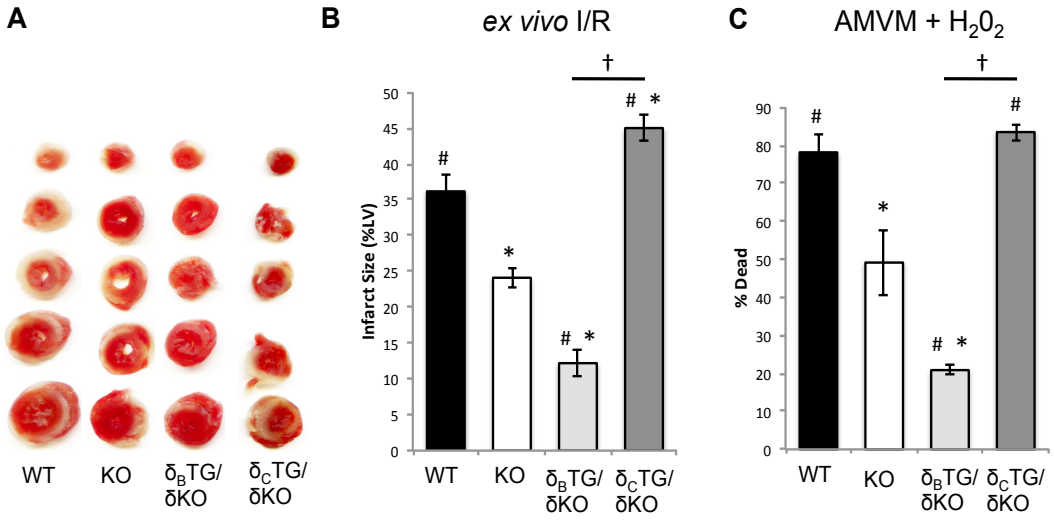

Figure 2-10. Differential effects of CaMKII subtypes on *ex vivo* I/R injury and oxidative stress. (A) Representative images hearts from WT, CaMKIIδKO, CaMKIIδ$_B$TG/δKO, and CaMKIIδ$_C$TG/δKO mice subjected to 25 minutes ischemia and 1 hour of reperfusion in the Langendroff mode. Hearts were sectioned and stained with TTC to reveal infarcted tissue. (B) Infarct size was quantified from TTC stained heart sections. Data are mean±SEM values from 14-16 mice. (C) Adult myocytes (AMVM) isolated from WT, CaMKIIδKO, CaMKIIδ$_B$TG/δKO, and CaMKIIδ$_C$TG/δKO mice were treated with 15μmol/L H_20_2. Data are mean±SEM n=3. *P<0.05 vs WT. #P<0.05 vs KO. †P<0.05 vs δ$_B$TG/δKO

CaMKII is known to be activated during *ex vivo* I/R, but previous experiments have not assessed subtype-specific CaMKII activation. Although we previously demonstrated that δ$_B$ and δ$_C$ can be equivalently activated by several pharmacological interventions[2], we wondered if I/R might lead to differential activation of the two subtypes. To assess I/R-induced activation of

CaMKII we analyzed phosphorylation of CaMKII at its autophosphorylation site threonine-286. Similar increases in CaMKII autophosphorylation were observed in whole cell lysates from hearts of δ_BTG/δKO and δ_CTG/δKO mice subjected to I/R (Figure 2-11A).

Many substrates of CaMKIIδ_C (e.g phospholamban, RyR2, and Histone deacetylase 4) localize to the cytosol or the cytosolic face of the cardiac sarcoplasmic reticular (SR) membrane. Thus we carried out further studies using a subcellular fraction enriched for cytosolic and membrane proteins. Strikingly in this fraction we observed differential activation of the δ_B and δ_C subtypes in response to I/R. Indeed whereas no increases in CaMKII autophosphorylation were observed in cytosolic/membrane fractions from δ_BTG/δKO mouse hearts, there was a more than 3-fold increase in autophosphorylated CaMKII in cytosolic/membrane fractions enriched from δ_CTG/δKO hearts (Figure 2-11B).

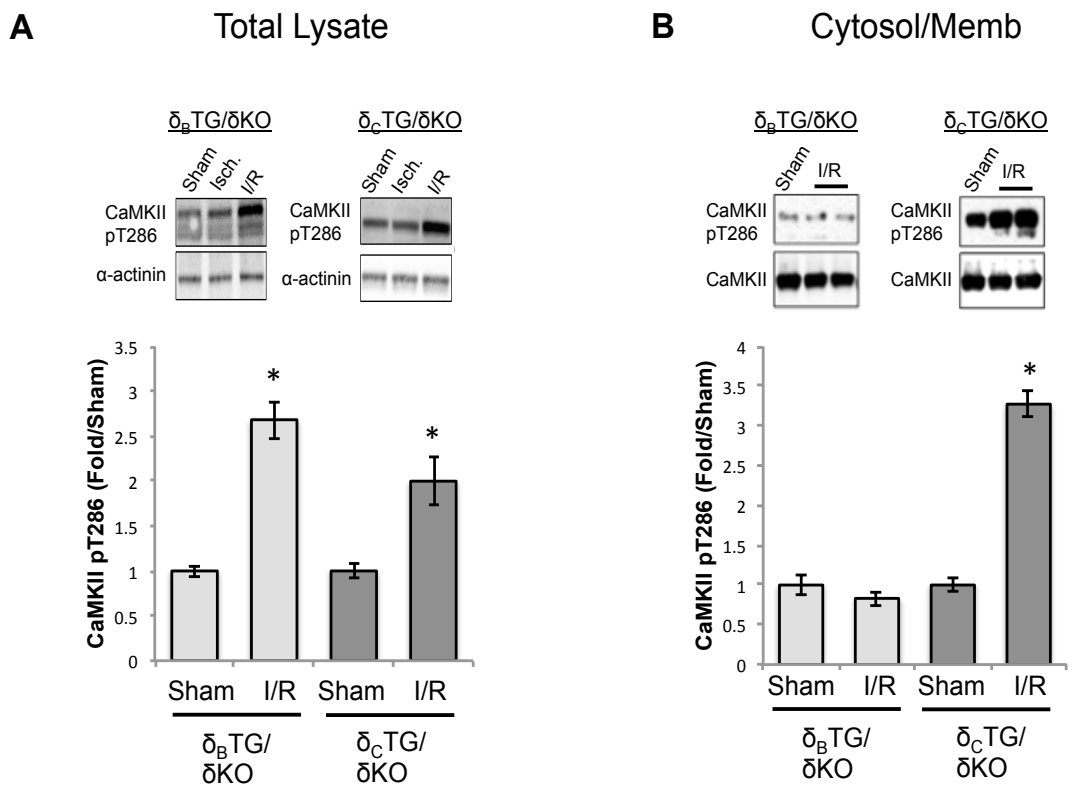

Figure 2-11. CaMKIIδ$_C$ is selectively activated in a subcellular fraction containing cellular membranes and cytosol. (A) Lysates from the hearts of CaMKIIδ$_B$TG/δKO and CaMKIIδ$_C$TG/δKO mice were subjected to western blot analysis using an antibody specific for auto-phosphorylated CaMKII. Data are mean±SEMS values from 4-6 mice. (B) Subcellular fractionation of hearts from CaMKIIδ$_B$TG/δKO and CaMKIIδ$_C$TG/δKO mice was carried out as described in methods yielding a fraction that includes cytosol and cellular membranes. Data are mean±SEM values from 4-6 mice. *P<0.05 vs sham.

2.4.2.3 CaMKIIδ subtypes differentially regulate NF-κB activation and subsequent upregulation of TNF-α during *ex vivo* I/R

Our earlier studies examining *in vivo* I/R damage linked the deleterious effects of CaMKII with activation of IKK and subsequent NF-κB nuclear accumulation. To determine which CaMKIIδ subtype was responsible, and

also assess involvement of systemic factors (e.g. leukocyte infiltration) in CaMKIIδ-mediated NF-κB activation, we examined regulation of IKK and NF-κB by I/R in the *ex vivo* heart. The phosphorylation of IKK was found to be elevated during *ex vivo* reperfusion in δ_CTG/δKO mice but not in δ_BTG/δKO mice (Figure 12A). Furthermore I/R mediated activation of IKK in δ_CTG/δKO mice was associated with an increased nuclear localization of the p65 subunit of NF-κB, which was not observed in δ_BTG/δKO (Figure 12B).

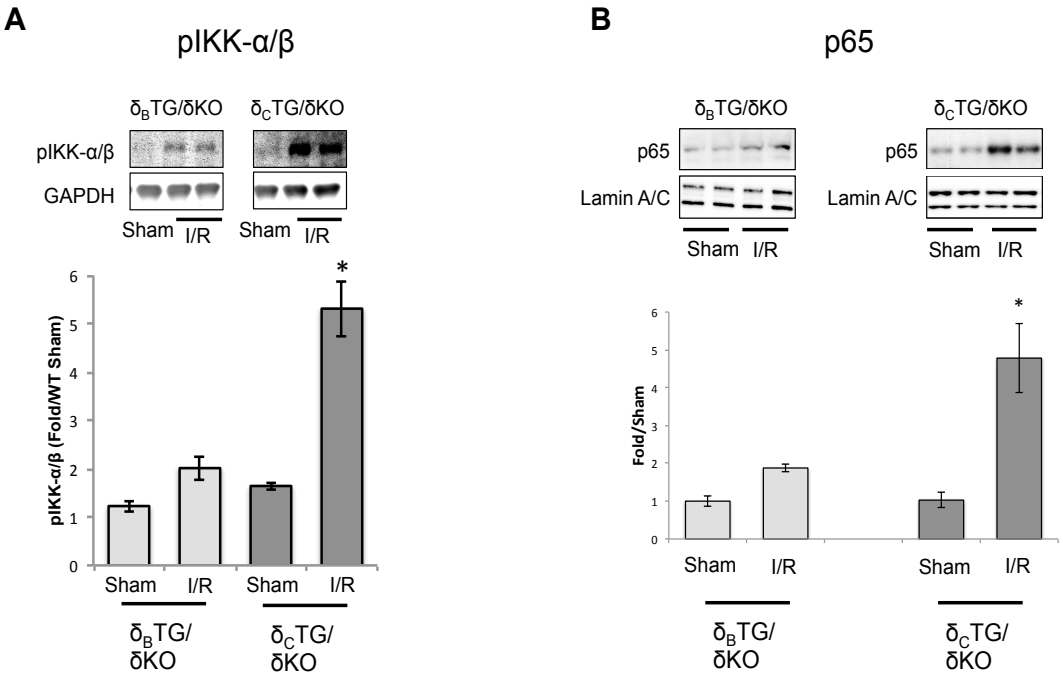

Figure 2-12. The NF-κB pathway is activated in CaMKIIδ$_C$TG/δKO mice following reperfusion. (A) Activation of IKKα/β was assessed by western blot using an antibody specific for auto-phosphorylated IKKα/β. (B) Nuclear translocation of p65 is downstream of IKK activation. We assessed nuclear p65 accumulation by western blot in nuclear fractions made from hearts subjected to *ex vivo* I/R. Data are mean±SEM values from 6 mice. *P<0.05 vs sham.

Nuclear p65 translocation would be expected to result in transcriptional activation of NF-κB target genes. Thus as further evidence that differences in NF-κB activation in δ_CTG/δKO and δ_BTG/δKO are functionally significant, we measured mRNA levels of genes regulated by NF-κB. Ischemia/reperfusion increased interleukin 6 (IL-6) mRNA in hearts from δ_CTG/δKO mice to a greater extent than in those from δ_BTG/δKO mice (Figure 2-13A). Even more striking was the robust increase in TNF-α expression in δ_CTG/δKO mice and the absence of upregulation of this gene in δ_BTG/δKO mice (Figure 2-13B).

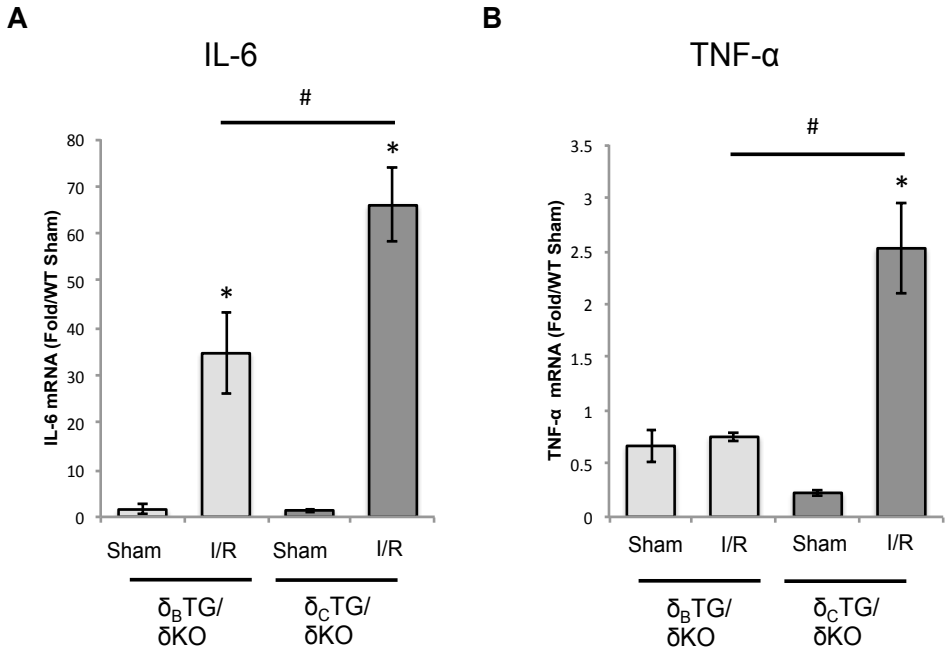

Figure 2-13. Genes downstream of NF-κB are upregulated in CaMKIIδ$_C$TG/δKO mice after *ex vivo* I/R. We assessed NF-κB activation by measuring mRNA levels of genes regulated by NF-κB. *A,* IL-6 *B,* TNF-α. Data are mean±SEM values from 4 mice. *P<0.05 vs sham.

To demonstrate that the deleterious effects of CaMKIIδ_C activation after I/R were mediated by IKK/NF-κB signaling as we observed in the *in vivo* studies presented in Chapter 2.2, we blocked IKK activation with the pharmacological inhibitor BMS-345541 (BMS)[21]. Since this drug had not, to our knowledge, been used in the *ex vivo* perfused heart we evaluated its efficacy and determined a dose (5μmol/L) that kept p65 sequestered in the cytosol/membrane fraction, i.e prevented its nuclear translocation. Hearts from δ_BTG/δKO, δ_CTG/δKO, WT, and KO mice were then exposed to 5μmol/L BMS or vehicle prior to and throughout the I/R protocol. Infarct size was significantly reduced by BMS administration in WT and δ_CTG/δKO mice while the already diminished infarct formation that was observed in δ_BTG/δKO animals was not affected (Figure 2-14A). CaMKIIδKO animals showed a modest but significant further reduction in infarct size.

In light of the selective elevation of TNF-α mRNA and evidence that active TNF-α can be produced in and secreted from the isolated perfused heart, we asked whether TNF-α mediated the deleterious effects of CaMKIIδ_C. For these studies we used etanercept, which inhibits TNF-α by preventing its interaction with TNF-α receptors[38, 39]. Etanercept was perfused at 5μg/ml prior to and throughout the I/R protocol. The results were similar to those obtained with IKK/NF-κB inhibition i.e. infarct size was significantly reduced in WT and δ_CTG/δKO mice and was not reduced further in δ_BTG/δKO animals. CaMKIIδKO animals showed modest but significant further reductions in

infarct size in the presence of etanercept (Figure 2-14B). These findings reveal a surprisingly important role for autocrine and/or paracrine TNF-α signaling in the *ex vivo* isolated perfused heart during I/R, demonstrate that this process is regulated by CaMKIIδ_C, and establish an essential cardiac-intrinsic role for NF-κB in I/R injury.

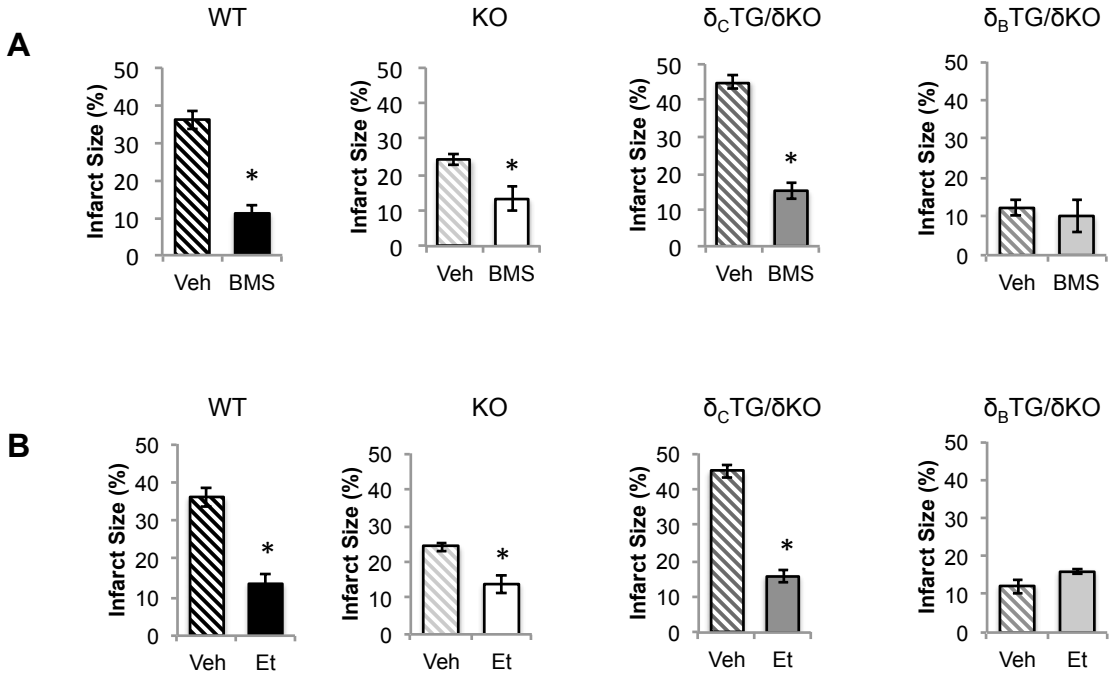

Figure 2-14. Inhibition of IKK or TNF-α ameliorates I/R damage. (A) Vehicle and BMS-345541 (5μmol/L) were perfused into hearts from WT, KO, CaMKIIδ_BTG/δKO, and CaMKIIδ_CTG/δKO animals. (B) Vehicle or etanercept (5μg/ml) were perfused into hearts from WT, KO, CaMKIIδ_BTG/δKO, and CaMKIIδ_CTG/δKO animals. Infarct size was measured via TTC staining following 1hr of reperfusion. Data are mean±SEM values from 4-8 mice. *P<0.05 vs veh.

2.4.3 Discussion

Our laboratory previously demonstrated that mice in which CaMKIIδ was selectively deleted from cardiomyocytes exhibited diminished infarct formation in response to *in vivo* I/R[1]. These experiments provided evidence that CaMKIIδ activation in cardiomyocytes has deleterious effects on infarct formation. This work did not, however, address the question of which subtype(s) of CaMKIIδ were responsible for cardiomyocyte death in response to I/R. Here we characterize and utilize mice in which the $CaMKII\delta_B$ or $CaMKII\delta_C$ isoforms are expressed in the CaMKIIδ KO animals to independently determine the role of each of these isoforms in cardiomyocyte CaMKII signaling. In addition we analyzed I/R damage in the *ex vivo* isolated perfused heart where the role of systemic inflammatory factors is eliminated. Our studies demonstrate that infarct development in the *ex vivo* heart is regulated by signals that are rapidly and locally generated and is significantly attenuated in the absence of CaMKIIδ. We further show that $CaMKII\delta_C$ re-expression in cardiomyocytes reverses the attenuation of infarct formation seen in the CaMKIIδKO mice. In contrast $CaMKII\delta_B$ re-expression does not exacerbate infarct formation and in fact further limits infarct development compared to that observed in KO mice. The significantly different effects of CaMKIIδ subtype expression in *ex vivo* I/R occur despite the fact that we see relatively similar levels of activation of $CaMKII\delta_B$ and $CaMKII\delta_C$ in response to I/R.

NF-κB has been implicated in *in vivo* I/R damage and myocardial infarction and our earlier studies demonstrated that CaMKIIδ activates NF-κB during *in vivo* I/R[1, 10, 40]. While activation of NF-κB in the heart was rapid, we assumed that systemic factors such as subsequent infiltration of non-cardiac inflammatory cells into the heart, occurring during the 24-48h time period of reperfusion, was a significant contributor to infarct development. Here, however, we demonstrate that cardiac-intrinsic signaling mediates CaMKIIδ$_C$ and NF-κB effects on infarct formation, i.e. activation of CaMKIIδ$_C$ and subsequent NF-κB mediated responses that occur in the isolated perfused heart regulate cardiomyocyte death and myocardial damage. It is possible that this cardiac intrinsic effect of CaMKIIδ is initiated by induction of cardiomyocyte cell death, leading to release of factors from necrotic cells that induce inflammatory signaling[41, 42]. We previously showed, however, that cyclosporine-A (CsA) treatment did not block NF-κB activation in response to *in vivo* I/R, indicating that this response was independent of necrosis [1] and have confirmed this in the current *ex vivo* study. A direct effect of CaMKII on NF-κB activation is also supported by our previous demonstration that adenoviral expression of CaMKIIδ$_C$ in neonatal rat ventricular myocytes induces IKK activation.

Strikingly, we show here that NF-κB activation is elicited by cardiac expression of CaMKIIδ$_C$ but not CaMKIIδ$_B$. This is consistent with the finding that IKK phosphorylation is increased by I/R in CaMKIIδ$_C$TG/δKO but not in

CaMKIIδ_BTG/δKO animals. Furthermore, we demonstrated that I/R increased activation of CaMKIIδ_C but not of CaMKIIδ_B in the cytosol/membrane, where IKK is localized. Thus in the isolated perfused heart activation of the CaMKIIδ_C subtype elicits inflammatory responses previously assumed to be generated through systemic signals *in vivo*.

Previous studies using pharmacological inhibitors to demonstrate involvement of CaMKIIδ in *ex vivo* I/R damage linked the effects of CaMKII to phosphorylation of PLN and RyR2 and subsequent Ca^{2+} dysregulation[16], supporting the hypothesis that rapid posttranslational modification of CaMKII targets was a driver of myocardial injury. Indeed, we originally considered it unlikely that transcriptional regulation by CaMKIIδ_C-mediated activation of NF-κB could play a significant role in determining cell viability over the course of only one hour of reperfusion. We observed, however, that inhibition of either IKK or TNF-α had a profound effect on infarct formation in WT mouse hearts exposed to 25 min *ex vivo* ischemia and 60 min reperfusion.

Recent studies have shown that receptor-interacting protein kinase 3 (RIP3) mediates TNF-α-mediated cell death through formation of the necrosome[43]. RIP3 phosphorylates and activates CaMKII during I/R[44]; accordingly a deleterious positive feedback loop involving CaMKIIδ_C, TNF-α, and RIP3 could be a major primary driver of myocardial damage during I/R. The experiments presented in this study clearly indicate that rapid TNF-α upregulation, downstream of myocardial NF-κB activation, is a mechanism by

which CaMKIIδ_C elicits infarct formation in the isolated perfused heart. This vicious cycle would likely be further enhanced and sustained by Ca^{2+} dysregulation resulting from CaMKII-mediated phosphorylation of SR substrates and increases in reactive oxygen species (ROS) during I/R[45].

While we demonstrated that cardiac TNF-α expression during I/R is mediated by CaMKIIδ_C activation in cardiomyocytes, we do not know the extent to which TNF-α is formed in and secreted from these cells. Cardiac-resident macrophages and other non-myocytes are potential sources of TNF-α and could thus act in an autocrine or paracrine fashion to affect cardiomyocyte survival. The mechanism and extent to which CaMKIIδ_C activation in myocytes signals to other cells is not clear, but our studies demonstrate that the cardiomyocyte initiates signals though CaMKIIδ that upregulate TNF-α. TNF-α inhibitors such as etanercept have been used to inhibit TNF-α signaling in patients with various autoimmune disorders[46]. Effects of TNF-α inhibitors on cardiovascular disease outcome have also been evaluated with uncertain results that could in part be due to systemic responses to sustained antibody administration[42]. These detrimental effects would not be expected to occur during short-term treatment thus one could consider use of TNF-alpha inhibitors at the onset of reperfusion following primary percutaneous intervention for myocardial infarction.

In summary, we demonstrate that the δ_C isoform of CaMKII contributes significantly to myocardial damage following *ex vivo* I/R. Signaling occurs

through NF-κB and TNF-α and acute inhibition of the generation or function of these molecules has a very robust protective effect in WT animals and in those expressing CaMKIIδ$_C$. Importantly, we show that these events occur during a much shorter timeframe than would have been predicted by previous studies of CaMKIIδ and NF-κB signaling in *in vivo* I/R, and that these events occur in the absence of systemic factors such as infiltration of cells originating outside of the heart. CaMKII inhibition is predicted to be of therapeutic benefit in a number of contexts. Our results indicate that selective CaMKIIδ$_C$ inhibition would confer the most benefit over blockade of all cardiac CaMKII isoforms although specific means of locally inhibiting the δ$_C$ isoform do not yet exist. An alternative approach would be to acutely block events that occur downstream of CaMKIIδ$_C$ activation during I/R such as IKK/NF-κB activation or TNF-α signaling.

2.5 Conclusions

Many studies have implicated the protein products of the CaMKII gene in various cardiac disease states, but very few investigate the individual functions of those proteins. The work presented in this chapter establishes that a single subtype of the CaMKIIδ gene, CaMKIIδ$_C$, is exclusively responsible for the deleterious effects of CaMKIIδ gene expression in I/R. Initial work established a central role for CaMKIIδ in I/R, and showed for the first time

direct activation of NF-κB by CaMKIIδ. A systematic biochemical analysis of CaMKIIδ subtypes revealed that they are not segregated within distinct subcellular compartments but in fact are expressed throughout the cell, and subsequently I led a research project to investigate the hypothesis that CaMKIIδ subtypes have differential roles in I/R. Our studies on NF-κB signaling downstream of CaMKIIδ$_C$ activation in I/R allowed us to recommend a novel therapeutic approach to treat I/R injury.

2.6 Experimental Procedures

In vivo I/R and hemodynamic measurements.

8-10 wk old mice were anesthetized by sodium pentobarbital (45 mg/kg, i.p.) and then subjected to myocardial ischemia induced by left anterior descending (LAD) coronary artery occlusion for 1hr followed by reperfusion for 24 hr. Sham operated mice were subjected to the same surgical procedures without LAD ligation. Buprenorphine (0.1 mg/kg) was administrated subcutaneously to reduce postoperative pain. In the animals with drug treatment, BMS- 345541 (2 mg/kg) (an inhibitor of I kappa B kinase) or cyclosporin A (CsA, 10 mg/kg) was intravenously administrated 10 min before reperfusion. At the end of reperfusion, the LAD artery was re-occluded. 2% Evans blue dye was injected into the carotid arterial root to stain the normally

perfused region blue and outline the area at risk (AAR). The left ventricle (LV) was rapidly excised and cut into 1-mm-thick transverse sections, which were then incubated in 1% 2,3,5- triphenyltetrazolium chloride (TTC) for 10 min at 37°C to differentiate the viable (red) and nonviable myocardium (pale) within the risk zone. The areas of infarction, at risk, and nonischemic LV were assessed by a blinded observer using Image J (NIH). The area at risk as a percent of the LV (AAR/LV) and the infarction size (IS) as a percent of the AAR (IS/AAR) were calculated. After 24-hr reperfusion, the hemodynamic measurements were performed. A 1.8-French high fidelity catheter tip micromanometer was inserted into the right carotid artery and then manipulated across the aortic valve into the left ventricle (LV). When a stable and reproducible pressure reading was obtained, LV pressure, Maximum positive and negative first derivative of left ventricular pressure ($\pm dP/dt_{max}$) were recorded and averaged from 12 beats.

Heart tissue preparation following *in vivo* I/R.

The left ventricles were cut transversely into two parts. Myocardium from half of the LV was frozen in liquid nitrogen for protein, gene array (RNA analysis) and DNA laddering. The other part was fixed for 24 hrs in 4% paraformaldehyde dissolved in 0.1 M PBS (pH 7.4), subsequently embedded in paraffin for histological analysis.

TUNEL assay and immunofluorescence staining

In situ DNA fragmentation was assessed using TUNEL assay on deparaffinized sections and the number of TUNEL positive cells was determined in 3 sections per group. Inflammatory cell infiltration after I/R was visualized by immunofluorescent staining of Cluster of Differentiation 68 (CD68).

Preparation of heart tissue extract and cell lysate for western blot analysis

Each mouse was humanely euthanized and placed in supine position. An incision was made parallel to the diaphragm and the rib cage was separated by cutting parallel to the sternum to visualize the heart. The heart was excised and immediately rinsed in ice cold PBS to remove blood. Atria were removed and the ventricles were snap frozen in liquid nitrogen. Frozen hearts were pulverized and powder was homogenized using a tissue homogenizer (Tissuemiser, Fisher Scientific) in ice cold RIPA buffer (150 mmol/L NaCl, 50 mmol/L Tris (pH7.4), 1% NP-40, 1 % of sodium deoxycholate,0.1 % of SDS, 0.2 mmol/L EDTA) supplemented with protease and phosphatase inhibitors (100 µmol/L Na_3VO_4, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mmol/L *p*-nitrophenyl phosphate, 1mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L NaF). Cardiomyocytes were washed 3 times in ice cold PBS and then harvested in the RIPA buffer formulation

described above. Tissue and cell lysates nutated 4°C for 20 min, and lysates were clarified by centrifugation at 14,000 RPM at 4°C. Protein concentration was measured using Bradford analysis.

Western Blotting

Lysates were mixed with LDS sample buffer and reducing agent, heated at 80°C for 10 minutes. Equal amounts of protein (10-40 µg), or equal volumes of fractions were loaded onto SDS-PAGE (invitrogen NuPage system), run, transferred to PVDF membranes (Millipore), blocked in 5% milk in TBS/Tween for 1 hour and the resulting blot probed using antibodies at a 1:1000 dilution in 3% BSA/TBS-tween. The antibodies used for immunoblotting were as following: rabbit CaMKIId antibody (a gift from Don Bers laboratory, University of California Davis, Davis, CA), mouse anti-phospholamban (Upstate Biotechnology), rabbit anti-phospho-phospholamban (Thr17) (Cyclacel, Dundee, UK), rabbit Rho-GDI (Cell signaling technology), rabbit VDAC/porin (Calbiochem), mouse SERCA2a (Thermo/Affinity Bioreagents), mouse Lamin A/C (Cell Signaling Technology), mouse anti-phospho-CaMKII (Thermo/Affinity Bioreagents), and rabbit anti-phospho-HDAC5 Ser498 (Signalway Antibody) CaMKIIδ, PLN, NF-κB and α-actinin (Santa Cruz); cleaved caspase-3; MCP-1, IκBα, Cytochrome C, phospho-IKKα/β Ser176/180, and VDAC (Cell Signaling); phospho-PLN at CaMKII site (Thr17) and PKA site (Ser16) (Badrilla); RyR and phospho-CaMKII (Affinity

Bioreagents); phospho-RyR2 at CaMKII site (Ser2814) and PKA site (Ser2808) (obtained from A.R.Marks, Columbia University, New York, New York, USA). Following primary antibody incubation, blots were washed with TBS- tween (10 min x 3 times) and incubated with secondary antibodies (1:5000) in 5% milk/TBS-Tween for 30-60 minutes. Anti- rabbit or anti-mouse secondary antibody (Sigma) was used at 1:10,000 dilution.

Nuclear fractionation

Mice were killed by cervical dislocation, and hearts were quickly removed and placed in ice-cold PBS. Following cannulation, hearts were perfused with PBS to remove blood, frozen in liquid N_2, and pulverized. Tissue was then homogenized in isolation buffer (70mmol/L sucrose, 190mmol/L mannitol, 20mmol/L HEPES, .2mmol/L EDTA) that was supplemented with various inhibitors: sodium vanadate, leupeptin, aprotinin, *p*-nitrophenyl phosphate, and phenylmethylsulfonyl fluoride. Homogenate was centrifuged at 600g for 10m at 4°C to yield cytosolic and nuclear fractions.

Affymetrix and transcription factor binding site enrichment analysis

Affymetrix Gene 1.0 ST v1 arrays were hybridized to labeled cDNA prepared from WT and CaMKIIδ KO hearts subjected to 1hr ischemia followed by 24hr reperfusion (n=3 replicates/condition). Affymetrix .cel files were normalized and RNA signal values generated with AltAnalyze. To determine

genes differentially expressed after I/R in WT and CaMKIIδ KO hearts, interaction P values were computed with the LIMMA package in R. We identified 3,129 probesets with differential expression (interaction $P<0.05$ and at least a 20% fold change between any one of the four groups). These genes were clustered with the HOPACH algorithm. To predict transcription factors that might regulate these 3,129 differentially expressed genes their 5kb promoters were scanned for enrichment (compared to frequency of enrichment in all annotated 5kb promoters) of evolutionarily conserved (human to mouse) transcription factor binding sites from the TRANSFAC database using Whole Genome RVista tool. This analysis yielded 973 transcripts that contained at least one conserved NF-κB site. These were HOPACH clustered and each cluster was annotated by enrichment for Gene Ontology terms using GO-Elite.

NF-κB activity assay

An enzyme-linked NF-κB immunosorbent assay (ELISA)-based method (Trans-AM NF-κB; Active Motif, Carlsbad, CA) was used to quantify NF-κB activation according to the manufacturers guidelines. Briefly, cardiac lysates were placed in 96-well plates coated with an oligonucleotide containing the NF-κB consensus sequence, and the presence of active NF-κB was detected by using antibodies specific for p65 subunits that are not complexed to IκB and thus able to bind the consensus sequence. A horseradish peroxidase

(HRP)-conjugated secondary antibody was used to quantify NF-κB binding by conversion of an applied chromogenic substrate.

CaMKII activity assay

CaMKII activity was measured in ventricular homogenate using Syntide-2, a synthetic CaMKII-specific substrate peptide. Briefly, following homogenization in lysis buffer (50mM HEPES, 10% ethylene glycol, 2mg/mlBSA,5mM EDTA, pH 7.5), ventricular lysates were incubated at 37°C for 10min in autonomous activity buffer (50mM HEPES, 10mM magnesium acetate, 1mM EGTA, 1mg/ml BSA, 20μM Syntide-2, 1mM DTT, 400nM ATP[γ-^{32}P], pH 7.5) or maximal activity buffer (50mM HEPES, 10mM magnesium acetate, 500nM calcium chloride, 1μM calmodulin, 1 mg/ml BSA, 20μM syntide-2, 1mM DTT, 400nM ATP[γ-^{32}P], pH 7.5). Labeled peptides were blotted onto Whatman P81 filter paper and washed extensively. CPM was quantified in a scintillation counter. Percent activation was calculated as the ratio of autonomous to maximal activity for each sample.

Adenoviral CaMKIIδ overexpression

Neonatal rat ventricular myocytes (NRVMs) were isolated from hearts of 2–3-day-old Sprague-Dawley rat pups and infected with 40 MOI Adv-GFP, 40 MOI constitutively active CaMKIIδ, or 40 MOI constitutively active CaMKIIδ in the presence of 15μM BMS-345541 for 24 hours. Nuclear p65 was

determined in nuclear extracts by Western blotting, and phospho-IKKα/β Ser176/180 was determined in whole-cell lysate by Western blotting.

Transgenic mice

Transgenic mice expressing either CaMKIIδ$_C$ or CaMKIIδ$_B$ in the heart were generated. CaMKIIδ$_B$ and δ$_C$ mice were crossed with CaMKIIδ KO mice and heterozygous offspring carrying the transgene were inbred with the ones without the transgene. Genotypes used for experiments were WT, δKO, CaMKII δ$_B$ and δ$_C$ TG and CaMKII δ$_B$ and δ$_C$ TG mice in the δ null background. All mice used in these studies were black swiss background, and WT littermates were used as controls. All mice used in the present study were of mixed gender and 5-6 weeks of age, unless otherwise noted. All procedures were performed in accordance with NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

Subcellular tissue fractionation

Flash frozen hearts were pulverized and homogenized using a Dounce glass tissue grinder. The homogenate was centrifuged for 10 min at 600×g, the pellet washed three times and then re-suspended in nuclear extract buffer (HEPES 20 mmol/L, 25% Glycerol, NaCl 420 mmol/L, $MgCl_2$ 1.5 mmol/L, EDTA 0.2 mmol/L). The resulting supernatant is the Nuclear Fraction. The

supernatant from the first centrifugation was centrifuged at 5000×g (to remove mitochondria) and the resulting supernatant then centrifuged at 100,000×g. The supernatant is the Cytosolic Fraction and the pellet, after being washed 3 times and re- suspended in RIPA buffer (NaCl 150 mmol/L, Tris 20 mmol/L, 1% Triton X 100, 0.1% SDS), is the SR/membrane fraction.

SR purification

Freshly isolated myocytes from 4 mouse hearts were suspend in homogenization buffer and using a dounce homogenizer, cells were homogenized using 15-20 strokes on setting B. Cell lysates were spun at 10,000 RPM to clarify lysates and pellets resuspended in homogenization buffer and homogenize for 10 strokes at setting B. Following a spin @10,000 rpm for 8min, supernatant was collected and loaded onto a fresh sucrose gradient consisting of 1ml of 20%, 2ml of 27%, 2 ml of 30%, 2ml of 34%, 3ml of sup with 72% sucrose (1.5 ml +1.5ml 72%). Gradients were spun at 22000rpm for 24hrs @4C. SR fraction was harvested from the interface of the 27% and 30% gradient.

Langendorff Perfusion of Heart

Mice (4 weeks old, weighing 23–25 g) were heparinized (500 units/kg, intraperitoneally) and cervically dislocated. Hearts were rapidly excised, washed in ice-cold Krebs- Henseleit solution (118 mmol/L NaCl, 24 mmol/L

NaHCO3,4 mmol/L KCl, 1 mmol/L NaH2PO4, 2 mmol/L CaCl2, 1.2 mmol/L MgCl2, 12 mmol/L glucose and 10 mmol/L Hepes,pH7.4), and cannulated via the aorta on a 20-gauge stainless steel blunt needle. Hearts were perfused at 80 mm Hg on a Langendorff apparatus using Krebs- Henseleit solution at 37 °C. Hearts were perfused with oxygenated buffer for 12 min to allow for equilibration, followed by 15 minutes perfusion with 100nmol/L phenylephrine or vehicle, or a bolus injection of 10mmol/L caffeine or vehicle directly into the heart. KN-93 (20mmol/L) was added to the Krebs-Henseleit buffer used for the 12 minute equilibration perfusion as a pre-treatment. Following this pre-treatment, KN-93 was administered simultaneously with PE or caffeine. Heart was removed following perfusion/treatment and immediately rinsed in ice cold buffer, atria removed, and then flash frozen in liquid nitrogen.

Membrane/cytosol fractionation procedure

Mice were killed by cervical dislocation, and hearts were quickly removed and placed in ice-cold PBS. Following cannulation, hearts were perfused with PBS to remove blood, frozen in liquid N_2, and pulverized. Tissue was then homogenized in isolation buffer (70mmol/L sucrose, 190mmol/L mannitol, 20mmol/L HEPES, .2mmol/L EDTA) that was supplemented with various inhibitors: sodium vanadate, leupeptin, aprotinin, *p*-nitrophenyl phosphate, and phenylmethylsulfonyl fluoride. Homogenate was centrifuged at 600g for 10m at 4°C, and the supernatant transferred to another tube. The

supernatant was centrifuged at 5,000g for 15m at 4°C to yield a cytosol/membrane fraction.

Transthoracic echocardiography

Echocardiography was performed using the VisualSonics VeVo 770 Imaging System (VisualSonics, Toronto, Canada) equipped with high-frequency 30 MHz probe, as described. All measurements were performed in the presence of exogenous isoproterenol. Body temperatures were maintained between narrow ranges (37.0 ± 1.0 °C) to avoid confounding effects of hypothermia. Heart rate varied per mouse in response to isoproterenol.

Isolated perfused (*ex vivo*) I/R

Mice were killed by cervical dislocation, and hearts were quickly removed and placed in ice-cold Ca^{2+}-free Krebs-Henseleit buffer. Hearts were then placed on a Langendorff perfusion system (Radnoti LLC), and perfused at a constant pressure of 80 mmHg at 37 °C with a modified Krebs-Henseleit buffer solution containing (in mmol/L); 2.0 $CaCl_2$, 130 NaCl, 5.4 KCl, 11 dextrose, 2 pyruvate, 0.5 $MgCl_2$, 0.5 NaH_2PO_4, and 25 $NaHCO_3$ and aerated with 95% oxygen and 5% carbon dioxide, pH 7.4. To measure infarct size, hearts were subjected to 25-minute global ischemia and 1-hour reperfusion; the ventricles were then frozen and cut transversely into 5 slices of equal thickness. The slices were then incubated in 1% TTC/PBS and fixed in 10%

formalin-PBS for 24 hours. Fixed slices were then scanned, and ImageJ was used to measure and calculate the size of the infarct area and the total area. For experiments utilizing BMS-345541 (Sigma-Aldrich) the drug was dissolved in Krebs-Henseleit buffer solution at a concentration of 5μmol/L and was present throughout the I/R protocol. For those using etanercept, the drug was present for the entire I/R procedure at a concentration of 5μg/ml.

Isolation of adult mouse ventricular myocytes

Cardiac ventricular myocytes were isolated from the ventricles of 8–12-weeks-old WT, CaMKIIδKO, CaMKIIδ$_B$, and CaMKIIδ$_C$ mice. Animals were killed via cervical dislocation, and hearts were removed, cannulated, and subjected to retrograde aortic perfusion at 37 °C, at a rate of 3 ml/min. Hearts were perfused for 4 min in Ca^{2+}-free buffer, followed by 8–10 min of perfusion with 0.25 mg/ml collagenase (Blendzyme 1, Roche). Hearts were removed from the cannula and the ventricle was dissociated at room temperature by pipetting with increasingly smaller transfer pipettes. Collagenase was inactivated once the tissue was thoroughly digested, by resuspending the tissue in medium containing 10% bovine calf serum. Calcium was gradually added back to a final concentration of 1 mmol/L and cells were plated on laminin-coated dishes in minimal essential medium/Hanks' balanced salt solution containing 5% serum. After 1 hr, cells were washed and serum-free

medium was added back. Cells were treated with 15μMOL/L H_20_2 to induce cell death.

RT-PCR

RNA extraction for real time analysis was performed using the solid-phase RNeasy purification kit from Qiagen (Venlo, Netherlands). First strand cDNA synthesis for Real time PCR was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grand Island, NY). Gene expression was determined using Taqman® Universal PCR master mix, Cy5-labeled Taqman® probe for TNF-α and IL-6 and FAM-labeled Taqman® probe for GAPDH (Applied Biosystems).

Statistical Analysis

All data are presented as means ± standard error of the mean (SE). Comparisons between groups were performed using the Student *t* test, the Mann Whitney U test, Kruskal-Wallis test or One-way ANOVA, followed by the Tukey post hoc test, where appropriate. A p value < 0.05 was considered statistically significant.

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Chapter 3

The role of CaMKIIδ in GPCR-mediated fibrosis, mitochondrial dysfunction, and heart failure development

3.1 Summary

The hypothesis that CaMKIIδ is activated in the stressed heart has been confirmed by a variety of observations, and we postulated that CaMKIIδ was a central mediator of signaling events during cardiac stress that result in dysfunction and cell death. We have demonstrated that CaMKIIδ expression and activity are central mediators of hypertrophy decompensation during TAC, heart failure development downstream of sustained G_q signaling, and the development of fibrosis and cardiac dysfunction during sustained β-adrenergic stimulation. Using mice that lack expression of CaMKIIδ, we demonstrated that hypertrophy decompensation does not occur in KO mice as it does in animals that express normal levels of CaMKIIδ, and described pathways by which this role of CaMKIIδ is achieved[1]. Mice with transgenic expression of G_q develop cardiac hypertrophy and subsequent decompensation leading to heart failure. By crossing KO animals with those that overexpress G_q, we were able to delineate a role for CaMKIIδ in G_q-induced cardiac stress and determine that altered ROS production associated with decreased expression of the

mitochondrial protein UCP3 underlies the deleterious role of CaMKIIδ in this paradigm. We further implicated CaMKIIδ in the pathogenesis of myocardial disease by demonstrating that mice lacking CaMKIIδ do not develop fibrosis in response to chronic β-adrenergic stimulation despite exhibiting normal physiological and hypertrophic responses to isoproterenol. I assisted and provided data for the studies presented in this chapter, which when viewed together allow us to conclude that CaMKIIδ is generally important in the manifestation of cardiac stress.

3.2 Mitochondrial reprogramming induced by CaMKIIδ mediates hypertrophy decompensation.

3.2.1 Introduction

The heart is a highly adaptive organ, often responding to stress by altering its physiology and function. In most cases, this adaptive response is beneficial in the short-term; it enables the heart to adequately supply the body with blood even under conditions that would otherwise diminish cardiac function[2-4]. Unfortunately, these adaptive responses often result in cardiac dysfunction, cell death, and arrhythmia when sustained for long periods of time. A central focus of cardiovascular science is to elucidate the molecular mechanisms by which this process occurs. Some of the most significant

changes during cardiac stress occur in the regulation of Ca^{2+} cycling, and such changes can precede development of cardiac dysfunction. This is the case not only in chronic forms of cardiac stress such as hypertension, but also in acute forms of cardiac injury such as myocardial infarction[5, 6].

Alterations in CaMKII activity and expression are known to occur during the pathogenesis of heart failure[7, 8]. In order to study whether these changes contribute to the development of cardiac disease, we generated animals that lack CaMKIIδ and subjected them to TAC. TAC simulates the type of cardiac stress that occurs in humans with hypertension, and animals subjected to TAC develop severe cardiac dysfunction over the course of several weeks. Animals lacking CaMKIIδ developed hypertrophy to the same degree as WT mice, but did not decompensate. KO animals had improved cardiac function and survived longer than mice with normal expression of CaMKIIδ[1]. These important experiments focused our efforts on studying pathways that regulate cell death and decompensation as opposed to those regulating hypertrophy. One such pathway is that downstream of G_q.

Prohypertrophic hormones such as α-adrenergic agonists and angiotensin II stimulate the heterotrimeric G protein G_q and mice with cardiomyocyte-specific overexpression of the catalytically active α subunit of G_q develop marked cardiac hypertrophy that recapitulates hypertrophy development and subsequent decompensation. Supporting the concept that G_q signaling is of central importance to the development of hypertrophy during

pressure overload induced by TAC are data showing that mice lacking G_q/G_{11} are unable to develop hypertrophy in response to TAC[9, 10].

G_q signaling has been linked to increases in intracellular calcium concentration, suggesting the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) as a potential downstream mediator of G_q-mediated hypertrophy and heart failure[11]. G_q-induced cardiac decompensation has also been associated with mitochondrial dysfunction and increased mitochondrial ROS generation[12-14]. Data indicating CaMKII signaling can lead to altered mitochondrial calcium handling[15-17] and ROS generation further suggested that CaMKIIδ might serve as the downstream mediator of the maladaptive mitochondrial effects of G_q signaling. To test this hypothesis we crossed CaMKIIδ KO animals with mice that overexpress G_q in order to determine to what degree CaMKIIδ signaling mediated the deleterious effects of G_q overexpression.

3.2.2 Results

3.2.2.1 CaMKIIδ deletion rescues Gq-induced cardiac dysfunction but does not affect Gq-induced hypertrophy.

Consistent with previously published reports, analysis of hypertrophic indices revealed significant increases in left ventricular mass and cardiomyocyte cross-sectional area in G_q mice (G_qTG) compared with WT

animals. Associated with these changes in the G_q mice were observations indicating increased CaMKII activity. Specifically, phosphorylation of CaMKII substrates at the SR was elevated in animals overexpressing G_q (Figure 3-1A). Consistent with our previous observations of KO mice during pressure overload, deletion of CaMKIIδ did not abrogate the development of hypertrophy in G_q transgenic mice (Figure 3-1B). Further studies in cardiac myocytes demonstrated that pharmacological inhibition of CaMKII did not block the ability of G_q to induce cellular hypertrophy (Figure 3-1C). Thus, CaMKIIδ is unlikely to be involved in the signaling events linking G_q activation to cardiac hypertrophy.

Compared with WT animals, G_qTG mice had diminished cardiac function by 8 weeks of age. This was evidenced by reduced fractional shortening and left ventricular systolic dilatation (Figure 3-1D and 3-1E). Another indicator of insufficient cardiac function is lung weight/body weight ratios, which increase as heart failure worsens (Figure 3-1F). This ratio was significantly increased in G_qTG mice. All of these changes were ameliorated by deletion of CaMKIIδ. Other characteristic heart failure–associated phenotypes of G_q mice, including cardiomyocyte apoptosis, fibrosis, and ventricular arrhythmias, were likewise improved by CaMKIIδ deletion. Thus, while structural G_q-stimulated cardiac hypertrophy was maintained after CaMKIIδ abrogation, deletion of CaMKIIδ prevented the associated cardiac decompensation.

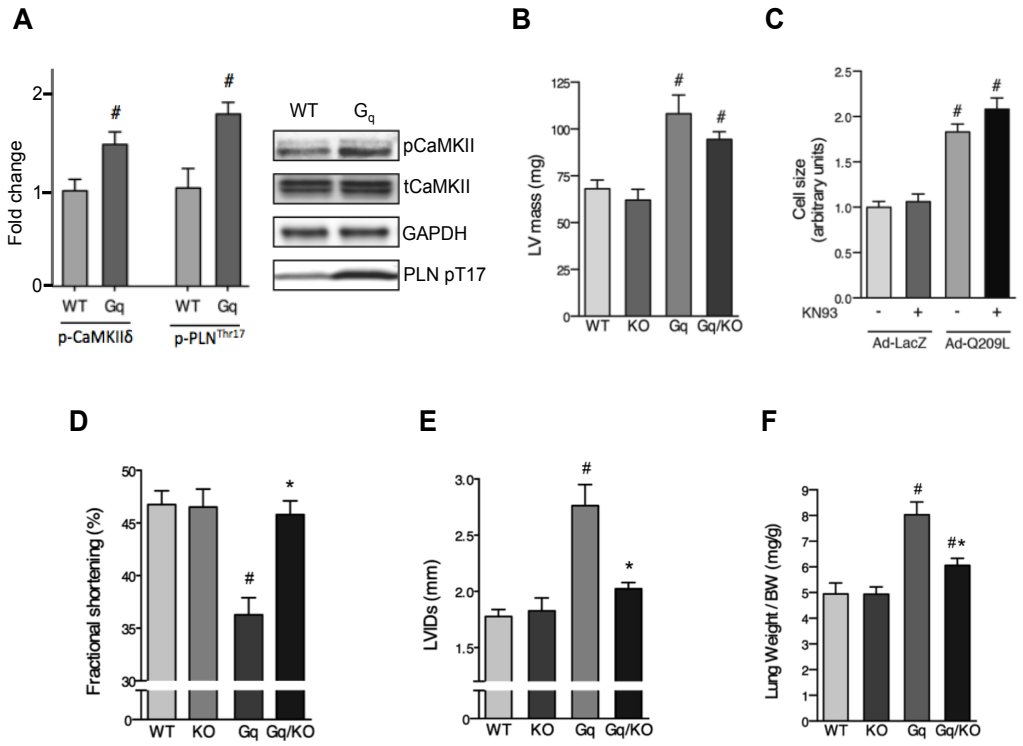

Figure 3-1 CaMKIIδ is not required for G_q-induced cardiac hypertrophy and CaMKIIδ knockout prevents G_q-induced cardiac dysfunction. (A) Phosphorylated CaMKIIδ in whole cardiac lysates, corrected for total CaMKIIδ or phosphorylation of phospholamban (PLN) at the CaMKIIδ specific site in whole cardiac lysates, corrected for GAPDH. The two bands visible with the total CaMKIIδ antibody represent the two splice variants δ_B and δ_C. #, $p < 0.05$ versus WT. (B) A Gravimetric and echocardiographic index of left ventricular (LV) hypertrophy, LV mass n=7 for wild type [WT], n=5 for CaMKIIδ knockout [KO], n=9 for Gq, and n=8 for Gq mice in a CaMKIIδ knockout background [Gq/KO]) (C) Neonatal rat ventricular myocytes stained with phalloidin after infection with an adenovirus expressing constitutively activated Gαq (Ad-Q209L) or β-galactosidase (Ad-LacZ) were cultured in the presence or absence of the CaMKII inhibitor KN93, and average cardiomyocyte circumference 24 hours after infection was assessed. Scale bar is 10 μm. All values are expressed as mean±SEM. #*P*<0.05 vs WT or Ad-LacZ. (D) and (E) Echocardiographic indices of left ventricular function dilatation, respectively. n=7 for WT, n=5 for KO, n=9 for Gq, and n=8 for Gq/KO **(**E**)** Lung weight/body weight ratio, an indicator of the severity of dysfunction (n=6–10 per group). All values are expressed as mean±SEM. #*P*<0.05 vs WT; **P*<0.05 vs Gq.

3.2.2.2 CaMKIIδ deletion limits G_q-induced mitochondrial dysfunction and oxidative stress

The deleterious effects of G_q are partially mediated through increases in mitochondrial oxidative stress[18]. To determine whether G_q signaling affects mitochondrial function via CaMKII activation, we measured mitochondrial ROS generation in WT, KO, G_qTG, and G_qTG/KO animals. ROS was assessed by dihydroethidium staining and mitochondrial protein carbonylation. G_q mice displayed a significant increase in mitochondrial ROS generation that was partially normalized by CaMKII deletion (Figure 3-2A). Further *in vitro* experiments linked these ROS increases to cardiomyocyte cell death. NRVM expressing an activated construct of G_q display increases in cell death, and pharmacological inhibition of CaMKII partially blocks this effect. However, in the presence of a strong antioxidant (N-Acetyl-Cysteine) inhibition of CaMKII no longer blocks cell death (Figure 3-2B). These data implicate ROS generation in the signaling pathways linking G_q activation to CaMKII and cell death.

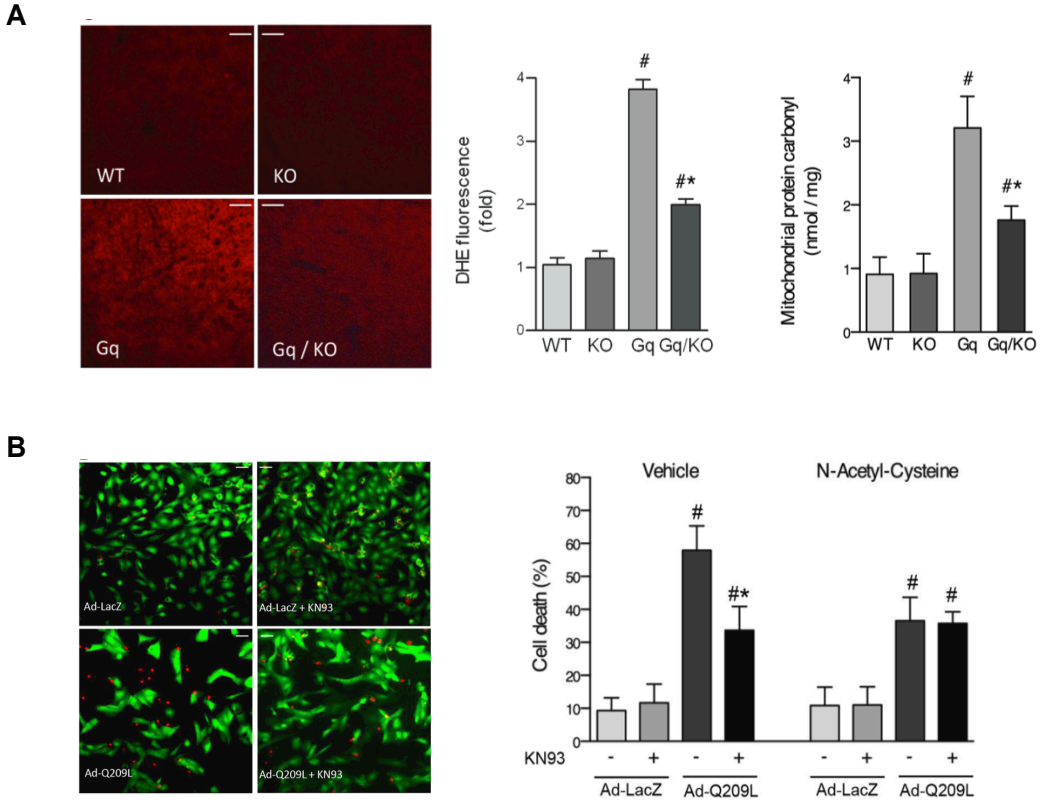

Figure 3-2. CaMKIIδ inhibition/deletion prevents mitochondrial ROS production and cell death mediated by G_q. (A) Cryopreserved myocardial sections were stained with dihydroethidium (DHE), an indicator of total myocardial ROS. Scale bar is 60 μm. Bar graph depicts average DHE fluorescence in the different experimental groups (n=5–7 per group). Carbonylation of mitochondrial proteins in the different experimental groups was measured by ELISA of cardiac mitochondrial homogenates (n=6–8 per group). All values are expressed as mean±SEM. #*P*<0.05 vs WT; **P*<0.05 vs Gq. (B) Typical example of NRVMs stained with the live cell indicator calcein (green) and the dead cell indicator propidium iodide (red) after infection with constitutively activated G_q (Ad-Q209L) or Ad-LacZ and cultured in the presence or absence of KN93. Scale bar is 40 μm. Cells were considered viable when positive for calcein and negative for propidium iodide. Bar graph depicts cell death expressed as the % total cells in the different experimental groups, with or without the addition of the ROS scavenger *N*-acetylcysteine or vehicle. #*P*<0.05 vs Ad-LacZ; **P*<0.05 vs Ad-Q209L.

3.2.2.3 CaMKIIδ deletion normalizes G_q-induced changes in UCP3 gene expression though regulation of PPARα

In order to understand how CaMKII leads to mitochondrial dysfunction, we utilized RNA sequencing to detect mitochondrial genes that are differentially regulated in G_qTG mice compared to G_qTG/KO mice. One of the mitochondrial gene transcripts found to be markedly downregulated in G_q mice and restored in G_q/KO mice was uncoupling protein 3 (UCP3). When activated, uncoupling proteins such as UCP3[19], can induce proton leak by dissipating the proton motive force across the inner mitochondrial membrane, thereby reducing ROS generation[20]. The decrease in UCP3 mRNA levels observed through RNA sequencing was confirmed by changes in UCP3 protein expression in mitochondria isolated from G_q and G_q/KO mice. To further explore the mechanism and significance of G_q regulation of UCP3, we examined the regulation of UCP3 in NRVMs expressing activated G_q (Q209L). UCP3 mRNA and protein expression were significantly decreased in cells expressing Q209L, and this was prevented by CaMKII inhibition with KN93 confirming a role for CaMKII in the regulation of UCP3 expression (Figure 3-3A).

Peroxisome proliferator–activated receptor α (PPAR-α) has been suggested to regulate the expression of UCP3[19, 21]. We analyzed the mitochondrial genes that were affected by G_q expression and normalized by CaMKIIδ deletion for transcription factor binding site enrichment. This analysis

showed significant enrichment for PPAR-α binding sites. Analysis of the RNA sequencing data described above demonstrated that PPAR-α mRNA levels were reduced in G_q mice compared with WT mice but not in Gq/KO versus WT mice. Western blot analysis revealed changes in PPAR-α protein expression; PPAR-α was decreased in the G_q, and this was normalized by CaMKIIδ deletion. Expression of Q209L in NRVMs also effectively decreased PPAR-α mRNA through CaMKII consistent with a direct effect of G_q activation on PPAR-α expression that requires or relies on CaMKII activation (Figure 3-3B). Further experiments established that UCP3 is required for CaMKII inhibition to ameliorate G_q -induced cell death, strongly implying that diminished UCP3 expression dependent on PPAR-α downregulation by CaMKII is an important mechanism by which G_q activation induces cell death and cardiac dysfunction.

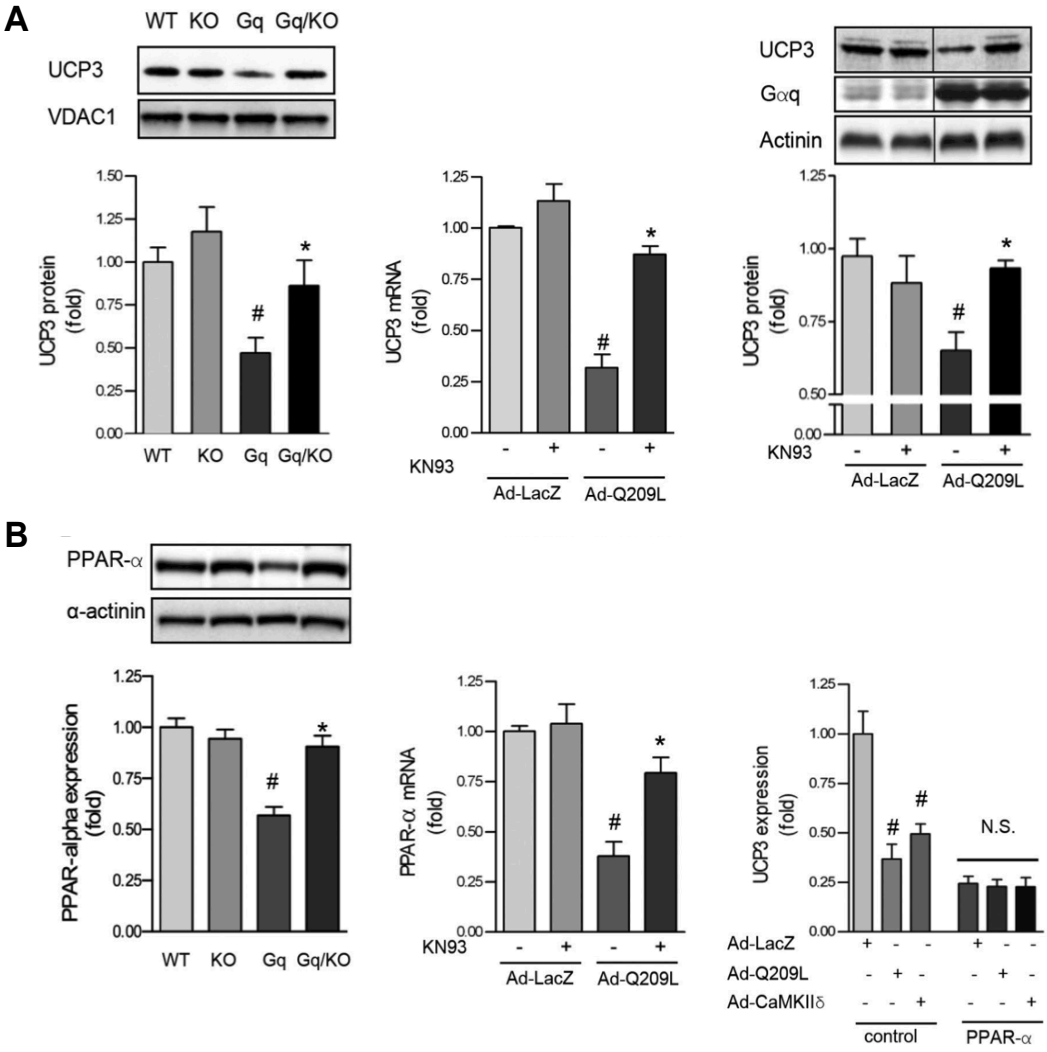

Figure 3-3. CaMKIIδ downregulates PPAR-α and UCP3 expression. (A) UCP3 protein expression in lysates of mitochondria from mouse ventricle and UCP3 mRNA expression in NRVMs infected with Ad-Q209L or Ad-LacZ and cultured in the presence or absence of the CaMKII inhibitor KN93. UCP3 protein expression was measured in whole cell lysates from these cells (B) PPAR-α protein expression in whole cell lysates from mouse ventricle and PPAR-α mRNA expression from NRVMs treated as in (A**).** UCP3 mRNA expression was also measured in NRVMs which were first transfected with control or PPAR-α siRNA and subsequently infected with Ad-LacZ, Ad-Q209L, or constitutively activated CaMKIIδc (CaMKIIδ). All values are expressed as mean±SEM. #*P*<0.05 vs wild type (WT) or Ad-LacZ; **P*<0.05 vs Gq or Q209L.

3.2.3 Discussion

CaMKIIδ, while dispensable for hypertrophic growth, plays a key role in the transition from hypertrophy to heart failure. How this occurs, and whether CaMKIIδ activation is an essential component of pathological hypertrophy downstream of G_q signaling, is not known. Here, we used combined genetic manipulation of G_q and CaMKIIδ to uncover changes in mitochondrial gene expression as a key mediator of CaMKIIδ and G_q-induced cardiac dysfunction, further emphasizing the potential for CaMKII as a therapeutic target.

Downregulation of UCP3 has been associated with increased cardiac ROS levels, and studies using UCP3 knockout mice demonstrate increased angiotensin-induced cardiac ROS levels and greater susceptibility to oxidative stress[22, 23]. These findings are consistent with and extended by our observation that G_q-induced mitochondrial ROS generation is mediated, at least in part, through transcriptional repression of UCP3 by CaMKII. We further demonstrate that CaMKII contributes to UCP3 downregulation in response to G_q and that this downregulation is functionally important in mediating G_q and CaMKII-induced cell death. Our findings suggest that activation of CaMKII, which occurs in response to multiple agonists and stressors, is a nodal point for regulation of mitochondrial ROS generation.

There is a broad precedent for transcriptional regulation of UCP3 by pharmacological interventions and physiological stress and the role of CaMKII in transcriptional regulation has also been well documented[11, 20]. Here, we

demonstrate CaMKII-dependent decreases in expression of both UCP3 and the transcription factor PPAR-α in hearts of G_q mice and in NRVMs expressing activated G_q or CaMKIIδ. siRNA-mediated knock- down of PPAR-α reduced UCP3 expression, and no further effects of G_q or CaMKII on UCP3 expression were observed in the absence of PPAR-α, supporting a role for PPAR-α in transcriptional control of UCP3. We also report decreases in both UCP3 and PPAR-α following pressure overload, which are rescued by CaMKIIδ deletion. PPAR-α has been shown to be reduced in experimental models of heart failure and PPAR-α agonists shown to preserve cardiac function[20, 24, 25]. How CaMKII downregulates PPAR-α and subsequently UCP3 remains to be determined. We used pharmacological inhibitors to test effects of both class I and class II histone deacylases on this process in NRVMs but failed to observe any block of CaMKIIδc-mediated PPAR-α or UCP3 downregulation.

3.3 CaMKIIδ mediates β-adrenergic effects on RyR2 phosphorylation and SR Ca(2+) leak and the pathophysiological response to chronic β-adrenergic stimulation.

3.3.1 Introduction

Hypertrophy decompensation as a result of sustained cardiac stress most likely involves CaMKII in a variety of contexts. In addition to our work

showing that this process requires CaMKIIδ in the context of chronic Gq stimulation, we have also demonstrated a similar relationship between CaMKIIδ and cardiac dysfunction in the context of chronic β-adrenergic stimulation. CaMKII is known to be activated following Ca^{2+}-dependent activation of calmodulin, through a variety of posttranslational modifications including autophosphorylation, oxidation, and O-linked glycosylation[11, 26-28]. In response to the activation of G protein–coupled receptors (GPCRs) by agonists such as endothelin or phenylephrine, CaMKIIδ is activated, possibly as a result of the release of Ca^{2+} from $InsP_3$-sensitive stores[29]. Other GPCR agonists such as angiotensin, which are known to induce oxidative stress, lead to CaMKII activation via oxidation of Met281/282(3). There is growing evidence that β-AR stimulation can also activate CaMKII, although the signaling mechanisms through which this occurs remain in dispute[30, 31].

A relationship between β-adrenergic stimulation and CaMKII activation is intriguing because both the β-adrenergic and CaMKII pathways have been independently implicated in the development of heart failure[1, 11, 30, 32, 33]. Sustained β-AR stimulation resulting from increased sympathetic tone underlies the efficacy of β-AR blockers in heart failure treatment[32]. Increases in CaMKII expression and activation have been widely observed in animal and human models of heart failure and inhibition of CaMKII in human myocytes from patients with heart failure increases their contractile function[7, 8, 33-36]. Thus, we hypothesized that CaMKII and its targets may represent major

downstream signals for the pathological remodeling induced by chronic β-adrenergic stress.

We utilized CaMKIIδ KO mice to determine the role of CaMKIIδ activity in the acute effects of β-AR stimulation. In addition, we used KO mice to examine the effects of chronic β-AR stimulation on the development of hypertrophy and maladaptive remodeling. We demonstrate that deletion of CaMKIIδ does not affect acute physiological β-adrenergic responses or the development of cardiac hypertrophy, but protects the heart against chronic isoproterenol (ISO)-induced fibrosis and heart failure development.

3.3.2 Results

3.3.2.1 Characterization of the response of CaMKIIδ KO mice to acute isoproterenol administration

CaMKIIδ KO mice have no obvious basal phenotype when compared to WT mice, and the loss of CaMKIIδ expression is not associated with compensatory upregulation of other CaMK isoforms[1]. Immunoblot analysis using an antibody specific for the δ isoform of CaMKII confirmed deletion of two major splice variants of CaMKIIδ. Lysates of ventricular tissue from CaMKIIδ-KO mice were also assayed for CaMKII catalytic activity (at maximal activating conditions) and shown to have less than 20% of the total CaMKII

activity seen in WT animals. This residual activity presumably reflects CaMKII isoforms other than CaMKIIδ that are present at low levels in myocytes and in non-myocyte cell types in the heart.

CaMKIIδ phosphorylates a number of cardiac proteins involved in Ca^{2+} handling, including phospholamban (PLN) and RyR2. CaMKII and protein kinase A (PKA) share RyR2 as a target, but the phosphorylation sites are distinct and can be phosphorylated independently[37, 38]. To examine the effects of CaMKIIδ gene deletion on the phosphorylation of RyR2 elicited by β-AR stimulation, we isolated cardiac myocytes from WT and CaMKIIδ KO hearts and treated them with 100 nmol/L isoproterenol (ISO) for 5 min. Immunoblot analysis confirmed that total expression of RyR2 was unchanged in hearts from CaMKIIδ KO animals compared to WT controls. Myocytes from CaMKIIδ KO mice showed decreases in basal phosphorylation of RyR2 at the CaMKII site (Ser2814) compared to WT. There was no detectable decrease in basal phosphorylation at the PKA site (pRyR Ser2808) in CaMKIIδ KO cells. Treatment of WT mouse myocytes with 100 nmol/L ISO lead to a robust increase in phosphorylation of PLN and RyR2 at both the CaMKII and PKA sites, but only the former was significantly attenuated by CaMKIIδ deletion (Figure 3-4A).

RyR2 phosphorylation at CaMKII sites has been linked to increases in diastolic SR Ca^{2+} leak through studies carried out in CaMKII transgenic mice[39-41]. Our collaborators isolated myocytes from WT and CaMKIIδ KO mice to

directly examine the role of CaMKII in ISO-mediated increases in Ca^{2+} sparks and diastolic Ca^{2+} leak. ISO treatment led to a 2.5-fold increase in Ca^{2+} spark frequency (CaSpF) in myocytes from WT mice but failed to significantly increase Ca^{2+} sparks in CaMKIIδ KO cells (Figure 3-4B). These data are consistent with the observed changes in RyR2-Ser2814 phosphorylation and its dominant role in regulating SR Ca^{2+} leak, and implicate CaMKII in ISO-induced dysregulation of SR Ca^{2+} homeostasis.

To examine the role of CaMKII in β-adrenergic effects on the intact heart, we isolated hearts from WT and CaMKIIδ KO mice and perfused them in the Langendorff mode. We first used an enzymatic CaMKII activity assay to demonstrate that ISO treatment of isolated hearts activates CaMKII, as assessed by increases in the percentage of autonomous (Ca^{2+} independent) activity. Indeed, autonomous CaMKII activity was increased by more than 2-fold (from 9% to 22% of total activity) at 10 min after the addition of 1 μmol/L ISO (Figure 3-4C). Baseline cardiac performance was identical in isolated hearts from WT and CaMKIIδ KO mice, and ISO increased various measures of cardiac performance similarly in hearts from both WT and CaMKIIδ KO animals[42] (Figure 3-4D) suggesting that these responses are mediated by ISO effects on CaMKIIγ and PKA signaling rather than on CaMKIIδ.

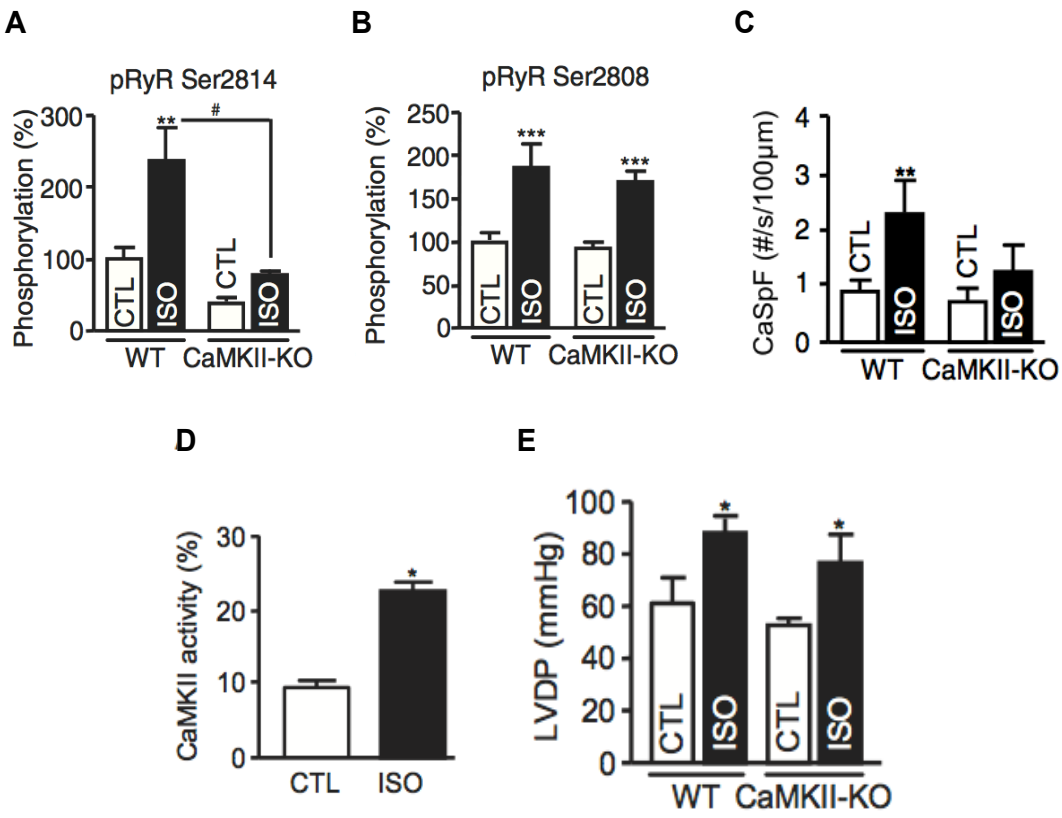

Figure 3-4. CaMKIIδ activation by ISO is associated by increased RyR2 phosphorylation and SR Ca^{2+} leak. (A) Quantification of RyR2 phosphorylation at CaMKII site Ser2814. (B) Quantification of RyR2 phosphorylation at PKA site Ser2808. Data are presented as mean ± SEM of the ratio of normalized phosphorylated protein over total protein (n = 3–4 each). *p<0.05; **p<0.01; ***p<0.001 vs. CTL; $^{\#}$p<0.05 vs. WT. (C) Mean Ca^{2+} spark frequency (CaSpF) in intact cardiomyocytes isolated from WT (n = 8 from 2 mice) and CaMKIIδ-KO mice (n = 7 from 2 mice), during control (CTL) conditions and after exposure to isoproterenol (ISO). (D) Autonomous CaMKII activity measured at steady state under control (CTL) conditions, and after 10 min of ISO perfusion. Activity of CaMKII was determined in fresh ventricular lysates from WT mouse hearts. (E) Group averages of left ventricular developed pressure (LVDP). Data are presented as mean ± SEM. n = 6 mice per group; *p<0.05 vs. CTL.

3.3.2.2 CaMKIIδ KO mice are protected from fibrosis and cardiac dysfunction following chronic β-adrenergic stimulation

The hypertrophic response of the mouse heart to β-AR stimulation with ISO is well described[43, 44]. We used repeated daily injections or osmotic pump infusion of isoproterenol to determine if adult mice lacking CaMKIIδ would be resistant to the hypertrophic effect of β-AR signaling. WT and CaMKIIδ KO mice were treated with ISO for 9 days, 2 weeks, or 4 weeks. WT mice exhibited significant increases in left ventricular mass in response to ISO. Notably, CaMKIIδ KO hearts showed increases in heart/body weight that were equivalent to those of WT at all times and routes of ISO administration. As a final measure, cardiac myocyte dimension was examined and found to be increased to the same extent in WT and CaMKIIδ KO hearts, confirming unchanged pathological hypertrophy at the cellular level and whole-organ level in CaMKIIδ KO mice (Figure 3-5A). The finding that the hypertrophic response to β-adrenergic stimulation is independent of CaMKIIδ activation extends and is consistent with our previous studies that showed no change in the hypertrophic response of CaMKIIδ KO mice to TAC or Gq overexpression.

The overall phenotypic changes induced by 2-week chronic infusion of ISO were relatively mild and did not result in heart failure development. However, infusion with ISO resulted in development of fibrosis in WT, which was significantly attenuated in hearts from CaMKIIδ KO mice (Figure 3-5B). Increased synthesis and secretion of fibrillar collagen types I and III from

cardiac fibroblasts plays a major role in the development of fibrosis. Accordingly, we assessed expression of collagen type I and type III mRNAs and found them to be markedly increased following chronic ISO treatment in hearts from WT but not CaMKIIδ KO[42]. These findings suggest that CaMKIIδ activation contributes to the development of interstitial tissue abnormalities that accompany the progression of heart failure in the setting of chronic β-adrenergic stress.

While infusion of ISO for two weeks was not sufficient to induce heart failure, this was achieved in experiments in which mice received 4-week daily injections of ISO. Pulmonary congestion, an indicator of heart failure assessed by relative lung weight, was significantly increased by ISO treatment in WT mice and this response was significantly attenuated in CaMKIIδ-KO mice. Furthermore, echocardiographic analysis revealed severe LV dysfunction, as indicated by reduced fractional shortening after four weeks of ISO treatment in WT, but not in CaMKIIδ-KO mice (Figure 3-5C). The CaMKIIδ KO mice subjected to ISO also showed a trend towards improved survival compared with WT.

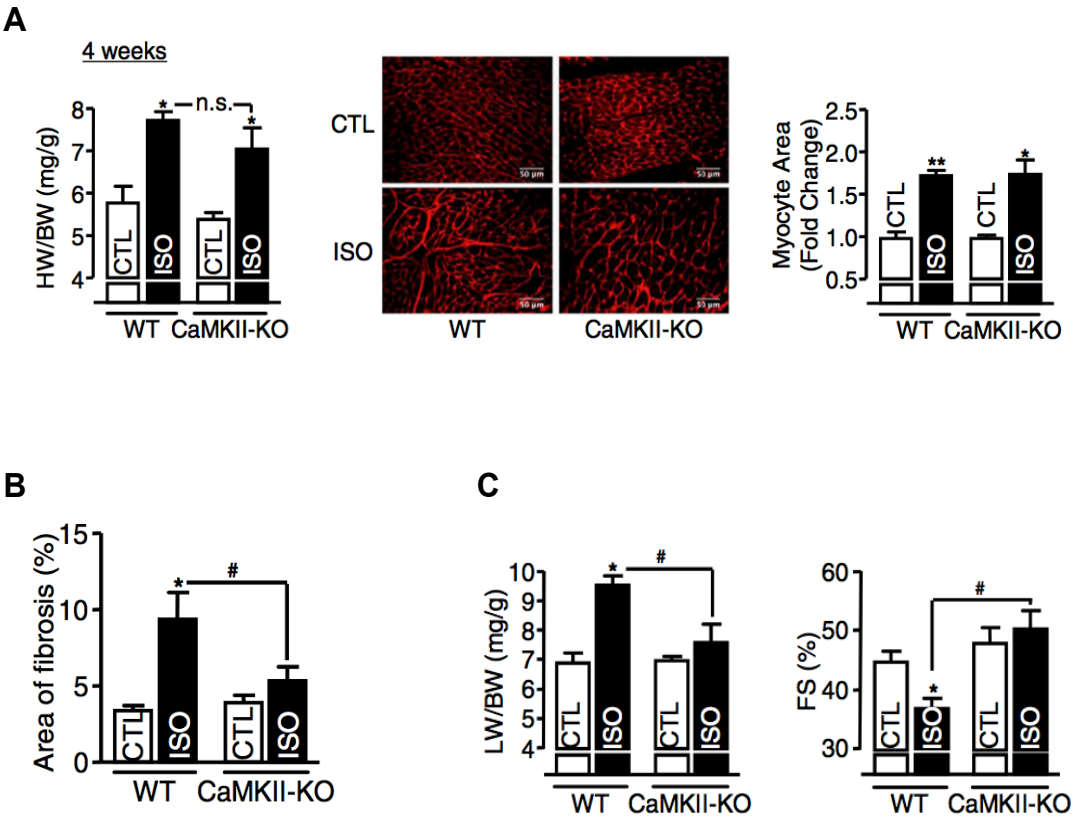

Figure 3-5. CaMKIIδ deletion limits the development of fibrosis and cardiac dysfunction in response to chronic ISO. (A) Indices of hypertrophy including heart weight normalized to body weight (HW/BW) and myocyte area measured following TRITC-labeled wheat germ agglutinin staining in WT and KO mice after 4 weeks ISO (10 mg/kg/d i.p.). (B) Quantification of the area of fibrosis in hearts stained with Masson's trichrome (n = 4). (C) Lung weight normalized to body weight (LW/BW) of WT and KO mice (n = 7–9 per group) and left ventricular fractional shortening (FS) measured by echocardiography.

3.3.3 Discussion

Inhibition of β-AR signaling is a widely used therapy in the treatment of heart failure, however, signaling events downstream of β-AR stimulation resulting in cardiac dysfunction have not been fully elucidated. CaMKIIδ is a mediator of several deleterious signaling pathways in the heart, and we

examined the role of CaMKIIδ in response to both acute and chronic β-AR stimulation. Acute β-AR activation effects on contractility and heart rate were unaltered in CaMKIIδ KO mice, and chronic ISO-induced stress resulted in comparable cardiac hypertrophy in WT and CaMKIIδ KO mice. Strikingly, however, the development of cardiac fibrosis and reduction in cardiac function were inhibited and reversed in CaMKIIδ KO subject to chronic treatment with ISO. All of these data implicate CaMKIIδ in β-AR-induced cardiomyopathy.

Pathological upregulation of fibrosis in cardiac tissue may predispose hearts to arrhythmias and cardiac arrest. This, along with acute arrhythmogenic effects of ISO, could explain the high mortality resulting from daily injection of ISO and the somewhat diminished mortality seen in CaMKIIδ KO mice. That significant mortality is still observed in the CaMKIIδ KO is likely due to the ability of ISO injection to still elicit β-AR and cyclic AMP-dependent inotropic and chronotropic responses.

RyR2 is one of the major downstream targets of CaMKIIδ in cardiac myocytes and is the main intracellular Ca^{2+} channel required for Ca^{2+} release from the SR. Enhanced CaMKIIδ activity in failing hearts has been linked to diastolic SR Ca^{2+} leak in both human patients and in various animal models of heart failure[35, 40, 45]. It may be challenging to accept, at face value, that increasing diastolic SR Ca^{2+} leak is sufficient to lead to heart failure development. It is likely, however, that this mechanism is critical because it initiates and contributes to a vicious cycle in which CaMKII is a central player.

Increased SR Ca^{2+} leak through CaMKII activation has been associated with an elevation of mitochondrial Ca^{2+} and subsequent development of heart failure and cardiomyocyte cell death[15, 46]. Elevated mitochondrial Ca^{2+} increases the production of reactive oxygen species (ROS), which can also lead to posttranslational modifications in CaMKII that render it constitutively active. Activated CaMKII can in turn affect mitochondrial Ca^{2+} and respiratory function through phosphorylation of a mitochondrial protein involved in mitochondrial calcium uniport, events which could further contribute to mitochondrial dysfunction and cardiomyocyte cell death[17]. CaMKII also has numerous other targets that could contribute to heart failure development.

The studies presented here use genetic deletion of CaMKIIδ to provide direct evidence that CaMKII mediates the maladaptive effects of increased β-AR stimulation. Disabling CaMKII-mediated RyR2 phosphorylation or inhibiting CaMKII would appear to be promising approaches, used alone or in conjunction with β-AR blockers, to attenuate ventricular dysfunction in response to excessive β-AR in chronic heart failure.

3.4 Conclusions

CaMKIIδ is a important and consequential nodal regulator of a variety of cellular processes in both normal physiology and disease states. The studies presented in the chapter represent a selection of the research projects

resulting in publication that I participated in during my thesis work. The data clearly demonstrate the benefit of CaMKIIδ gene deletion in the context of G_q overexpression and chronic isoproterenol treatment.

3.5 Experimental procedues

Animal Models

To study the role of CaMKIIδ in $G\alpha_q$-induced heart failure, $G\alpha_q$ transgenic mice were crossed with CaMKIIδ knockout mice to generate CaMKIIδ heterozygous mice with or without Gαq overexpression. WT, KO, $G\alpha_q$ transgenic, and G_q-TG mice in a CaMKIIδ knockout background were generated by the crosses of CaMKIIδ heterozygous mice with or without $G\alpha_q$ overexpression. Between 6 and 14 male mice were included in each experimental group. Echocardiography, left ventricular hemodynamic measurements and cardiac histology were performed.

Biochemical and histological analysis

Cardiac mitochondrial protein carbonyl was measured using OxiSelect protein carbonyl ELISA kit (Cell Biolabs, San Diego, CA). Apoptosis was measured with the Roche DNA fragmentation ELISA. Cardiac ROS levels were measured with dihydroethidium (DHE) staining of cryopreserved

myocardium. Cardiac left ventricles were fixed for 24 h in 4% paraformaldehyde dissolved in 0.1 M PBS (pH 7.4), embedded in paraffin, cut transversely into 5-μm sections, and stained with Masson's trichrome. Images (10× magnification) from sections were analyzed in Adobe Photoshop CS3 with the fuzziness of the color range (blue or red) adjusted to the value 150. The percentage area of fi- brosis was calculated using the equation: % fibrosis = blue pixels / (blue pixels + red pixels) × 100. To assess myocyte cross-sectional area, serial 5 μm sections were stained with TRITC-labeled wheat germ agglutinin (WGA; Sigma, St Louis, MO, USA) and nuclei with DAPI (Vector Laboratories, Burlingame, CA, USA). Myocyte cross-sectional area was quantitated using ImageJ software (version 1.48 g, NIH, Bethesda, MD, USA).

Neonatal Ventricular Cardiomyocyte Culture and live cell imaging

Neonatal rat ventricular myocytes (NRVMs) were isolated, cultured, infected and transfected as described previously. Live cells were loaded with MitoSox (5 μmol/L), tetramethylrhodamine ethyl ester (TMRE, 50 nmol/L) Mitotracker green (1 mmol/L) calcein-AM (1 mmol/L) or propidium Iodide (5 mmol/L) and measured with a Zeiss observer, inverted microscope.

Isolation of adult mouse ventricular myocytes

Cardiac ventricular myocytes were isolated from the ventricles of 8–16-

week-old WT or CaMKIIδ-KO mice. Animals were anesthetized with pentobarbital, and hearts were removed, cannulated, and subject- ed to retrograde aortic perfusion at 37 °C, at a rate of 3 ml/min. Hearts were perfused for 4 min in Ca^{2+}-free buffer, followed by 8–10 min of perfusion with 0.25 mg/ml collagenase (Blendzyme 1, Roche). Hearts were removed from the cannula and the ventricle was dissociated at room temperature by pipetting with increasingly smaller transfer pipettes. Collagenase was inactivated by resuspending the tissue in medium containing 10% bovine calf serum once the tissue was thoroughly digested. Calcium was gradually added back to a final concentration of 1 mmol/L and cells were plated on laminin-coated dishes in minimal essential medium/Hanks' balanced salt solution containing 5% serum. After 1 h, cells were washed and serum-free medium was added back.

Line scan confocal microscopy

Cells were isolated with the retrograde Langendorff perfusion technique. Spontaneous Ca^{2+} sparks and SR Ca^{2+} load were assessed in freshly isolated cardiomyocytes loaded with 5 μmol/L Fluo-4 AM (Molecular Probes). Before each recording, cells were paced at 1 Hz for 1 min to reach the steady state. Ca^{2+} sparks were assessed in quiescent intact cells. SR Ca^{2+} load was assessed as the amplitude of the Ca^{2+} transient induced by rapid application of 10 mmol/L caffeine after 1 min of steady state 1 Hz pacing. Recordings were performed using confocal microscopy (Bio-Rad, Radiance

2100, ×40 oil immersion objective) in line scan mode (3 ms/line). Excitation was via Ar laser (488 nm) and emission was collected at N505 nm. Image analyses were performed with Image J software and homemade routines in Interactive Data Language (IDL; ITT). All experiments were made at room temperature. All procedures were approved by the University of California Davis Institutional Animal Care and Use Committee.

CaMKII activity assay

CaMKII activity was measured in ventricular homogenate using Syntide-2, a synthetic CaMKII-specific substrate peptide. Hearts were isolated and ventricles homogenized in lysis buffer (50 mmol/L HEPES, 10% ethylene glycol, 2 mg/ml BSA, 5 mmol/L EDTA, pH 7.5), and assayed immediately without freezing. The assay buffer contained 50 mmol/L HEPES, 10 mmol/L magnesium acetate, 1 mg/ml BSA, 20 μmol/L Syntide-2, 1 mmol/L DTT, 400 nmol/L [γ-^{32}P]ATP, pH 7.5 and either 1 mmol/L EGTA (for autonomous activity) or 500 μmol/L $CaCl_2$, plus 1 μmol/L calmodulin (for maximal activity). The reaction was carried out at 30 °C for 10 min and blotted onto Whatman P81 phospho- cellulose paper. Percent activation was calculated as the ratio of auton- omous to maximal activity for each sample.

Isolated heart studies

Mice were treated with heparin (500 U/kg i.p.) and anesthetized with

pentobarbital (150 mg/kg i.p.). Animals were then killed, and hearts were quickly removed and placed in ice-cold Ca^{2+}-free Krebs– Henseleit buffer. Hearts were then placed on a Langendorff perfusion system (Radnoti LLC), and perfused at a constant pressure of 80 mmHg at 37 °C with a modified Krebs–Henseleit buffer solution con- taining (in mmol/L); 2.0 $CaCl_2$, 130 NaCl, 5.4 KCl, 11 dextrose, 2 pyru- vate, 0.5 $MgCl_2$, 0.5 NaH_2PO_4, and 25 $NaHCO_3$ and aerated with 95% oxygen and 5% carbon dioxide (pH 7.4). The left atrium was removed, and a water-filled balloon connected to a pressure transducer (MLT844, ADInstruments) was inserted into the left ventricle and inflat- ed to 10 mmHg of LV end-diastolic pressure (LVEDP). Pre-agonist base- line data were recorded following a 20-min equilibration period. Subsequently, an ISO (1 μmol/L) infusion was initiated, and peak re- sponse was recorded. LV developed pressure (LVDP), the maximum rate of positive and negative change in LV pressure (±dP/dt), and heart rate were continuously recorded via a data acquisition system (Powerlab/8SP, ADInstruments). Hearts were flash-frozen in liquid ni- trogen and stored at −80 °C until processed. For assay of CaMKII activity, hearts were homogenized and assayed immediately.

Immunoblotting

Western blot analysis from snap-frozen ventricular tissue was performed. The antibodies for immunoblotting were as follows: CaMKIIδ (D.M. Bers, UC Davis); PLN and α-actinin (Santa Cruz); GAPDH, total Akt, and

Ser473 pAkt (Cell Signaling); phospho-PLN at CaMKII site (Thr17) and PKA site (Ser16) (Badrilla); RyR (Affinity Bioreagents); phospho-RyR2 at CaMKII site (Ser2814) and PKA site (Ser2808) (obtained from A.R. Marks, Columbia University, New York, New York, USA); ANP (US Biological); HDAC5 and Ser498 pHDAC5 (Assay Biotech).

Isoproterenol injection and osmotic pump delivery

Mice were treated with daily intraperitoneal injections of isoproterenol for 9 days (15 mg/kg/day) or 4 weeks (10 mg/kg/day) or isoproter- enol was delivered for 2 or 4 weeks using implantable osmotic pumps for the continuous in vivo dosing of unrestrained mice (Alzet, Model 2004, Cupertino, CA). Pumps were loaded with isoproterenol to deliver 30 mg/kg/day (2 weeks) or 20 mg/kg/day (4 weeks), the animal was anesthetized, and a small incision was made for subcutaneous pump implantation on the back. The pump was inserted, delivery portal first, in order to minimize interactions between the isoproterenol and the healing of the incision, and staples or surgical sutures were used for closure of the wound. In 4-week experiments, cardiac performance was evaluated every week for four weeks in the presence of isoproterenol then reevaluated during the fifth week without drug infusion.

Transthoracic echocardiography

CaMKIIδ-KO and WT control mice were anesthetized by isoflurane, 2%

induction and 1%–2% maintenance and echocardiography performed using an Agilent Technologies, Sonos 5500 with a 15 MHz linear probe. At least three independent M-mode measurements were obtained for each animal. Studies comparing S2814A and WT control mice were car- ried out in the Wehrens laboratory at Baylor College of Medicine using VisualSonics VeVo 770 Imaging System (VisualSonics, Toronto, Canada) equipped with a high-frequency 30 MHz probe. All measurements were performed at a steady-state sedation level throughout the procedure (1.0% to 1.5% isoflurane mixed with 0.5 L/ min 100% O_2) and in the presence of exogenous isoproterenol. Body temperatures were maintained between narrow ranges (37.0 ± 1.0 °C) to avoid confounding effects of hypothermia.

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Chapter 4

Conclusions and future studies

4.1 Summary of findings

Ca^{2+}/CaM-dependent protein kinase II delta (CaMKIIδ) is the predominant cardiac isoform of CaMKII, and is alternatively spliced to generate the δ_B and δ_C subtypes. CaMKIIδ_B and CaMKIIδ_C are similar in terms of catalytic activity and sensitivity to Ca^{2+}. Additionally, both subtypes are regulated by posttranslational modifications (PTMs) in that they acquire a similar degree of Ca^{2+}-independent or autonomous activity when PTMs are appended to residues in the regulatory domain of the kinase. When inserted into the variable domain of CaMKIIα, the 11-amino acid insert in CaMKIIδ_B can confer nuclear localization. Furthermore, mutagenesis of the first two lysines in the insert abrogates the nuclear localization of these constructs. Thus CaMKIIδ_B contains a nuclear localization signal. Despite the inclusion of an NLS only within CaMKIIδ_B, both δ_B and δ_C are expressed throughout the cell, and are not restricted to separate subcellular compartments. CaMKIIδ has been implicated in a host of cardiac pathologies, but the contributions of individual CaMKIIδ splice variants to these disease states have not been fully elucidated.

At the start of this thesis project, work from our laboratory demonstrated that deletion of CaMKIIδ protects the heart from pressure-overload-induced cardiac dysfunction, without altering basic hypertrophic responses. These data suggested a key role for CaMKIIδ in processes regulating cell death and cardiac remodeling during prolonged cardiac stress, and we hypothesized that CaMKIIδ was a mediator of the deleterious effects of a wide range of stimuli, including chronic signaling through G protein-coupled receptors (GPCRs).

Transgenic expression of the α subunit of the heterotrimeric G protein G_q induces a hypertrophic cardiomyopathy that results in failure and premature death, and we tested the role of CaMKIIδ in this process by crossing G_q transgenic mice with those lacking CaMKIIδ. Studies using these animals demonstrate that CaMKIIδ is required for heart failure development induced by G_q. Following transcriptional analysis, we determined that CaMKIIδ induces altered mitochondrial gene expression associated with mitochondrial dysfunction, mitochondrial oxidative stress, and ROS-driven cell death, implicating CaMKIIδ as a central mediator of the mitochondrial reprogramming and cardiomyocyte loss associated with heart failure development downstream of G_q. CaMKIIδ expression was associated in decreased in the expression of uncoupling protein 3 (UCP3), a mitochondrial protein whose expression is correlated with reduced ROS levels. Our studies demonstrate that CaMKIIδ-mediated downregulation of PPARα leads to UCP3 downregulation, and that mitochondrial ROS generation is an important

mediator of the deleterious effects of CaMKIIδ expression. We used a similar approach to evaluate the role of CaMKIIδ in chronic β-adrenergic stimulation and found that CaMKIIδ KO mice have normal hypertrophic responses to isoproterenol stimulation, as was the case for G_q overexpression. Additionally, KO mice had normal PKA-mediated physiological responses to ISO. KO mice were significantly protected form heart failure and fibrosis development, however. The findings of these studies strongly implicated mitochondrial Ca^{2+} dysregulation as an important mediator of pathological CaMKIIδ signaling.

In addition to our studies regarding the role of CaMKIIδ in cardiomyopathies induced by chronic GPCR stimulation, we also investigated the regulation of ischemia/reperfusion (I/R) injury by CaMKIIδ. KO mice were subjected to *in vivo* I/R and the area of dead tissue or infarct was measured. Deletion of CaMKIIδ conferred significant protection from I/R, and was associated with a diminished inflammatory response. Our experiments confirmed that this inflammatory activation was mediated by CaMKIIδ and was not secondary to necrosis. We subsequently hypothesized that CaMKIIδ might regulate I/R damage through the transcription factor NF-κB, and demonstrated that NF-κB activation during I/R was regulated by CaMKIIδ. Lastly, we used pharmacological inhibition of NF-κB to verify its important role in CaMKIIδ-mediated I/R damage.

The role of CaMKIIδ subtypes in this process was not clear. Prior research indicated that $CaMKII\delta_B$ and $CaMKII\delta_C$ elicited different and opposing

effects *in vitro* and when expressed in mice, and our initial hypothesis centered on the theory that CaMKIIδ$_B$ and CaMKIIδ$_C$ were segregated in different subcellular compartments. In order to evaluate this possibility, we performed subcellular fractionation on transgenic animals expressing CaMKIIδ$_B$ and CaMKIIδ$_C$ and measured their expression in several subcellular compartments. Surprisingly, CaMKIIδ$_B$ and CaMKIIδ$_C$ were found throughout the cell and were distributed similarly. Heteromultimerization with endogenous CaMKIIδ subtypes might have explained these results, but we measured similar distributions in animals that overexpressed CaMKIIδ$_B$ or CaMKIIδ$_C$ in the KO background where heteromultimerization cannot occur.

The main focus of this thesis, the role of CaMKIIδ subtypes in *ex vivo* I/R injury, builds on the findings of the aforementioned studies by expanding the study of CaMKIIδ subtypes to the context of I/R. WT, KO, CaMKIIδ$_B$TG/δKO, and CaMKIIδ$_C$TG/δKO mice were subjected to *ex vivo* I/R, and infarct size was measured. δ$_C$TG/δKO animals had larger infarcts than those observed in δ$_B$TG/δKO mice and also displayed robust increases in NF-κB activation. Using pharmacological inhibition of NF-κB and TNF-α we demonstrate that CaMKIIδ$_C$-mediated inflammatory signaling during I/R underlies the pathological consequences of CaMKIIδ expression and activation during I/R.

4.2 Ongoing and future studies

Current experiments are designed to reveal basic mechanistic information about the different properties of the CaMKIIδ$_B$ and CaMKIIδ$_C$ subtypes and to investigate whether these properties are still evident in the absence of overexpression. Since CaMKIIδ$_B$ and CaMKIIδ$_C$ are encoded by the same gene, traditional genetic approaches to study the involvement of endogenous molecules are not applicable. In lieu of traditional KO animals in which an entire gene is deleted or abrogated, we generated animals in which exon 14 of the CaMKIIδ gene was floxed and deleted only in cardiomyocytes. Exon 14 encodes for the 11-amino acid insert in CaMKIIδ$_B$ that differentiates it from CaMKIIδ$_C$, and so deletion of this exon would prevent its insertion into transcripts of the CaMKIIδ gene. Thus, these animals are conditional CaMKIIδ$_B$-specific knockouts.

Initial experimentation has revealed that these animals, as expected, do not express any CaMKIIδ$_B$ mRNA. CaMKIIδ$_C$ mRNA expression is approximately similar to that seen in WT animals, but more quantitative measurements are ongoing. CaMKIIδ$_B$-specific knockouts have no overt phenotype though we plan on performing echocardiographic analysis of these animals to assess heart function at various ages. In support of our conclusions from the studies presented in Chapter 2.4 using δ$_B$TG/δKO and δ$_C$TG/δKO mice, deletion of CaMKIIδ$_B$ does not confer protection from *ex vivo* I/R injury. These preliminary results indicate that endogenous expression of CaMKIIδ$_C$

alone is sufficient to mediate the deleterious effects of CaMKIIδ in I/R. Interestingly, the protective phenotype observed in CaMKIIδ$_B$TG animals does not appear to be robust enough to confer protection from infarct formation when CaMKIIδ$_B$ is expressed at endogenous levels. We plan to assess more sensitive measures of myocardial damage during I/R in order to fully evaluate the effect of CaMKIIδ$_B$ deletion in regard to cardiomyocyte death during I/R. An alternative method to study the role of endogenous levels of CaMKIIδ$_B$ and CaMKIIδ$_C$ would be to use AAV9 vectors to elicit relatively low levels of expression of δ$_B$ and δ$_C$ in cardiomyocytes. We are have generated and amplified AAV9 encoding δ$_B$ and δ$_C$ and are currently optimizing our protocols to ensure consistent CaMKIIδ subtype expression.

We identified selective activation of CaMKIIδ$_C$ in the cytosolic compartment as an important mediator of NF-κB activation and infarct formation during *ex vivo* I/R, but this mode of regulation is unlikely to be the only factor in the distinct properties of δ$_B$ and δ$_C$. While not well appreciated, CaMKIIδ$_B$ localization can be regulated by phosphorylation of Ser^{332} immediately adjacent to the NLS of CaMKIIδ$_B$ which limits localization of CaMKIIδ$_B$ to the nucleus. Remarkably this mode of regulation is also seen when the NLS is moved from the middle of the protein to the N-terminus, suggesting that conformational changes are not required for phosphorylation to block the NLS. We have generated viral constructs in which Ser^{332} has been mutated to Ala, and plan to use this mutagenesis approach to evaluate the

role of CaMKIIδ_B NLS inhibition in I/R injury.

4.3 Concluding remarks

Over one million people in the United States have a heart attack every year. The work presented here provides a novel approach for the treatment of cardiac damage that occurs during a heart attack, and provides mechanistic information regarding how such therapies might elicit a beneficial outcome. This dissertation represents a small but significant contribution to the ongoing efforts to mitigate the tremendous impact of heart disease and should encourage further research into the relationship between Ca^{2+} signaling and inflammatory signaling in the context of ischemia/reperfusion injury.